Jameel M. Al-Khayri · Shri Mohan Jain
Dennis V. Johnson *Editors*

Advances in Plant Breeding Strategies: Fruits

Volume 3

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Editors
Jameel M. Al-Khayri
Department of Agricultural Biotechnology
King Faisal University
Al-Hassa
Saudi Arabia

Dennis V. Johnson
Cincinnati, OH
USA

Shri Mohan Jain
Department of Agricultural Sciences
University of Helsinki
Helsinki
Finland

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Preface

Contemporary plant breeders no longer need to rely solely on traditional methodologies in their work of assuring a sustainable and elastic level of world food production. However, human population is increasing at an alarming rate in developing countries and food availability could gradually become a serious problem. Agriculture production is severely affected because of environmental pollution, rapid industrialization, water scarcity and quality, erosion of fertile topsoil, limited remaining arable land to expand production area, lack of improvement of local plant types, erosion of genetic diversity, and dependence on only few crop species for food supply worldwide. According to FAO, 70% more food must be produced over the next four decades to feed a projected population of 9 billion people by the year 2050. Currently, only 30 plant species are used to meet 95% of the world's food requirements, which are considered as the *major crops.* The breeding programs of these crops have been very much dependent on the ready availability of genetic variation, either spontaneous or induced. Plant breeders and geneticists are under constant pressure to sustain and increase food production by using innovative breeding strategies and introducing minor crops that are well adapted to marginal lands and can provide source of nutrition through tolerance of abiotic and biotic stresses. In traditional breeding, introgression of one or a few genes into a cultivar is carried out via backcrossing over several plant life cycles.

With the development of new molecular tools, molecular marker-assisted backcrossing has facilitated rapid introgression of a transgene into a plant and reduced linkage drag. Continued development and adaptation of plant biotechnology, molecular markers, and genomics have established ingenious new tools for the creation, analysis, and manipulation of genetic variation for the development of improved cultivars. For example, molecular breeding has great potential to become standard practice in the improvement of several fruit crops. Adopting a multidisciplinary approach comprised of traditional plant breeding, mutation breeding, plant biotechnology, and molecular biology would be strategically ideal for developing new improved crop varieties. This book highlights the recent progress in the development of plant biotechnology, associated molecular tools, and their usage in plant breeding.

The basic concept of this book is to examine the best use of both innovative and traditional methods of plant breeding to develop new crop varieties suited to different environmental conditions to achieve sustainable food production and enhanced food security in a changing global climate.

Two volumes of this book series were published in 2015 and 2016, respectively: Volume 1 subtitled *Breeding, Biotechnology and Molecular Tools* and Volume 2 subtitled *Agronomic, Abiotic and Biotic Stress Traits*. This Volume 3, subtitled *Fruits*, focuses on advances in breeding strategies for the improvement of individual fruit species using both traditional and modern approaches. It consists of 23 chapters grouped according to climate zones: Part I, Temperate Fruits; Part II, Subtropical Fruits; and Part III, Tropical Fruits.

Chapters are written by internationally reputable scientists and subjected to a review process to assure quality presentation and scientific accuracy. Each chapter begins with an introduction covering-related backgrounds and provides in-depth discussion of the subject supported with high-quality color photos, illustrations, and relevant data. The chapter concludes with an overview of the current status of breeding and recommendations for future research directions. A comprehensive list of pertinent references is provided to facilitate further reading.

The book is an excellent reference source for plant breeders and geneticists engaged in breeding programs involving biotechnology and molecular tools together with traditional breeding. It is suitable for both advanced undergraduate and postgraduate students specializing in agriculture, biotechnology, and molecular breeding as well as for seed companies.

We are greatly appreciative of all chapter authors for their contributions toward the success and quality of this book. We are proud of this diverse collaborative undertaking, especially since this volume represents the efforts of 72 scientists from 22 countries. We are also grateful to Springer for giving us an opportunity to compile this book.

Al-Hassa, Saudi Arabia — Jameel M. Al-Khayri
Helsinki, Finland — Shri Mohan Jain
Cincinnati, OH, USA — Dennis V. Johnson

Contents

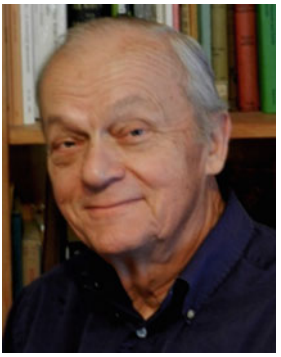
Editors and Contributors

About the Editors

Prof. Jameel M. Al-Khayri is a Professor of Plant Biotechnology, Department of Agricultural Biotechnology, King Faisal University, Saudi Arabia. He received B.S. in Biology in 1984 from the University of Toledo, M.S. in Agronomy in 1988, and Ph.D. in Plant Science in 1991 from the University of Arkansas. He is a member of the International Society for Horticultural Science and Society for In Vitro Biology as well as the National Correspondent of the International Association of Plant Tissue Culture and Biotechnology. His graduate work resulted in the establishment of in vitro regeneration protocols for spinach and zoysiagrass. For the last two decades, he dedicated his research efforts to date palm. He has authored over 50 research articles in refereed international journals, over 20 review chapters, and co-edited several journal special issues and seven reference books on date palm and advances in plant breeding. He has been involved in organizing international scientific conferences and contributed numerous research presentations. In addition to teaching, advising students, and research, he held administrative responsibilities as the Assistant Director of Date Palm Research Center, Head of the Department of Plant Biotechnology, and Vice Dean for Development and Quality Assurance. He served as a member of Majlis Ash-Shura (Saudi Legislative Council) for the 2009–2012 term. Currently, he is maintaining an active research program

on date palm focusing on genetic transformation, secondary metabolites, and in vitro mutagenesis to enhance tolerance to abiotic and biotic stress.

Prof. Shri Mohan Jain is a Consultant and Plant biotechnologist, Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland. He has received M.Phil. in 1973 and Ph.D. in 1978 from Jawaharlal Nehru University, New Delhi, India. He was a Postdoctoral Fellow in Israel, USA, and a Visiting Scientist/Professor in Japan, Malaysia, Germany, and Italy. He was a Technical Officer, Plant Breeding and Genetics, International Atomic Energy Agency (IAEA), Vienna, Austria, 1999–2005. He is a member of the International Association of Plant Tissue Culture and Biotechnology and Editorial Board member of Euphytica, in Vitro, Propagation of Ornamental Plants, Emirates J. Food and Agriculture, and a series on Forest Biotechnology. His publications are more than 160 in peer-reviewed journals, book chapters, and conference proceedings, and edited 55 books; invited speaker and acted as a chairperson in several international conferences worldwide. He was awarded Nobel Peace Prize in 2005 in commemoration of the awarding to IAEA of the Nobel Peace Prize for 2005; also former consultant to IAEA, the European Union, The Government of Grenada, Iranian Private Company and the Egyptian Government. Currently, his research interests are somatic embryogenesis, organogenesis, haploidy, somatic cell hybridization, somaclonal variation and mutagenesis mainly in medicinal plants, date palm, and banana genetic improvement; genetic diversity, erosion, conservation, and utilization in the context of climate change and food and nutritional security.



Prof. Dennis V. Johnson is a Consultant and former University Professor, and he is a graduate of the University of California Los Angeles where he completed his B.A. (1966), M.A. (1970), and Ph.D. (1972) degrees in geography, with specialization in agriculture and biogeography. He has taught at several colleges and universities including the University of Houston and was a Visiting Professor for 2 years at the University of Ceará, Fortaleza, Brazil. He also has worked extensively with international development agencies providing technical assistance to agriculture and forestry on projects and programs in Africa, Asia, Europe, and Latin America. He has published numerous articles on palm utilization and conservation and has edited or written books for FAO, IUCN, and UNEP. He has also translated into English plant science books from Portuguese and Spanish. A decade ago, he began to focus his research on date palm, in particular its introduction to non-traditional areas such as Spain, North and South America, and Australia. He co-authored a book on date growing in the USA and has made presentations at five recent international date palm conferences, and co-edited the book Date Palm Biotechnology.

Contributors

Jameel M. Al-Khayri Department of Agricultural Biotechnology, College of Agriculture and Food Sciences, King Faisal University, Al-Hassa, Saudi Arabia

Pablo Aleza Tissue Culture Laboratory, Center of Citriculture and Vegetable Production, Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, Spain

Fateh Aljane Arid Land and Oasis Cropping Laboratory, Institute of Arid Regions of Medenine, Medenine, Tunisia

Elijah Miinda Ateka Department of Horticulture, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

Ather-uz-Zaman Department of Agro-Biotechnology, GETCO Agro Vision, Dhaka, Bangladesh

Ian S. E. Bally Department of Agriculture, Fisheries (DAF), Queensland Government, Mareeba, QLD, Australia

Irit Bar-Ya'akov Unit of Deciduous Fruit Tree Sciences, Newe Ya'ar Research Center, Agricultural Research Organization, Ramat Yishay, Israel

Diego Barranco Departamento de Agronomía, Edificio Celestino Mutis, Universidad de Córdoba, Córdoba, Spain

Isaura Castro Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal

Carlos Bernard Moreno Cerqueira-Silva Laboratório de Genética Molecular Aplicada, Departamento de Ciências Exatas e Naturais, Universidade Estadual do Sudoeste da Bahia (UESB), Itapetinga, Bahia, Brazil

José Cuenca Tissue Culture Laboratory, Center of Citriculture and Vegetable Production, Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, Spain

Dayanand Dalawai Department of Botany, Karnatak University, Dharwad, India

Glacy Jaqueline da Silva Molecular Biology Department, Paranaense University, UNIPAR, Umuarama, PR, Brazil

Pricila Santos da Silva Agricultural Sciences Center of Lages, University of Santa Catarina State, Florianópolis, SC, Brazil

Vijayalaxmi S. Dandin Department of Botany, Karnatak University, Dharwad, India

Anete Pereira de Souza Departamento de Biologia Vegetal, Centro de Biologia Molecular e Engenharia Genética, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil

Onildo Nunes de Jesus Centro Nacional de Pesquisa em Mandioca e Fruticultura, Empresa Brasileira de Pesquisa Agropecuária (Embrapa Mandioca e Fruticultura), Cruz das Almas, Bahia, Brazil

Natalie L. Dillon Department of Agriculture, Fisheries (DAF), Queensland Government, Mareeba, QLD, Australia

Luca Dondini Dipartimento di Scienze e Tecnologie Agro-Alimentari, Università di Bologna, Bologna, Italy

Elisa Susilene Lisboa dos Santos Laboratório de Genética Molecular Aplicada, Departamento de Ciências Exatas e Naturais, Universidade Estadual do Sudoeste da Bahia (UESB), Itapetinga, Bahia, Brazil

Concepción M. Díez Departamento de Agronomía, Edificio Celestino Mutis, Universidad de Córdoba, Córdoba, Spain

Awatef Essid Arid Land and Oasis Cropping Laboratory, Institute of Arid Regions of Medenine, Medenine, Tunisia

Fábio Gelape Faleiro Centro de Pesquisa Agropecuária do Cerrado, Empresa Brasileira de Pesquisa Agropecuária (Embrapa Cerrados), Brasília, Distrito Federal, Brazil

Bilquees Fatima Plant Tissue Culture Cell, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan

Manfred Fischer Fruit Genebank, Institute for Plant Genetics and Crop Plant Research (IPK), Dresden, Pillnitz, Germany

Andrés Garcia-Lor Tissue Culture Laboratory, Center of Citriculture and Vegetable Production, Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, Spain

Fernanda Grimaldi Agricultural Sciences Center of Lages, University of Santa Catarina State, Florianópolis, SC, Brazil

Zac Hanley The Institute of Plant and Food Research New Zealand Limited, Auckland, New Zealand

Doron Holland Unit of Deciduous Fruit Tree Sciences, Newe Ya'ar Research Center, Agricultural Research Organization, Ramat Yishay, Israel

José I. Hormaza Department of Subtropical Fruit Crops, Instituto de Hortofruticultura, Subtropical y Mediterránea La Mayora, (IHSM La Mayora-CSIC-UMA), Algarrobo-Costa, Málaga, Spain

Burhanettin Imrak Department of Horticulture, Faculty of Agriculture, University of Cukurova, Adana, Turkey

Amjad Iqbal Department of Agriculture, Abdul Wali Khan University Mardan, Mardan, Pakistan

Rafiul Islam Department of Botany, Rajshahi University, Rajshahi, Bangladesh

Shri Mohan Jain Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland

Dennis V. Johnson Consultant, Cincinnati, OH, USA

Nesibe Ebru Kafkas Department of Horticulture, Faculty of Agriculture, University of Cukurova, Adana, Turkey

Salih Kafkas Department of Horticulture, Faculty of Agriculture, University of Cukurova, Adana, Turkey

Ali Kuden Department of Horticulture, Faculty of Agriculture, University of Cukurova, Adana, Turkey

Nerea Larranaga Department of Subtropical Fruit Crops, Instituto de Hortofruticultura, Subtropical y Mediterránea La Mayora, (IHSM La Mayora-CSIC-UMA), Algarrobo-Costa, Málaga, Spain

Jorge Lora Department of Subtropical Fruit Crops, Instituto de Hortofruticultura, Subtropical y Mediterránea La Mayora, (IHSM La Mayora-CSIC-UMA), Algarrobo-Costa, Málaga, Spain

Stefano Lugli Dipartimento di Scienze e Tecnologie Agro-Alimentari, Università di Bologna, Bologna, Italy

Mouaad Amine Mazri Institut National de la Recherche Agronomique, CRRA de Marrakech, UR Agro-Biotechnologie, Marrakech, Morocco

Nebojša Milošević Department of Pomology and Fruit Breeding, Fruit Research Institute, Čačak, Republic of Serbia

Tomo Milošević Department of Fruit Growing and Viticulture, Faculty of Agronomy, University of Kragujevac, Čačak, Republic of Serbia

Naomi Nzilani Mumo Department of Horticulture, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

Hosakatte Niranjana Murthy Department of Botany, Karnatak University, Dharwad, India

Sabrine Nahdi Department of Plant Breeding and Crop Protection, Higher School of Agriculture, El Kef, Tunisia

Poornananda M. Naik Department of Agricultural Biotechnology, College of Agriculture and Food Sciences, King Faisal University, Al-Hassa, Saudi Arabia

Luis Navarro Tissue Culture Laboratory, Center of Citriculture and Vegetable Production, Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, Spain

Francis Kweya Ombwara Department of Horticulture, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

Kee-Yoeup Paek Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju, Republic of Korea

So-Young Park Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju, Republic of Korea

Santiago Pereira-Lorenzo Department of Crop Production and Engineering Projects, Universidad de Santiago de Compostela, Lugo, Spain

Van The Pham Department of Plant Resources, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Ha Noi, Vietnam

Fernando Pliego-Alfaro Departamento de Biología Vegetal, Facultad de Ciencias, Instituto de Hortofruticultura Subtropical y Mediterránea (IHSM) "La Mayora" (UMA-CSIC), Málaga, Spain

Ilknur Polat Bati Akdeniz Agricultural Research Institute (BATEM), Antalya, Turkey

Rashad Qadri Institute of Horticultural Sciences, University of Agriculture Faisalabad, Faisalabad, Pakistan

Luis Rallo Departamento de Agronomía, Edificio Celestino Mutis, Universidad de Córdoba, Córdoba, Spain

Pilar Rallo Departamento de Ciencias Agroforestales, Universidad de Sevilla, ETSIA, Seville, Spain

Ana María Ramos-Cabrer Department of Crop Production and Engineering Projects, Universidad de Santiago de Compostela, Lugo, Spain

Gopalapillai Ravikumar Seri-Biotech Research Laboratory, Central Silk Board, Bangalore, Karnataka, India

Fredah Karambu Rimberia Department of Horticulture, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

Silviero Sansavini Dipartimento di Scienze e Tecnologie Agro-Alimentari, Università di Bologna, Bologna, Italy

Abdulkadir Sarıer Department of Horticulture, Faculty of Agriculture, University of Cukurova, Adana, Turkey

María Paz Suárez Departamento de Ciencias Agroforestales, Universidad de Sevilla, ETSIA, Seville, Spain

Amalendu Tikader Aranya Enclave, Hongasandra, Bangalore, Karnataka, India

Carlos Trapero Departamento de Agronomía, Edificio Celestino Mutis, Universidad de Córdoba, Córdoba, Spain

Muhammad Usman Plant Tissue Culture Cell, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan

Kunjupillai Vijayan Central Silk Board, BTM Layout, Madiwala, Bangalore, Karnataka, India

Fabíola Villa Horticulture Department, Western Paraná State University, UNIOESTE, Marechal C. Rondon, PR, Brazil

Juliana Fátima Welter Agricultural Sciences Center of Lages, University of Santa Catarina State, Florianópolis, SC, Brazil

Yaodong Yang Hainan Key Laboratory of Tropical Oil Crops Biology, Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences, Wenchang, Hainan, China

Part I
Temperate Fruits

Chapter 1
Apple (*Malus* spp.) Breeding: Present and Future

Santiago Pereira-Lorenzo, Manfred Fischer, Ana María Ramos-Cabrer and Isaura Castro

Abstract Apple breeding has been extremely successful in providing a highly diverse fruit crop. Recent (>50 millions years ago) genome-wide duplication (GWD) resulted in the 17 chromosomes in the Pyreae and confirmed the origin of cultivated apple on *Malus sieversii* being the same species as *M.* × *domestica*. *Malus*, as many other species in the family Rosaceae, shows gametophytic self-incompatibility (GSI), which forces outcrossing. GSI at the pistil is regulated by extacellular ribonuclease, S-RNase, which is encoded by S locus. Growers and agronomists have provided multiple cultivars with different colors, shapes, resistances, climatic adaptation or industrial aptitudes. The aim in apple breeding was the combination of different kinds of resistance and good fruit quality to produce dessert cultivars and cultivars for processing. Some of the best of these cultivars display resistance to scab (*Venturia inaequalis*), mildew (*Podosphaera leucotricha*), fire blight (*Erwinia amylovora*), bacterial canker (*Pseudomonas syringae*), red spider mite (*Panonychus ulmi*), winterfrost and good fruit quality. Different scab resistance sources of wild species (Vf, Vr, VA) were combined in the new series of cultivars. Multiple efforts worldwide have conserved most of that variation, the pillar for the traditional and new techniques profiting from the analysis of the apple genome, the genome-wide association studies (GWAS), identifying SNPs and genes, the analysis of genes differentially expressed

S. Pereira-Lorenzo (✉) · A. M. Ramos-Cabrer
Department of Crop Production and Engineering Projects, Universidad de Santiago de Compostela, Campus de Lugo, 27002 Lugo, Spain
e-mail: santiago.pereira.lorenzo@usc.es

A. M. Ramos-Cabrer
e-mail: ana.ramos@usc.es

M. Fischer
Fruit Genebank, Institute for Plant Genetics and Crop Plant Research (IPK), 01326 Dresden, Pillnitz, Germany
e-mail: manfr.fischer@googlemail.com

I. Castro
Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Universidade de Trás-os-Montes e Alto Douro, 5000-801 Vila Real, Portugal
e-mail: icastro@utad.pt

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(GDE) identified by qRT-PCR and microarray analysis, and the recent molecular genetic tool CRISPR/Cas9 to edit and correct the genome.

Keywords Germplasm · Diversity · Breeding · Hybridization · Genomics Assisted selection · Molecular markers · Phenotypic traits

1.1 Introduction

The apple stopped being a semi-luxury a long time ago, and is now a basic foodstuff. After all, at 56 million mt harvested annually, it is in twelfth place in world production of foodstuffs (maize 604 million mt, citrus fruits 103 million mt, bananas 57 million mt) (FAO 1998). The trend is increasing. The reason for this development is the nutritional-physiological value of the fruits as well as their good handling in harvesting, storage, transportation and marketing. The apple has become a product which is traded worldwide.

The physiological value of the apple lies in its high vitamin, antioxidant, and mineral content. The apple contains all important free amino acids in harmonious combination. The vitamin content, it is true, varies substantially between the varieties and years, but one is still able to cover a large part of the daily vitamin requirement with just the regular consumption of apples. The dietary effect of apples stems also from the high content and combination of fruit sugars, in which varieties with increased fructose content are particularly recommended for diabetics. On the basis of these figures, one can probably only has an inkling of the variety of different flavors that are available from apples. But this also explains the interest in maintaining many different varieties, in order to also be able to appraise and enjoy the fine differences (Hartmann 2015). The nostalgia for old varieties is great at present, as a certain degree of simplicity of taste is unmistakable in the few varieties available in supermarkets. But the breeding work is going on worldwide.

Apples have been known as a foodstuff for about 10,000 years. They spread from their center of diversity, an area corresponding roughly to present-day southwest China, Kazakhstan and Kirgizia and the bordering foothills of the Himalayas, via central Asia, from there via the historic Silk Road in the Near East, and from there through the Greeks and Romans to Europe. The wild species *Malus sieversii*, found in Central Asia, is today regarded as the original form. On the way to Europe, natural cross-fertilization with *M. orientalis*, found in the Caucasus, took place. In an easterly direction, it spread via southern China to eastern Asia, where it is likely that cross-fertilization took place, most likely with *M. prunifolia* and *M. baccata*. In a northerly direction, dissemination took place via the Trans-Caucasus to southern Russia. It is possible that cross-fertilization took place here with the wood-apple native to Europe, *Malus sylvestris praecox*. In the Persian Empire, there was already plantation-like cultivation of fruit in the seventh to fifth centuries before Christ. The dissemination of the apple, which, as everyone knows, is distinguished by a very high genetic variability, was always accompanied by an appropriate selection, so

that in the end tasty and large-fruited forms were multiplied, which can be seen as the archetypes of today's varieties. A precondition of this was that one understood how to vegetative multiply fruit coppices, i.e. refine them, in order to be able to keep and propagate the selected best in this manner, again and again. This was possibly deduced by Babylonian gardeners roughly 2500–3000 years ago. This is how apples began to be *domesticated*. This took place about 3000–4000 years later on the old Silk Road, when goats and sheep were domesticated between the Euphrates and the Tigris, which happened about 7000–8000 years earlier (Büttner et al. 2000a; Juniper et al. 1999).

Thanks to Homer, we know from the Odyssey (800 B.C.), that Greeks were already naming different types of apple (Petzold 1990). The Romans also knew many varieties of apples by name, and spread these to the North and West in Europe (Hurt 1916). Stock containers were able to be excavated from sunken Viking ships (1000–500 A.D.), which, among other things, contained apples as an ingredient of preserved food. Today many of the *old* varieties still known, thus came from the seeds of these varieties in the Middle Ages and later. It is clear that the first varieties of apple to reach America came with emigrants from Europe in the late Middle Ages.

Genuine pomology, that is to say the scientific treatment of varieties of fruit, began in the eighteenth century, and the science of fruit cultivation first appeared as a specialist subject within plant cultivation at the end of the nineteenth century. Some important varieties from this time are still being cultivated, such as cvs. Delicious (USA before 1880), Prince Albrecht of Prussia/Prinz Albrecht von Preussen (Germany around 1865), Cox Orange (England around 1850), Boskoop (Holland 1856), Gravenstein (Italy before 1670), McIntosh (Canada 1796), Granny Smith (Australia before 1870) or Goldparmäne (France before 1700) (Fischer 2010; Way et al. 1991). The form taken by the production of fruit 100–200 years ago was the standard tree. Intensive plantation cultivation of fruit with small-crowned spindle-bush trees according to strict economic parameters was only possible after the introduction of dwarfing rootstocks. Its development only began in the middle of the twentieth century—only about 70 years ago!

More and more integrated and biological (*organic*) cultivation methods are becoming increasingly important in all main fruit production areas of the world. The situation concerning the authorization of pesticides is becoming more critical as increased attention is focused on the conservation of the environment. The control of certain pests or diseases with chemicals is already being questioned to some degree. An alternative solution here is the cultivation of resistant varieties which can be grown with much less pesticides. Unfortunately, however, there is still a very low level of acceptance of resistant varieties in the commercial cultivation of apples.

At present, the essential conditions that must be met before a new variety can be introduced on the market are:

(a) With respect to the varieties:

1. Two colors with attractive shiny red shades as an over color
2. Sweet, aromatic taste
3. Firm, crisp fruit flesh with a long shelf life.

(b) With respect to the trade:

1. The willingness to introduce a new variety
2. Substitution of an established variety
3. Considerable advertising expenditure.

In the final analysis, the grower must and will produce what the trade wants, not the other way round, whereby it does not matter to the trade at present how the varieties are produced, as long as they conform to the required quality standards. There will be, however, certain changes with the introduction of trademarks such as Eco Label or Certified Organic labels. The demand for organically-grown products will increase even if the price is higher (Fischer and Fischer 2008; Kellerhals and Gessler 1995).

Producing fruit, especially apples, organically—and to the best quality in the long term—is not very easy and requires experienced specialists. In addition, pest control is still necessary in organic cultivation; the more sensitive the varieties grown, the more protection is needed. All these factors are only of little interest to the consumer, who wants the following criteria:

(a) High-quality fruits, free of pesticide residues (the consumer who is not well informed in this area feels this is more probable in organic cultivation, which explains the increased demand for this)
(b) Continuously, consistently good supply
(c) Diversity in varieties, (whereby the demand here keeps within limits).

The resistance of one variety to specific diseases or pests resulting from the breeding work for decrease in the use of plant protection agents in cultivation is still not a sales argument. This requires more intensive explanation and corresponding advertising (Stehr 2000).

1.1.1 Origin

Recent (>50 million years ago) genome-wide duplication (GWD) resulted in the 17 chromosomes in the Pyreae (Velasco et al. 2010) and confirmed the origin of cultivated apple on *Malus sieversii* being the same species as *M.* × *domestica*; closely related to *M. orientalis* and to *M.* × *asiatica* (a Chinese cultivated apple form), and less closely to *M. sylvestris*, *M. baccata*, *M. micromalus* and *M. prunifolia*.

The wild apple *Malus sieversii* (Ldb.) Roem is found in the Tian Shan Mountains in Central Asia, and it is thought to be originated about 4000–10,000 years ago. Apples were brought to west by the Silk Route where they hybridized with other wild apples, such as *M. baccata* (L.) Borkh. in Siberia, *M. orientalis* Uglitz. in the Caucasus and *M. sylvestris* Mill. in Europe (Cornille et al. 2014).

Grafting an apple produced instant domestication as in other perennial crops, with further hybridizations but without important genetic introgressions from other species as was believed, such as *Malus sylvestris* (Velasco et al. 2010).The specific

fruit of the Pyreae, the pome, is formed by the growth of the lower part of the sepals, which was related to MADS-box genes, involved in flower and fruit development and in the metabolism of sorbitol.

Over 7000 apple cultivars have been cited, but only a few are relevant in the world production. Those cultivars originated in the nineteenth century, such as McIntosh (1800s), Jonathan (1820s), Cox's Orange Pippin (1830s), Granny Smith (1860s), Delicious (1870s) and Golden Delicious (1890s) (Janick et al. 1996; Noiton and Alspach 1996). Most of them are chance seedlings from which clones were selected, or frequently from controlled hybridizations.

Local cultivars, many of them conserved in germplasm banks, were cited so early as the eighteenth century, such as those in Spain (Sarmiento 1986), where hybridization, selection of polyploids and introgression were the main origin of variability which was fixed, instant domestication, by grafting (Pereira-Lorenzo et al. 2017). The use of molecular markers determined that clonality in Spanish collections accounted up to 30%. In some important varietal groups intra-cultivar variability was found, with seedlings derived from a main clone and some polyploids (21%) derived from diploids as a result of the union of an unreduced 2n-gamete with a normal n-gamete (Einset 1952).

1.2 Development of Molecular Markers and Marker-Assisted Selection

Marker-assisted selection (MAS) is based on the use of genetic markers to select specific traits or quantitative trait loci (QTL) for which they are associated, normally named *marker-trait association* and *marker-locus-trait association*, respectively) (Ru et al. 2015).

Early selection on progenies of tree breeding programs is one of the main interests of Marker-assisted seedling selection (MASS), which uses DNA markers to perform an early DNA-based evaluation of genetic traits on seedlings obtained from designed biparental crosses to combine selected traits in order to produce a potential novel cultivar (Ru et al. 2015). MASS shortens the period of seedling selection due to juvenility and large plant sizes of most fruit trees. MASS can also have advantages for: (i) the identification of individuals with multiple resistance alleles to a disease; (ii) when most of the observed phenotypic variance is due to environmental factors; and (iii) when there are unexpected genotype × environment interactions, G × E), which involves to replicate seedlings in one or more locations. Even though DNA markers improve the genetic gain of seedling selection, especially for traits with low heritability, no study has been done in rosaceous tree fruit on the genetic gain of MASS.

Some examples of MASS on apples are (Ru et al. 2015) related with resistances: (i) screening Rvi6 (Vf) gene for scab apple resistance; and (ii) fire blight (FB-F7QTL) in combination with scab (alleles at the Rvi6 and Rvi4 loci), and powdery mildew

(alleles at the Pl2 locus); and a marker-locus-trait association between a gene-based SCAR marker, the Md-ACS1-indel marker designed based on the Md-ACS1 gene) and apple fruit postharvest storability.

1.3 Genetic Diversity Within Genus *Malus* and *Malus* × *Domestica*

Although *Malus sieversii* has been identified has the main contributor to the genome of the cultivated apple (*M.* x *domestica*), multiple species have contributed to the genetic makeup of domesticated apples, in particular the wild European crabapple *M. sylvestris* which is genetically more closely related to *M. domestica* than *M. sieversii* (Cornille et al. 2014). In fact, estimated genetic differentiation based on the analysis of the apple genome (Velasco et al. 2010) was considered *moderate* between *M.* x *domestica* and *M. sieversii* (Fst = 0.14) and *great* between *M.* x *domestica* and *M. sylvestris* and between *M. sieversii* and *M. sylvestris* (Fst = 0.17 and Fst = 0.21, respectively). Chloroplast genome diversity corroborates the close relationship between *M.* x *domestica* and *M. sylvestris* with the existence chlorotypes shared by both species (Coart et al. 2006).

Considering domesticated apples *Malus* x *domestica*, allelic diversity in genetic resources from mainland Portugal were reported as 11.5 alleles per locus (Ferreira et al. 2016), similar to that reported in northwestern Spain (Galicia and Asturias) by Pereira-Lorenzo et al. (2007) (11.4), to local germplasm from Aragon, northeastern Spain (12.4) (Pina et al. 2014) in Iranian landraces (10.7) (Gharghani et al. 2009), and for Sarajevo and eastern Bosnia accessions (13.5) (Gasi et al. 2013), but lightly lower than those reported for the Portuguese Azores Islands apple germplasm (15.2 alleles/locus) (Foroni et al. 2012), northeastern Spain (Aragon, Navarre, Basque Country, La Rioja and Catalonia regions) local material by Urrestarazu et al. (2012) (16.7), for the local accessions of Italian apple germplasm maintained at Bologna (16.9) (Liang et al. 2015), by Van Treuren et al. (2010) in the Netherlands (18.5) and that found for whole Spain (18.6) (Pereira-Lorenzo et al. 2017), for whole Europe (23.2) (Urrestarazu et al. 2016) and in the American germplasm bank for *Malus* x *domestica* with 19.1 alleles per locus (Gross et al. 2014).

Heterozygosity in local cultivars was 0.75 in mainland Portugal (Ferreira et al. 2016), about the same level of the American germplasm bank for *Malus* x *domestica*, 0.73 (Gross et al. 2014), and slightly higher than the level of diversity found in Aragon-Spain, 0.68 (Pina et al. 2014) but slightly lower than in north-eastern and north-western Spain, 0.80 and 0.83 respectively (Pereira-Lorenzo et al. 2007; Urrestarazu et al. 2012) and in Italy, 0.81 (Liang et al. 2015), Portuguese Azores Islands, 0.82, (Foroni et al. 2012), in in French germplasm (0.83) (Lassois et al. 2016) and broad European apple germplasm (0.81) (Urrestarazu et al. 2016).

Both triploids and tetraploid varieties are known to exist for apple (Brown 1998). Van Treuren et al. (2010) reports 21% genotypes with 3 alleles at single locus or

multiple loci, 10% of them known triploids and also 4% of samples with even 4 alleles per locus. In Portuguese apple germplasm, among 64 unique genotypes, 12 (≈19%) were putative triploids (Ferreira et al. 2016). Higher values, 24 and 29%, were detected in Spanish germplasm by Urrestarazu et al. (2012) and Ramos-Cabrer et al. (2007), respectively. Recently, Urrestarazu et al. (2016) in germplasm of the whole of Europe reported lower numbers of triploid genotypes, 162 in 2031 unique genotypes (8%).

1.4 Genome-Wide Selection

The use of microarrays was useful to identify genes related to fruit development from pollination to ripeness, with a high correlation with the expression of those genes in tomato (Janssen et al. 2008).

An extremely useful tool for further genetic studies was provided by the genome of cultivated apple (Velasco et al. 2010). Domesticated apple showed a high number of putative genetic genes respecting to other crops and their gene sequences are being used for genome-wide functional studies. Many of genes have ben related to resistances, flavor and other agronomy characteristics, such as the *MADS-box* genes, involved in flower and development the pome and in the metabolism of sorbitol. 4.4 SNPs per kb were found and they are useful for association studies with phenotypic traits.

Genome selection (GS) is thought to overcome marker-assisted selection (MAS) and marker-assisted recurrent selection (MARS), but some authors (Jonas and de Koning 2013) pointed out that crops show a great diversity related to breeding cycles which makes GS more complex than in cattle breeding.

A genome-wide association study (GWAS) was already used to differentiate cider and dessert apples and to identify SNPs and genes related to that differentiation (Leforestier et al. 2015). Genetic differentiation between both groups of cultivars was low, explained because of the mixed types used for both cider and dessert. No genes were reported in the regions related to the SNPs found differentiating both groups, perhaps due to the still scarce knowledge about the genetic control of traits such as bitterness on apples, related to a higher content in polyphenols.

Analysis of genes differentially expressed (GDE) were identified by qRT-PCR and microarray analysis in fruited and deflowered cv. Gala adult trees to understand biennial bearing (Guitton et al. 2016). Some of them were differently expressed, such as Tempranillo (TEM), Floral Transition at Meristem (FTM1) and Squamosa Promoter Binding PROTEIN-Like (SPL), and homologs of SPL and leucine-rich repeat proteins were found in QTLs previously related to biennial bearing.

1.5 Mating Systems in *Malus*

Malus, as many other species in the family Rosaceae, shows gametophytic self-incompatibility (GSI), which forces out-crossing since the pistil blocks closely related pollen (De Nettancourt 2001). GSI at the pistil is regulated by extacellular ribonuclease, S-RNase, which is encoded by S locus (Sassa et al. 2010). There is another pollen S gene, tightly linked to the *S-RNase*, which determines the specificity of the pollen. Both genetic units are named S haplotype. Pollen S genes are F-box genes called SLF/SFB. The interaction of Pistil S (*S-RNase*) and pollen S (SLF/SFB) proteins determine incompatibility and, therefore, pollen tube growth in the pistil.

The unlikeness of recombination between *S-RNase* and SFB makes to identify both *S-RNase* and SFB alleles for *S*-genotyping (Yamane and Tao 2009).

1.6 Hybridization Between Species

Fruit breeding aspires toward cultivating new varieties, which will have stable resistance and comply with the demands of the wholesale trade and of consumers. None of the resistant varieties which have become known internationally yet meet these stringent requirements sufficiently, and this is why there are still reservations about their large-scale cultivation. However, resistant varieties have already been accepted for a long time in the self-supply and home garden sector.

It remains the aim of apple breeding to bring together in new varieties improvements in fruit quality + yield + resistance to different pathogens. Another new challenge is establishing a durable resistance in field cultivation, now that observations from different parts of Europe on the breakdown in the monogenic scab resistance sources from the *Malus floribunda* (Baumgartner et al. 2016; Büttner et al. 2000b; Fischer et al. 2000; Kellerhals and Gessler 1995; Lespinasse 2001) have come to light. So the use of old *vital* cultivars for breeding is under discussion (Bannier 2011; Sattler and Bannier 2016). Positive results do not yet exist. The fruit quality of progenies after crossings with cvs. Rote Sternrenette, Früher Victoria, Kardinal Bea or Prinzenapfel was unacceptable and unsuitable for recommendation (Fischer and Fischer 2008; Rueß 2016).

The main breeding trends are dessert apple varieties for fresh consumption. Fruit quality is the first and foremost priority. The strategy of resistance breeding is characterized by its integration into a system of measures for plant protection in the broadest sense. While doing so, the total success is not aspired to, but rather a type of field resistance which allows both organisms—variety (host) and pathogen—to possess a chance of survival, and the host-parasite ecosystem maintains a natural balance. It assumed that there is the possibility of living with the pathogens, not complete resistance (immunity), but rather the stable limiting of the damage caused by the pathogens to an economically-acceptable level (Fischer 2011; Koller et al. 1995). It is particularly important also to keep a constant low variation of the pathogens and

in this way to keep constant as long as possible the environment-related dynamics of the host-pathogen-relationship. As a result, mutations arising among the harmful organisms are respectively prevented or delayed, or develop straits which are able to overcome the resistance in culture varieties. Therefore, variety, environment and harmful organisms must be *managed* in resistance breeding and later on in cultivation. Even for specialists this requires a high degree of knowledge and considerable instinct (Baumgartner et al. 2015; Fischer and Fischer 2006; Kellerhals and Gessler 1995).

Up to now only a few wild *Malus* species have been used as sources of resistance traits. Distantly-related species like *M. hupehensis, M. sargentii*, *M. coronaria*, *M. fusca*, *M. florentina* and *M. trilobata* should be considered as new sources for future breeding for resistance. However, using these species brings along problems with fertility and crossability because of polyploidy and apomixis. A number of backcrossings in conventional breeding methods will be necessary to get excellent fruit quality. Biotechnological methods (marker-assisted selection, gene mapping, genetic engineering, work with double haploids (DH) are possibilities for the future to help integrate resistance genes directly into susceptible varieties (Baumgartner et al. 2016; Dunemann 2017; Emeriewen et al. 2014; Flachowsky et al. 2014). However, this work must be started with creation of a broad base of pyramiding genes for resistance to various diseases. It would be much easier to use the *M. sieversii* germplasm, which was collected from the American expeditions to Kazakhstan and is being now evaluated at the Geneva (USA), Havelook North (New Zealand) and the Dresden-Pillnitz (Germany) fruit gene banks. Resistance to scab and mildew has been found in progenies from different habitats. Up to now, the base of this resistance is unknown (Baumgartner et al. 2015; Büttner et al. 2000a; Geibel et al. 2000; Hatton 1954; Fischer and Fischer 2004, 2007; Fischer et al. 2005).

1.7 Cross-Pollination to Produce Interspecific Hybrids

Malus species and apple cultivars are usually diploid and have good crossability. Triploid forms are rare and not much suited for breeding. Technical problems at the time of crossing can be the blooming time, but it is solvable today.

In the apple breeding the aim is the combination of different kinds of resistance and good fruit quality in two directions: dessert cultivars and cultivars for processing. The best of these cultivars display resistance to scab (*Venturia inaequalis*), mildew (*Podosphaera leucotricha*), fire blight (*Erwinia amylovora*), bacterial canker (*Pseudomonas syringae*), red spider mite (*Panonychus ulmi*), winter frost and good fruit quality. Different scab resistance sources of wild species (V_f, V_r, V_A) were used to combine it in the new series of cultivars (Baumgartner et al. 2016; Büttner et al. 2004; Fischer and Fischer 1996, 2002, 2006; Kellerhals et al. 2009).

By utilization of all acquired evaluation data of *Malus* species and apple varieties, the following were used mostly as donors in cross-combination programs (Fischer 2011):

Fig. 1.1 Apple cultivars **a** Rewena, **b** Regia

As sources for high yield and fruit quality. At the first step: cvs. Cox Orange, Oldenburg, Baumann Renette, Hammerstein and others. At the following steps: Alkmene, Auralia, Clivia, Elstar, Golden Delicious, Helios, Jonathan, Pilot, Pinova, Piros, Undine and others.
As sources for scab resistance. At the first step: cv. Antonova kamienna (V_A). At the following steps: *Malus* x *floribunda* (V_f), *M.* x *micromalus* (V_m), *M.* x *atrosanguinea* (V_f ?), *M. pumila* (V_r), and the cvs. James Grieve, Cox Orange, Oldenburg and others.
As sources for mildew resistance. *Malus* x *zumi* cv. Calocarpa, *M.* x *robusta* cv. Persicifolia, *M.* x *floribunda, M.* x *micromalus*, and the cvs. Dülmener Rosen, James Grieve, Helios, Alkmene, Lord Lambourne, Worcester Parmain, among others.
As sources for fiber blight resistance. *Malus* x *robusta* cv. Persicifolia *M.* x *sublobata, M.* x *floribunda, M. prunifolia, M. fusca* (Emeriewen et al. 2014; Flachowsky et al. 2014), and a resistant clone from the Pillnitz breeding program Pi-A 44,14, and last also the cvs. Remo, Rewena, Reanda, Regia, Rekarda, Recolor and Rebella (Fischer 2011) (Fig. 1.1).

New sources for resistance breeding, especially for pyramiding resistance genes, were found in the Fruit Gene Bank Dresden-Pillnitz in some *Malus* species and old apple cultivars. However, if the V_f-gene is to overcome, new resistance sources and cultivars with two or more different sources of resistance will be required in the future to stabilize healthiness in the field. The evaluation of the *Malus* wild species in the Pillnitz Fruit Gene Bank showed that only 4% of all accessions had no symptoms of scab and mildew. Only a few of these are used as donors in breeding up to now, like some *Malus domestica* (scab, mildew), *M. baccata* (scab, mildew, winter frost), *M.* x *robusta* (scab, mildew, fire blight), *M. micromalus* (scab), *M.* x *zumi* (mildew) and *M. floribunda* (scab, fire blight). But the potential of wild species is much higher,

especially by using modern breeding methods. Furthermore the following species are carriers of resistance genes: *M. coronaria* (scab, mildew), *M. florentina* (scab), *M. fusca* (immune to fire blight), *M. hupehensis* (mildew, scab), *M. sargentii* (mildew, scab), *M. sylvestris* (mildew), *M. trilobata* (mildew, scab), *M.* x *halliana* (scab), *M. sieversii* (scab, mildew, fire blight in offsprings) (Büttner et al. 2000b).

To find donors of polygenic resistance to both apple scab and mildew, the assortment of apple cultivars in the Fruit Gene Bank remained untreated with fungicides for 2 years. It was found that 3.5% of 850 cultivars were not infected with scab or mildew. This result is important for the breeding of resistant apple cultivars with polygenic sources of resistance after the first results of overcoming monogenic scab resistance in apple cultivars. The most susceptible cultivars were found to be those grown all round the world, such as Delicious, Golden Delicious, Granny Smith, Gala, Rubinette and others. The group that showed both scab and mildew resistance includes: cvs. Bittenfelder Sämling, Börtlinger Weinapfel, Engelsberger Weinapfel, Gewürzluiken, Hibernal, Jacob Fischer, Kardinal Bea, Kirschweinling, Merton Prolific, Peasgoods Nonsuch, Rote Sternrenette and Spätblühender Taffetapfel. These cultivars can be regarded as carriers of polygenic scab and mildew resistance but crossing experiments are required to evaluate these assumptions. Of the scab resistant cultivars with V_f-resistance only Cidor, Juliane and Rebella were found to be without mildew infection (Fischer and Dunemann 2000). The results are listed in Table 1.1 for the cultivars with the best evaluation after 2 years without fungicide use, Table 1.2 presents the results of scab resistant cultivars. For nonresistant, noninfected cultivars a marker analysis gave no indication of the existence of the well-known genes for monogenic resistance to scab or mildew. These old and new cultivars are suitable for use in landscape improvement, for small orchards and home gardens, and as parents for transferring polygenic resistance in fruit breeding (Fischer and Fischer 2007; Kellerhals and Gessler 1995). Last but not least, Table 1.3 presents the results of apple breeding in Dresden-Pillnitz for cultivars with multiple resistances for professional production, landscaping and home gardening.

1.8 In Vitro Culture

Tissue culture is most often used to describe all types of aseptic plant culture procedures leading to plant growth. Tissue culture techniques can involve any of: (a) protoplasts, (b) callus, (c) tissues (apical or root meristems or pith segments), (d) organs, including buds or roots, (e) embryos and (f) plantlets (having both roots and stem/leaves) (De Filippis 2014).

Fruit species breeding based on biotechnological methods employs mainly embryo culture, regeneration from protoplasts, somatic hybridization, in vitro mutant selection, genetic transformation and haploid production (Germanà 2006).

These tissue culture protocols have been used in most of fruit trees with different objectives (Fig. 1.2) (Deberegh and Zimmerman 2002; Magyar-Tabori et al. 2010; Sedlak and Paprstein 2016; Xu et al. 2008), such as commercial propagation of elite

Table 1.1 Apple cultivars two years without using of fungicides—cultivars with the best evaluation, suitable for using in breeding new *vital* cultivars and for home gardening

Cultivars	Resistance level in two years				
	Mildew	Scab/leaf		Scab/fruit	
	1997	1999	1997	1999	1997
Altländer Pfannkuchen	2	5	2	1	1
Bellefleur krasnij	2	2	1.5	2	2
Bittenfelder Sämling	2	1	1	1	1
Börtlinger Weinapfel	2	2.5	1	1	1.5
Cidor	1.5	1	2	1	1
Dayton (V_f)	2	4	1	1	1
Discovery	2	3	2	1	1.5
Engelsberger Weinapfel	2	2	1	1	1
Engelshofer	2	1	1	1	1
Erbachhofer Mostapfel	2	2	1	1	1
Früher Victoria	1.5	3.5	2	1	1
Gewürzluiken	2	2	1	1	1
Golden resistent	2	2	2	1	1
Hagloe crab	2	2.5	1	1	1
Hibernal	2	1.7	1	1	1
Ilga	2	2	2	1	2
Jacob Fischer	2	3	2	2	1.5
Juliane	1	1	1.5	1	1
Kardinal Bea	2	2	2	2	1.5
Kirschweinling	2	2	1	1	1
Malus robusta 5 (Pl1)	1	1	1	1	1
Merton Prolofic	2	2	1	1	1
Peasgoods nonsuch	1	1	2	4	1
Präsident decour	2	1	1	1	1
Prinzenapfel	2	2	2	1	1
Rebella (V_f)	1	2	1	1	1
Remo (V_f)	3	3	1	1	1
Reglindis (V_A)	3	5	1	1	1
Rewena (V_f)	2	2	1	1	1
Riesenboiken	2	2	2	2	2
Rote Sternrenette	2	2	2	1	1
Spätblühender Taffetapfel	1.5	1.5	1	1	1

(continued)

Table 1.1 (continued)

Cultivars	Resistance level in two years				
	Mildew	Scab/leaf		Scab/fruit	
	1997	1999	1997	1999	1997
Gala	3.8	5	5	4.8	8.5
Golden delicious	4.8	5.3	8.8	8.3	8.8
Granny Smith	7.8	6.5	9	7.8	9
Idared	8.7	8	8.2	7.5	9

Evaluation: 1 = without infection, 9 = extreme susceptible
Source Fruit Genebank, Inst for Plant Gen and Crop Plant Research (IPK), Dorfplatz 2. O1326 Dresden—Pillnitz

rootstocks and cultivars (Sarwar and Skirvin 1997; Yepes and Aldwinckle 1994) production of stock for propagation of new obtentions from breeding programs; for genetic transformation (Krens et al. 2015); for production of haploids to overcome some problems related with apple long reproductive cycle, a long juvenile phase, and highly heterozygous (Höfer 2005); or eradication of viruses (Lizarraga et al. 2017; Paprstein et al. 2008); cryopreservation of genetic resources and development of synthetic seeds (Dobránszki and Teixeira da Silva 2010).

Mass micropropagation of different apple cultivars, mainly rootstocks, has been widespread in order to have healthy, uniform propagation materials in large quantities within a short time (Zimmerman 1991). For micropropagation, explants are surface sterilized, inoculated on culture establishment/initiation medium, multiplied on the medium mostly consisting of cytokinins and are subjected to rooting (Bhatti and Jha 2010). The success of micropropagation depends on various factors: (1) shoot tips or nodal shoot segments are most commonly used explants for shoot culturing in apple (Bhatti and Jha 2010); (2) BAP is the preferred cytokinin for apple-shoot multiplication; (3) other sugars than the most common sucrose can produce better caulogenic response, namely sorbitol in the rootstocks M9 and M26 (Yaseen et al. 2009); (4) rooting medium, mainly auxins, sugar, ethylene inhibitors and antioxidant content (see review of apple genotypes in Dobránszki and Teixeira da Silva 2010).

The interest of fruit breeders in haploids and doubled haploids (DH), lies in the possibility of shortening the time needed to produce homozygous lines compared to conventional breeding. With tree fruit crops, characterized by a long reproductive cycle, a high degree of heterozygosity, large size, and, sometimes, self-incompatibility, there is no way to obtain haploidization through conventional methods (Germanà 2006). Induction of embryogenesis and regeneration of pollen-derived plants from anther culture and induction of gynogenesis through unpollinated ovaries and ovules in *Malus domestica* (L.) Borkh, has been reported by several authors. Higher embryogenesis has been reported for microspore culture in the apple cvs. Remo, Rene and Realka than in anther culture although not correspondent in the regeneration phase (Höfer 2004, 2005).

Table 1.2 Apple cultivars two years without fungicides—scab resistant cultivars, suitable for organic production

Cultivars	Origin	Resistance level in two years				
		Mildew	Scab/leaf		Scab/fruit	
		1997	1997	1999	1997	1999
Gavin	CAN	6	6.3	1	1	1
Nova Easygro	CAN	3	4.5	1	1	1
Angold	CZ	6.3	6	1	1	1
Jolana	CZ	4.5	4	1	1	1
Melodie	CZ	8.5	5.5	1	1	1
Nela	CZ	2.5	1	1	1	1
Otava	CZ	6.5	5	1	1	1
Rayka	CZ	2.5	2.5	1	1	1
Resista	CZ	3.7	5	1	1	1
Rosana	CZ	5.5	5	1	1	1
Rubinola	CZ	2.5	2	1	1	1
Topaz	CZ	2	2	1	1	1
Vanda	CZ	6	6	1	1	1
Ahra	GER/Ahr	6.3	6	1	1	1
Ahrista	GER/Ahr	6	5.7	1	1	1
Reanda	GER/Pill	3	3	1	1	1
Rebella	GER/Pill	1	2	1	1	1
Reglindis	GER/Pill	3	4.7	1	1	1
Reka	GER/Pill	2.5	2	1	1	1
Releika	GER/Pill	4	4	1	1	1
Relinda	GER/Pill	2.5	3	1	1	1
Remo	GER/Pill	3	3	1	1	1
Remura	GER/Pill	3	3.5	1	1	1
Renora	GER/Pill	5	4.5	1	1	1
Resi	GER/Pill	5	7.5	1	1	1
Retina	GER/Pill	5.5	5	1	1	1
Rewena	GER/Pill	2	4	1	1	1
Baujade	FRA	4	4	1	1	1
Cidor	FRA	1.5	1	2	1	1
Florina	FRA	3.3	4	1	1	1
Judaine	FRA	2.5	3	1	1	1
Judeline	FRA	2.5	4		1	1
Juliane	FRA	1	1	1.5	1	1
Antonovka	RUS	5	4	1	1	1

(continued)

Table 1.2 (continued)

Cultivars	Origin	Resistance level in two years				
		Mildew	Scab/leaf		Scab/fruit	
		1997	1997	1999	1997	1999
Antonovka kamienna	RUS	3	4	1	1	1.5
Antonovka poltora fundova	RUS	2.5	2	1	1	1
Dayton (Coop 21)	USA	4.5	4	1	1	1
Enterprize (Coop 30)	USA	5.5	5	1	1	1
Freedom	USA	4	5.5	1	1	1
Gold Rush (Coop 38)	USA	9	6	1	1	1
Golden Resistent	USA	2	2	1.5	1	1
Jonafree (Coop 22)	USA	5.5	4	1	1	1
Liberty	USA	5	6.2	1	1	1
Macfree	USA	6	7	1	1	1
Priscilla	USA	5.3	5.3	1	1	1
Redfree (Coop 13)	USA	5	4.5	1	1	1
Sir Prize	USA	6	6.5	1	1	1

Evaluation: 1 = without infection, 9 = extreme susceptible
Source Fruit Genebank, Inst for Plant Gen and Crop Plant Research (IPK), Dorfplatz 2. O1326 Dresden—Pillnitz

Tissue culture is used in all present practical transformation procedures. Therefore, improvement in tissue culture systems is essential to achieve a more efficient gene transfer, selection and growth of selected transformant lines and so develop a more efficient transformation (De Filippis 2014). Moreover, often the ability to obtain fruit tree plants with new traits or mutations by genetic engineering or by new biotechnological tools depends on the existence of a well-established in vitro regeneration protocol, which depends on the genotype and the type of starting plant tissue used (Rai and Shekhawat 2014).

1.9 Rootstock Production

Rootstock has been used to graft apple cultivars since Roman times (Tubbs 1973). Dwarf apple trees were firstly referred in the UK as cv. Paradise in 1597, which were a mixture (Ferree and Carlson 1987). Extensive reviews on apple rootstocks can be found in Brown and Maloney (2005), Ferree and Carlson (1987), Masseron (1989), Webster and Wertheim (2003) and Wertheim (1998). Rootstock breeding has traditionally focused on dwarfing, hardiness, resistance to pathogens and pests, and better adaptability to soil conditions (Brown and Maloney 2005). Size control with

Table 1.3 Multiple resistance in the Pillnitz Re-cultivars® (Fischer 1999; Fischer et al. 2000)

Re-Sorte®	Scab	Source of resistance	Mildew	Fire blight	Bacterial cancer	Red spider mite	Frost damages of blossoms	Winter-frost
Reanda	X[a]	V_f	(X)	X	(O)	O	X	(O)
Rebella	(X)	V_f	X	(X)	X	X	X	X
Regine	(X)	V_f	(X)	(X)	(X)	X	X	X
Rekarda	(X)	V_f	(X)	#	X	X	(X)	O
Releika	(X)	V_f	(O)	(X)	X	X	X	O
Relinda	X	V_f	(X)	(O)	X	O	(X)	X
Remo	(X)	V_f	X	(X)	(O)	(O)	X	X
Rene[b]	(X)	V_f	O	X	(X)	O	X	(O)
Renora	X	V_f	(X)	(O)	(O)	(O)	(X)	(X)
Resi	(X)	V_f	O	(O)	X	O	X	O
Retina	(X)	V_f	(X)	(O)	(O)	(X)	X	O
Rewena	X	V_f	X	X	X	(O)	X	(O)
Recolor	X	$VA + V_f$	O	O	X	(X)	O	(X)
Realka[b]	X	V_r	O	X	(O)	(O)	O	(O)
Regia	X	V_r	X	X	(X)	(O)	(O)	X
Reka	X	V_r	(X)	(O)	X	O	O	(X)
Releta[b]	X	V_r	O	(O)	X	(O)	(O)	(O)
Remura[b]	X	V_r	(X)	(O)	(O)	O	(O)	X
Reglindis	X	V_A	(X)	(X)	(O)	X	X	X

Cultivars are suitable for organic production and home gardening, some cultivars very good for processing

[a]X = resistent, (X) = schwach resistent, (O) = schwach anfällig, O = anfällig

[b]no longer recommended

rootstocks of cv. M.27 is about 30–40% with respect to the maximum vigor provided by seedlings as rootstocks (Masseron 1989).

Some remarkable attempts to transform apple rootstocks were those on M26 by using *Agrobacterium rhizogenes* A4 (Lambert and Tepfer 1992), using plasmid binary vector plDB15 (Norelli et al. 1994), and using *A. rhizogenes* to control vigor (Holefors 1999); M6 using *A. tumefaciens* strain C58SZ707 (Norelli and Aldwinckle 1993); M7 using *A. tumefaciens* (Ko et al. 1998); Jork 9 using *A. tumefaciens* EHA101A and C58C1 (Sedira et al. 2001); Alnarp2 integrating the rolA gene to reduce vigor shortening the internodes (Zhu et al. 2001); and MM106 using *A. tumefaciens* strain C58C1 (Radchuk and Korkhovoy 2005).

Transgenic plants are limited in some countries by ethical constrains due to the fact that some of the traits introduced into a plant did not occur in the species (wild

Fig. 1.2 Laboratory for production of plants in vitro

and domesticated); therefore, cisgenic plants are considered the future in breeding, because in cisgenesis the introduced gene of interest previously existed as a native promoter in the domesticated or wild species for centuries (Jacobsen and Schouten 2008). Moreover, this technique allows inclusion of new traits to already-selected cultivars maintaining their excellent quality (Krens et al. 2015).

1.10 Germplasm Conservation

1.10.1 In Vitro Germplasm Conservation

Conservation of plant genetic resources prevents the loss of plant species with economic and ecological importance, namely vegetatively-propagated plants (Benelli et al. 2013).

In vitro conservation is currently mainly based on cryopreservation in liquid nitrogen (LN-196 °C) which ensures safe and cost-efficient long-term conservation of plant germplasm (Benson 2008; Engelmann 2012; Reed 2008). Apples have the advantage of being perennial woody species amenable to dormant bud cryopreservation techniques, and apple dormant bud methods have been successfully implemented. The National Center for Genetic Resources Preservation (NCGRPP, Fort

Collins, USA) has cryopreserved dormant buds of around 1900 accessions of apple. In the cryopreservation of dormant buds, branches or scions are harvested after mother plants have been exposed to a sufficient duration of cold temperatures. The optimum dehydration level of dormant buds is very important to achieve high recovery after cryopreservation (Benelli et al. 2013). Dehydration is usually performed at −5 °C for several days and in most cases up to moisture contents around 25–35% fresh weight. Before immersion in liquid nitrogen, samples are cooled slowly. In apple nodal sections, Towill et al. (2004) used a cooling rate of 1 °C/h from −5 to −30 °C.

There are some conditions for which the dormant bud cryopreservation method is not applicable, namely when buds do not reach a sufficient level of dormancy or apple material only exists in tissue culture (Li et al. 2015). Shoot tips excised from apical or axillary buds of in vitro-grown shoot cultures are the organs most commonly used in cryopreservation for vegetatively-propagated species (Lambardi and De Carlo 2003), and a good alternative to dormant buds. Indeed, in fruit tree cultivars shoot apex freeze-preservation in liquid nitrogen is the most suitable method to maintain viability and phenotypic and genetic stability, thereby ensuring long-term germplasm storage (Paul et al. 2000). Shoot tips are differentiated organs composed of organized tissues and thus not prone to somaclonal variation (Panis and Lambardi 2006).

The cryopreservation protocols established and most frequently employed in apple are encapsulation-dehydration, vitrification, encapsulation-vitrification and droplet-vitrification.

In the encapsulation-dehydration procedure shoot tips are coated in alginate gel, pregrown in liquid medium with sugar, partially desiccated in the air (current or laminar flow) or with silica gel before immersion in liquid nitrogen. Preculture on modified MS medium containing 0.75 M sucrose followed by 6 h of dehydration (21% residual water) led to the highest shoot regrowth of frozen, coated shoot tips of apple cv. Golden Delicious (Paul et al. 2000).

Vitrification involves treatment of shoot tips with cryoprotectants (usually glycerol and sucrose) followed by dehydration with highly concentrated vitrification solutions before cooling. Encapsulation-vitrification combines encapsulation-dehydration and vitrification, where shoot tips are encapsulated in calcium alginate beads and then treated with vitrification solutions. With the droplet-vitrification methodology, shoot tips treated with vitrification solution are placed on aluminum foil in minute droplets of vitrification solution which allows very high cooling rates and the direct contact between samples and the liquid nitrogen. In apple cv. Gala and 6 other genotypes of 4 different *Malus* species, Li et al. (2015) comparing the droplet-vitrification and encapsulation-dehydration procedures (Feng et al. 2013) and found that mean shoot regrow levels of the 7 genotypes were lower (48%) in the droplet-vitrification method than those (61%) in the encapsulation-dehydration procedure.

Several authors designate the encapsulation-dehydration technique for routine cryopreservation of apple germplasm (Li et al. 2015; Wu et al. 1999).

1.10.2 In Vivo Germplasm Conservation

A local cultivars is created at the moment when a farmer selectively propagates using plant material of the greatest interest. A very significant event had to correspond with initial propagation by grafting. In this sense, it is known that the Romans dominated and promoted grafting for the propagation of different varieties (unknown to us) in Roman times. Greeks and Romans spread the practice across Europe. During the Middle Ages, apple culture was promoted around monasteries. By the end of twelfth century, some famous cultivars were already named, such as Pearmain and Costard (Morgan and Richards 1993).

Most cultivars grown by farmers have been collected and conserved in germplasm collections. In Europe there are more than 30,000 accessions (IPGRI 1996), the majority held in ex situ vegetative field collections. Minimal data reported in each bank were collected in a European *Malus* database (Maggioni et al. 1997). Recently, most germplasm banks have been evaluated at the European level by using microsatellites (Ferreira et al. 2016; Lassois et al. 2016; Pereira-Lorenzo et al. 2017; Urrestarazu et al. 2016) in order to clarify the diversity, eliminate duplicates and to refine true-to-type characterization.

Information about apple genetic resources in the USA can be easily accessed (www.ars-grin.gov). A total of 4179 accessions are maintained in repositories; 1456 corresponding to *Malus* x *domestica*.

In Spain, major collections are located in Asturias, Galicia, Navarra, País Vasco y Zaragoza (Dapena 1996; Itoiz and Royo 2003; Pereira-Lorenzo et al. 2003, 2007). In those regions research programs are focused on local selection and breeding of ancient cultivars, which contain important resources for dessert and cider production.

1.11 Mutation Breeding

Fruit trees, including apples, have been considered attractive material from the viewpoint of mutation breeding because conventional breeding: (i) is time consuming, (ii) requires large space; and (iii) results are unpredictable due to their long juvenile phase and high degree of heterozygosity (Broertjes and van Harten 1988; van Harten 1998).

The most important apple-breeding objectives focus on: (i) fruit quality (firm, large and juicy fruits with good flavor and attractive colors without traces of russetting and good storage quality); (ii) pest and disease resistance; and (iii) shortening the juvenile phase and obtaining high yields in early years.

Natural apple mutants of major significant have been those of compact or spur-type growth, fruit color and time-to-fruit maturity (Spielgel-Roy 1990). Several bud sports displaying important characters have been described, for example skin color sports of several widely grown apple cultivars. The cv. Jonagored, for instance, is a spontaneous sport of the well-known cv. Jonagold, with much more intense early-red-

colored fruit, which currently is cultivated in dozens of countries (van Harten 1998). Apple ploidy chimeras are also known to occur leading to *giant* sports. Although the majority of cultivated apples are diploid, triploid cultivars arising from unreduced gametes and even tetraploid sports are known.

The increased impact of natural mutants provides incentives for mutation breeding. Only few induced mutants in fruit trees have been commercialized and are being planted on a large scale, contrary to the situation in ornamentals (Spiegel-Roy 1990). Currently, the FAO/IAEA Mutant Varieties Database (www.mvd.iaea.org) comprises 13 apple varieties. In this database a mutant variety is a new plant variety that is bred through: (i) direct use of a mutant line that is developed through physical and chemical mutagenesis, or somaclonal variation and activation of endogenous transposable elements; (ii) indirect use of a mutant line/lines, which is/are used as a parental variety/varieties in crossbreeding (crosses between mutant lines or with a commercial variety/varieties); and (iii) use of wild species' genes translocated into plant genomes through irradiation-facilitated translocations (www.mvd.iaea.org).

The first apple (*Malus pumila* Mill.) mutant variety, Mori-hou-fu 3A, was officially approved in 1963. It was developed in Japan by irradiation of scions with gamma rays (30 Gy) the main improved attribute being the mutant variety resistance to diseases. In 1970, 4 apple mutant varieties were officially approved 3 in France, and in Canada the mutant variety of McIntosh, McIntosh 8F-2-32, with improved skin color, was developed by irradiation of shoots with gamma rays. In 1995 the apple mutant variety, James Grieve Double Red, was approved, obtained by irradiation with gamma rays (62 Gy) and with the main improved attributes a good well-balanced taste, altered acids to sugars ratio, high yield (www.mvd.iaea.org).

From all new cultivars there arise new natural mutations; the difficulty is to identify them in the field. From the Dresden-Pillniz collection's cv. Pinova there exist nearly 12 mutations, 9 of them are in official testing, possibly 2 or 3 will be new protected cultivars in time (Fig. 1.3).

1.12 Genetic Transformation

The genome-wide sequence information and functional genomics of fruit trees have established the basis for crop genome engineering to improve fruit trees with unprecedented precision, maintaining most of the genetic identity of the main cultivars. Moreover, there is the recent technology CRISPR/Cas9, a molecular genetic tool used for genome edition and correction (Osakabe and Osakabe 2015). This technique is expected to be the method of choice for tree breeding in the near future. In comparison with traditional breeding, this technique can reduce the process, normally affected by the long juvenile period, gametophytic self-incompatibility (GSI), the high heterozygosity and the time consumed in phenotypic evaluation and lack of precision. Some commercial cultivars have been released in papaya and plum tree, genetically engineered for resistance to viral diseases (Scorza et al. 2013; Tripathi et al. 2007). But there are concerns about the higher cost of these new protected cul-

Fig. 1.3 One of the best mutations of the Pillnitz Pinova apple, the cultivar Evelina, presented at the Fruitlogistica in Berlin 2016 (Photo: Fischer)

tivars, along with environmental safety considerations such as outcrossing, because of the risk of transferring genetic traits and secondary and non-target adverse effects such as in proteins which are not known to display any toxic or allergenic properties. In apples, the first report using a single gRNA and Cas9 to produce a first-generation transgenic apple (Nishitani et al. 2016) by modifying an apple phytoene desaturase (PDS), which is required for chlorophyll biosynthesis and, therefore, transformed plants showed albino phenotype. It was the first reported genome editing in apple and in the Rosaceae. Genome editing was applied to the apple semi-dwarfing rootstock cultivar JM2 (*Malus prunifolia* (Wild.) Borkh. Seishi × *M. pumila* Mill. var. *paradisiaca* Schneid. M.9).

1.13 Conclusions and Prospects

Apple is an ancient worldwide crop in which new technologies such as genome-wide selection and genetic transformation are providing promising new cultivars combining high fruit quality with resistances to pests and diseases, and adapted to new climate conditions.

The recent molecular genetic tool CRISPR/Cas9 to edit and correct the genome will surely be the method for tree breeding in the near future, to overcome the long juvenile period, gametophytic self-incompatibility (GSI), the high heterozygosity and the time consumed in phenotypic evaluation and lack of precision of traditional breeding techniques. CRISPR/Cas9 is an extremely useful tool for further genetic studies of the genome of cultivated apples (Velasco et al. 2010) as it accelerates genome-wide association studies (GWAS), identifies SNPs and genes (Leforestier et al. 2015), and analyzes those genes which are differentially expressed (GDE) by using qRT-PCR and microarray analysis (Guitton et al. 2016).

All these new techniques will enable full use of the genetic diversity provided by nature; benefit growers and agronomists; and facilitate collecting, conserving and evaluating old selections, all of which will assure that apples continue to be one of the most desirable of fruits.

Appendix 1: Research Institutes and Online Resources

Julius-Kühn-Institut für Züchtungsforschung an Obst. D01326 Dresden-Pillnitz, Pillnitzer Platz 3a, Germany
https://www.julius-kuehn.de/dresden-pillnitz/
https://www.bundessortenamt.de

Appendix 2: Genetic Resources

www.deutsche-genbank-obst.de
https://www.julius-kuehn.de/zo/obstsorten-des-jki/
https://www.genres.de/kultur-und-wildpflanzen/erhaltung/deutsche-genbank-obst

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Chapter 2
Cherry Breeding: Sweet Cherry (*Prunus avium* L.) and Sour Cherry (*Prunus cerasus* L.)

Luca Dondini, Stefano Lugli and Silviero Sansavini

Abstract This chapter describes the cherry's origins, botanical classification (taxonomy) and domestication of the only two species cultivated for food and industrial processing: sweet and sour cherry. Cherry breeding programs worldwide have focused on trees, fruit quality traits and resistance to biotic and abiotic factors as well as on specific local characteristics. A detailed discussion of breeding techniques (crossbreeding, early selection, seedling screening and field assessment, embryo culture and mutagenesis) follows. A section is dedicated to sour cherry breeding, whose problems differ from those of the sweet cherry. We underline the importance of the knowledge and conservation of genetic resources for their use in genomic approaches. Then, discuss breeding strategies and the new traits that have been introduced in new genotypes through genomics. Germplasm biodiversity is analyzed in its phylogenetic context. Then, the molecular breeding approaches are extensively described with particular attention for gene mapping and the development of marker linked to monogenic and polygenic traits. A section is dedicated to the gametophytic incompatibility of the sweet cherry, with an updated summary of the research conducted to identify the 57 incompatibility groups (the cultivars for each of these are reported). Finally, we take into account other aspects related to breeding in respect to how functional genes affect some fruit characteristics, the strategies used after the cherry genome was sequenced and the potential of genetic engineering. The Appendix provides pomological profiles of 44 of the most important innovative

L. Dondini · S. Lugli · S. Sansavini (✉)
Dipartimento di Scienze e Tecnologie Agro-Alimentari, Università di Bologna,
v.le G. Fanin 46, 40127 Bologna, Italy
e-mail: silviero.sansavini@unibo.it

L. Dondini
e-mail: luca.dondini@unibo.it

S. Lugli
e-mail: stefano.lugli@unibo.it

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cultivars, according to the descriptive standard of the Brooks and Olmo official lists with each accompanied by original photos to aid in their identification.

Keywords Cherry taxonomy · Biodiversity · Fruit quality · Functional genomic Incompatibility · Molecular markers · Resistance · Sequencing

2.1 Origins

This species has a long history, as old as Western civilization. Cherry seeds have been found in many European sites dating back to 3–4 thousand years B.C., thus before the Greek and Roman civilizations. This is one of the reasons why the cherry is considered native to Europe (Faust and Surányi 1997), considered in its vast entirety without specifying between north and south; however, others (Iezzoni et al. 2017; Webster 1996) believe that the center of origin of the cherry can be located to the east, in Asia Minor somewhere between the Black and the Caspian seas. Afterwards, the species would have dispersed to the west (through the migration of people and birds). In any case, the first evidence of cultivated cherry orchards begins around the first millennium B.C. Overseas (USA), the species only arrived in the 1900s, although some authors believe it already was present around the mid 1500s (Jajasankar and Kappel 2011).

This migratory route was followed by the sour cherry (*Prunus cerasus* L., allotetraploid $2n = 4x = 32$), which, differing from the sweet cherry (*P. avium*, diploid $2n = 16$), is derived from natural hybridization between the sweet and ground cherry (*P. fruticosa* L.), which is indigenous to the same Caucasus area. Indeed, it seems that the sour cherry was repeatedly crossed with the ground cherry, given their intercompatibility (Olden and Nybom 1968).

For this reason, the sour cherry differentiated into a group of subspecies, as well as the Morellos (Griottes), Amarelles (Kentish) and the Italian Marasca (Hedrick 1915) cherries distinguished according fruit type and geographical distribution (Schuster et al. 2017). Ease of vegetative propagation (including suckering) made possible the development of various clones to be used as rootstock for the sweet cherry. The sweet cherry, instead, spread principally as a fruit species. However, since it is common in Europe, it also spread spontaneously as a wild species in natural woodlands; numerous ecotypes for lumber have also been selected, propagated and spread as forestry plants.

2.2 Taxonomy of the Cherry

In brief, both *Prunus avium* L. and *P. cerasus* L. taxonomically belong to the *Prunus* subg. *Cerasus* Pers. according to the classification of Rehder (1974); Section *Cerasus* Koehne. *P. fruticosa* Pall. (used as rootstock) also belongs to this subgenus as well

as the Chinese species *P. canescens* Bois (which is also diploid, like *P. avium* L.), used alone or as a hybrid, as a rootstock.

Three other important sections of the subgenus *Cerasus* are: *Mahaleb* Facke (to which the different species of European rootstock belong), *Pseudocerasus* Koehne (a Japanese ornamental flowering species) and *Microcerasus* Webb (to which other rootstocks belong, such as *Prunus tomentosa* Thunb.).

A second subgenus is made up of *Prunus* subg. *Padus* (Moench) Koehne of which only one alternative rootstock species is known (*P. padus* L.), aside from *P. serotina* Ehrh. and *P. virginiana* L. (ornamental species).

Cherry systematics is quite vast and complicated, so much so that many ornamental flowering species that do not fruit or bear small inedible fruits have proliferated in some Asian countries (e.g. Japan).

For bibliographic references, consult the works of Faust and Surányi (1997) and Iezzoni et al. (2017).

2.3 Evolution of Sweet Cherry Breeding

The sweet cherry (*Prunus avium* L.) is widespread throughout Europe, as well as in other areas of the world with a temperate climate where it is used for fresh consumption as well as for industrial uses. In contrast, the sour cherry is especially widespread in the Nordic countries, Eastern Europe and North America, generally used for the juice industry and in any case for transformation. Fresh consumption of sour cherries is quite limited, given the high level of organic acids, phenols and other bitter compounds from which the name *sour cherry* is derived.

Prunus avium L. is a very flexible fruit species, spread throughout the European latitudes, as much as in the countries farther north (due to the elevated adaptability to cold like no other species, except for the sour cherry and the apple) as much as in those of the south (due to its moderate need for winter cold and resistance to high summer temperatures, which are generally post-harvest). There is, however, a wide genetic variability that was under evaluated at the beginning of the twentieth century with the launching of the first public breeding programs. Actually, the first private seedling and selecting breeders only obtained slow albeit progressive improvements of the phenotype, aiming first of all at the adaptability of a single cultivar to each growing environment. An American cultivar of this series still grown today is Bing, obtained privately in Oregon in 1875 as well as two other historic cultivars of indeterminate origin such as the Turkish Ziraat and the very early Burlat, both also quite appreciated in contemporary markets.

The market classification of the cherry up until the mid-1900s was quite simple, there was a subdivision between soft flesh cherries and firm cherries, then yellow cherries with clear juice and red-black cherries with red or burgundy juice; a distinction was also made between short- and long-stemmed cherries. In any case, the variability of cultivar assets has always been large from one zone to another.

However, the international market and the growing affirmation of consumer-grade cherries has induced both the public institutions and the same passionate cherry growers. This is especially the case in countries that instituted patent rights on cultivars through the International Union for the Protection of New Varieties of Plants (UPOV) during the post-WWII period to launch specific programs aiming to form a foundation of characters common to the most popular cultivars. The characters are high and constant productivity, elevated qualitative characteristics, greater size, firm flesh, excellent gustatory characteristics, with a balanced sugar/acid ratio, environmental adaptability. The first public breeding program of INRA-Bordeaux (1965–1980), for example, was concluded without releasing any new cultivars because only French germplasm was used when choosing the parents, providing too narrow a genetic base (Quero-García et al. 2017a).

In the last 50–60 years, numerous breeding programs (Table 2.1) have been developed throughout the world, with spectacular results. Kappel (2008) and Sansavini and Lugli (2008) have described most of them at the Fifth International Cherry Symposium, held in Bursa, Turkey in 2005, with aims, purposes and achievements pursued throughout the world: a surprising revolution that has led to the re-modernization of the cultivar assets in various countries. Kappel (2008) referred to 7 breeding programs in the USA, Canada and Australia, while Sansavini and Lugli (2008) conducted an investigation in 27 European and Asian research centers to analyze the situation and deduce indications of current tendencies. Regarding the breeding methods followed, only 62% of the new cultivars came from controlled crosses, 17% from selected seedlings derived from open-pollinated parents, 16% from the clonal selections of cultivars that are already the object of cultivation and 4% from induced mutagenesis with the successive selection of the mutants.

2.4 Breeding Objectives

The release rate of new cultivars was quite high up until the decade of 2000, even if exclusively limited to the innovations made public (see Appendix and the Brooks and Olmo listings), because between 1990 and 2005 the annual average exceeded 10–15 new cultivars, but only a few were patented; these requirements were such as to widen the area of adaptability of each cultivar, taking advantage of the heterozygosity of the different characters as well as the introduction of genes studied and taken from new cultivars or recovered from historic germplasm; cherry species, in fact, are distributed and conserved in collections established in public research centers in many nations, with the aim of exploring the genetic potentiality of single cultivars, even those no longer grown. The interspecific variability is enormous; it suffices to cite one fact: for the single incompatibility locus, there are more than another 50 allelic groups (Table 2.4).

This is the case, for example, of cv. Stella, bearer of the self-fertile mutation of the S gene responsible for gametophytic self-incompatibility (GSI). Thus, in the group of programs encompassed by our investigation, aside from that of self-fertilization,

Table 2.1 Public and private sweet cherry breeding programs

Regions and Institutes	City	Country
North America		
Agriculture and Agri-Food Canada	Summerland	Canada
Washington State University	Prosser	WS USA
Cornell University	Geneva	NY USA
Zaiger's Inc Genetics*	Modesto	CA USA
Bradford Farms & BQ Genetics*	Le Grand	CA USA
South America		
Politecnica Universidad Catolica	Valparaiso	Chile
Instituto Nacional de Investigaciones Agropecuarias & Biofrutales	Santiago	Chile
Europe		
Institute National de la Recherche Agronomique & CEP Innovation	Bordeaux	France
Research and Breeding Institute of Pomology	Holovousy	Czech Republic
Julius Kuhn Institut—Institute for Breeding Research on Fruit Crops	Ahrensburg Dresden	Germany
University of Bologna, Department of Agricultural Sciences	Bologna	Italy
National Agricultural Research and Innovation Centre, Fruitculture Research Institute	Budapest	Hungary
Research Institute for Fruit Growing	Bistrita, Pitesti Jasi	Romania
Fruit Growing Institute	Plovdiv	Bulgaria
East Malling Research	East Malling	UK
Institute of Horticulture of National Academy of Agrarian Sciences	Kiev	Ukraine
Investigaciones Cientificas y Tecnologicas de Extremadura	Jerte Valley	Spain
Murcia Institute of Agri-Food Research and Development	Murcia	Spain
Asia		
Ataturk Horticultural Central Research Institute	Ataturk	Turkey
Horticultural Experiment Station, Yamagata Integrated Agricultural Research Center	Yamagata	Japan
Dalian Academy of Agricultural Sciences, Institute of Pomology	Dalian	China
Chinese Academy of Agricultural Sciences, Fruit Research Institute	Zhengzhou	China
Beijing Academy of Agricultural and Forest Sciences, Institute of Pomology Institute of Pomology an Forestry	Beijing	China

*Private companies

there were always three characteristics improving the quality of the fruit: size, firmness and flavor. However, one-half of the programs included self-fertilization or an increase in specific fertility (fruit set), the resistance to susceptibility to adverse environmental or climactic events (e.g. resistance to cracking, and to disease in post bloom and preharvest time), and also the extension of the seasonality of the harvest with new early- and late-ripening cultivars, to increase the harvesting and consumption calendar by up to 3 months.

The benefits were often resounding, obtained with innovations that were then introduced and spread through international nursery channels; for example, the average weight of the cherry fruit went from 6–8 to 12–14 g. Also, there were drastic changes in the market image of the cherry; a strong and decisive aid to this renewal was provided by the combination of new cultivars with new types of orchard layouts, essentially the introduction of dwarfing rootstock together with a high planting density as well as breeding forms suitable to obtaining short-structured and high-fruiting trees, and the use of anti-rain and anti-hail protective nets.

In all of these innovations, varietal evolution has played a decisive role that has not yet been exhausted; it continues to play an increasing and preponderant role because, thanks to increased genetic knowledge, the most recent being the sequencing of the genome. The new objectives can now be achieved by more targeted breeding, in a much shorter time period and with lower costs.

Today, breeding can be best improved with the support of genetic analyses conducted with markers; first iso-enzymatic ones were used; now only molecular ones. Over the period of a few years, the transition was made from RAPD and AFLP, equipped with a low level of polymorphism (Boskovic and Tobutt 1998; Wünsch and Hormaza 2002) to very precise markers such as SSR, SNP and the same sequences of the functional genes (Iezzoni et al. 2017).

In the past, in doing crosses, too few cultivars were used that were also too similar to one another, since they were in part derived from common parental lines, e.g. only five varieties were used in the current four American programs (Choi and Kappel 2004). Now, on the whole the situation is quite changed, thanks to the availability of a genetic pool that lets us greatly enlarge the gene flow of the rather recent past. An analysis of the European germplasm has shown approximately two distinct genetic areas corresponding to the two great distribution areas of northern and southern Europe. Another significant example was given by the self-fertility of the Stella cv. that, because it was used in almost all of the programs, made it possible to widen the allelic base of the GSI character. However, recently, self-fertilization was discovered to be present even in other autochthonous genotypes (landrace selections) such as in the Spanish Cristobalina cv. (Cachi and Wünsch 2011), in the Italian Kronio cv. (Marchese et al. 2007) and in the cv. Axel in Hungary, which makes it possible to increase the potentiality of the self-fertilizing character, reducing the number of inter-incompatibility groups (Kappel et al. 2012).

During the last two to three decades, other phenotypic characters have been compared and genetically studied for improvement. These include susceptibility to cracking, with the relative resistance index or tolerance and the parental lines on which to orient the programmed crosses having been reasonably recognized: these are cul-

tivars whose susceptibility to cracking should not exceed 10–15% of the fruit mass (individually immerging them in water). This was possible thanks to the experimental tests conducted by research stations that had extensive cherry varietal collections, such as those in Denmark (Christensen 1972) and Norway (Meland et al. 2014). Two cultivars with a low susceptibility to cracking are Regina and Fermina. Because, in any case, it is impossible to have resistant cultivars, nets or non-woven protective fabrics are used to prevent crop loss and avoid damage by cracking near the time of harvest (in fact, it is not worthwhile to pick the fruits when the damage affects more than 20–40% of them. Hand grading fruits in the field or in packing house would cost too much).

Another important aspect of breeding directed towards the prevention of environmental adversity damage is that of resistance to winter frost. This objective was addressed in programs in Latvia, Russia, Ukraine and other countries with frequent winter risks of this kind. We refer here to Quero-García et al. (2017b), who reported that in Germany a study by Fischer and Hohfeld, in Pillnitz-Dresden, reported the severity of the damage in a collection of 11 cultivars of sweet cherries, indicating that the least affected (cold hardy) were the Black Agle, Kristin, Hudson, Vic and Windsor cvs.

In Latvia, the German Donnissens Gelbe cv. was used successfully, as in Russia, to obtain cultivars with relatively large fruits (in any case, not as small as in the native landrace plants). These cultivars include Aiya, the Russian Radica and others.

On the other hand, in southern countries with mild winters, climatic conditions thus diametrically opposite, most varieties cannot be cultivated; it is necessary to thoroughly explore the germplasm potential. Already, attempts have been made in North Africa, Andalusia, California and Central Chile that seem successful or promising, with the aim of identifying cultivars adapted to Mediterranean or subtropical climates. Furthermore, current cherry cultivation in these areas is almost always based on cultivars that have been selected in the north, including Lapins, Brooks and Rainier and the Spanish cv. Cristobalina, as well as the Tunisian Bonjargoub. Also, there are other genotypes with extremely early blooms within the widespread cultivars, which belong to the *low-chilling* cultivar group.

In California, the private company, Zaiger's Inc Genetics, has launched cultivars of low-chilling cherries such as Royal Hazel and Royal Marie which are already commercially available.

Regarding resistance or tolerance to disease, in the past progress made through breeding was rather limited because the discovery of resistance genes is very difficult without the help of genomics. For example, in the areas where the cherry was attacked by the bacterial canker *Pseudomonas*, such as in Russia and in Canada, one can refer to the various experiences cited by Kappel et al. (2012) and the experiences of Krzesinska and Azarenko (1992) using various inoculation methodologies, as well as those of the John Innes Institute (UK) that licensed three cherry cultivars (Merla, Mermat and Merpet) that are totally resistant to *P. syringae* pv *morsprunorum* (Matthews and Dow 1978, 1983), but that are of little commercial interest.

Against the desiccation of apical shoots and fruit rot caused by *Monilinia* spp. (in humid areas with heavy rainfall), many researchers have worked from the pre-WWII

period to the present without identifying plants with resistance genes (Kappel and Shalberg 2008); however, still citing Jajasankar and Kappel (2011), some cultivars are tolerant enough of brown rot, the mold that can cause total crop loss in extreme cases (wet or rainy areas). Regina cv. has a certain tolerance, and is quite appreciated in markets throughout Europe, as well as the Early Korvik and Valery Chkalov cvs.

Finally, regarding insects, the fruit fly *Rhagoletis* spp. is still the most widespread and dangerous insect for the late-cherry cultivars, even if the attacks vary greatly from year to year. It is followed by the black aphid (*Myzus cerasi* Fabr.) and, most recently, a fruit midge (*Drosophila suzukii* Mats.), which has become uncontrollable for some years, quickly becoming universally widespread and damaging and still lacking natural enemies. At least in Italy, this has become the most dangerous pest regarding fruit damage. Chemical control is quite difficult and there are no genetically-resistant cultivars. Even in this case, then, indirect defense methods are being tested with tightly woven net coverings. Programs of genetic improvement are currently still far from offering solutions. To obtain truly useful results, research should be directed at the resistances present in ornamental species. However, it is unknown how many generations of re-crossings will be necessary if it is not possible to resort to bio-engineering (GMO) methods or to new biotechnological crops not presently allowed into Europe.

Currently *Drosophila* fruit flies attack affect all fruit species, except for rare cases. Nevertheless, it is always possible to find ranges of natural genetic preferentiality in the host plants of various insect species, which almost never correspond to the resistance range of the different genotypes.

An agronomic characteristic of past cherry cultivars that contributes to making harvesting difficult and costly is the detachment of the peduncle. Yields of 15/20 kg/h by manual harvesting of traditional cultivars with medium-small fruits are very low and can be doubled with new large-fruited cultivars. Cherries, unlike other stone fruits, have a peduncle that does not detach when the fruit ripens. Breeding has provided an alternative solution for the market, which bypasses the lack of abscission of the peduncle from the branch, through the easier and more natural detachment of the drupe from the peduncle when the fruit is mature.

Bargioni (1996), an Italian breeder, developed a new cultivar (Vittoria) with a new characteristic, ease of the fruit falling at maturity. It was sufficient to lay out net platforms or have a harvester equipped with a trunk vibrator and two independent wings to catch the cherries, and carry them on a conveyor belt to a collection and transport bin, filled or not with water.

The success of this new system, which requires consumer acceptance of stemless cherries, was rather modest or absent in Italy, while it was applied with greater success in Spain (Serradilla et al. 2014), with markets more open to the consumption of stemless cherries created and selected for this use. See the cvs. Picota series (Ambrunes, Pico Negro, Pico Colorado; Quero-García et al. 2017b).

2.5 Genetic Improvement Techniques: Crossing and Selection

The most common technique to genetically improve sweet cherry is intra-species crossing; it is the ideal method to exploit the combinative capacity that regulates the transmission of polygenic quantitative characters. If floral compatibility exists between two genotypes, the technique involves taking pollen from a genotype to fertilize the ovule (egg cell) of the other genotype. If genotypes are self-compatible, to investigate the genetic basis of specific characters or to obtain the segregation of some of these, self-pollination can be used, which involves self-fertilization. In some breeding programs, backcrossing is also used, i.e. crossing F1 genotypes with one of the two parents, generally the one with greater potential. Interspecies crossing, or more properly hybridization, is often used for the cherry, although exclusively for the genetic improvement of rootstock; it has also been used for the qualitative improvement of sour cherries, crossing the sour cherry (*P. cerasus* L.) with the sweet cherry (*P. avium* L.), not easily done due to ploidy differences.

In general, a cherry breeding program requires 12–20 years, depending on what phases have been chosen for the selection process and the techniques used to carry them out. The normally-applied model involves 4 steps: parental choice, carrying out the crossing and raising the seedlings to young plantlets (2 years); planting the seedlings and their evaluation in the field (S1 state ± 8 years); propagation by grafting the seedlings selected in the previous phase and their evaluation (S2 ± 6 years); propagation by grafting the selected genotypes in the two previous phases and their final evaluation (S3 ± 6 years). In some breeding programs, such as that of the University of Bologna, a very strict screening was applied during the S1 phase, to select very few seedlings which go directly to S3, thereby reducing the length of the selection process by at least one-third. If all goes well, this should all lead to the diffusion of a new plant variety that can be patented. In other programs (e.g. INRA in France and Biofrut in Chile), molecular selection techniques have been introduced with the help of SSR markers to shorten the cycles and to also make the selection process more targeted and efficient (marker assisted selection).

Sections 2.5 and 2.6 describe the direct experience of the cherry breeders who are active at Bologna University. For further details related to these activities, refer to the works by Bassi et al. (2012), Iezzoni et al. (2017) and Kappel et al. (2012).

2.5.1 Parental Selection

Once the general and specific objectives of a breeding program have been identified, the next step is choosing the parent plants to be crossed. This choice requires thorough knowledge of the qualitative and quantitative characters of the cultivar and of the chosen selections as potential parents and, when possible, a knowledge of the capacity of the individual parents to transmit their own characters to their progeny, both those

controlled by a single gene (e.g. self-fertility) as well as those that are polygenic and quantitative, which are the majority.

When choosing parents, most of all for organizational and logistic reasons, some general criteria must be taken into consideration regarding their phenological stages, floral genesis and productive potential, and some aspects of the floral biology of the parents. In the first place, where possible and starting from other considerations, the choice of parents to use as seed carriers should fall on the maternal parent with a later bloom time than that of the paternal parent; this is to make the preparation of the pollen to use for the cross more simple and efficient. Regarding the maturation period of the fruit, if the primary objective is not the creation of early and very-early cultivars, the parental choice to use as mother plant should fall towards genotypes with a medium-late or late maturation, this is to increase the probability that the hybrid seed embryo will be as developed as possible. If, instead, you are working with two parents with a contemporary flowering time, a useful technique is to anticipate the collection and preparation of the pollen the year before and then store it at a low temperature. The hardiness of the tree together with its floral aptitude and productive potential are also important criteria to consider when choosing female or male parents. For example, genotypes with a low-bearing capacity should be chosen as the male parent, or possibly avoided altogether. Another important aspect to be assessed before choosing the parents concerns the characters linked to the incompatibility groups that characterize the cherry genotypes (alleles of self-incompatibility).

2.5.2 Preparation of Pollen and Mother Plant Inflorescences

Pollen should be harvested from flowers that have not yet opened, at the white button or visible corolla phenological stage. Anthers are removed by hand with scissors or by rubbing the flowers on a suitably-gaged metal mesh and collecting on a tray. Subsequently, the stamen filaments or other flower parts are removed and the anthers are exposed to air in a controlled environment at a temperature of at least 20–22 °C for a maximum period of 24–36 h so that they dehydrate. It is best not to filter the mature pollen from the anther residues when placing it in the test tube, to prevent excessive compaction. During the pollination period, the pollen must be stored in test tubes under refrigeration at a temperature of 5–10 °C. If the pollen is also to be used in subsequent years, it can be stored after dehydration in liquid nitrogen or at a temperature of −80 °C. In general, it is good practice to check germination before beginning pollination by placing the pollen on an artificial liquid substrate containing 15–20% sucrose and 10 ppm of boron and to check the germination percentage after a few days. It should exceed at least 60–70% of the granules; if it is too low there is a risk of failure.

The preparation of a plant bearing self-incompatible seeds requires the isolation of the flowers before anthesis by covering the branchlets with small paper or cloth bags. For self-compatible cultivars, the corolla must be removed in order to avoid visits from pollinators, while the anthers must be removed before their dehiscence (emas-

culation). The branchlets carrying emasculated flowers do not need to be bagged. The elimination of the male flower structure is also currently advised for seed-bearing plants of self-incompatible cultivars so that possible contamination of the maternal pollen with paternal pollen can be avoided and the efficiency of the pollination can be increased.

The best stage to carry out pollination generally coincides with full bloom. The functional pollination period of the cherry tree, although slightly variable according to the annual climate conditions, is 24–48 h after emasculation and in any case not over the 24 h after full anthesis to coincide with the receptivity of the stigma, germination of the pollen granules and the pollen tubes arrival in the still-fertile ovule. Pollen can be applied to the stigma with a brush or fingertip.

Instead of isolating the single portions of the plant, it is possible to isolate the entire tree by caging it in a structure covered in nets or plastic film to impede external contact; a surreptitious method is that of inserting branches or blooming branchlets from the paternal parent into the cage at the right moment, as long as they are soaking in a container with water. In this case, pollination must be ensured by using bumblebees as pollinators. Another variation of this technique is the cultivation within a controlled environment (greenhouse or tunnel) of multiple plants raised in containers holding two cultivars that are self-incompatible and non-fertile cultivars together (this means they are from different allelic groups). In this way, it is possible to harvest the fruits of both cultivars since they are progeny obtained through identical and reciprocal crosses (A × B and B × A). Other solutions adopted in some breeding programs is to annually obtain large quantities of fruits and seeds derived from controlled crosses consisting of the preparation of actual cherry orchards with two or more non-self-fertile cultivars, planted in completely isolated areas outdoors far from other cherry orchards. Finally, it is possible to collect fruits from commercial plants made up of valuable cultivars to be used as the offspring in open pollinations. In this case fruits are simply harvested and the seeds extracted; the seedlings will be classified as OP, i.e. derived from open pollination of a chosen cultivar.

2.5.3 Fruit Harvest and Seed Planting

To guarantee the highest seed germination percentage, the fruits from which they are extracted must be collected at full maturity. However, studies have demonstrated that it is also possible to obtain good seed germination rates in some genotypes by harvesting the fruit not more than 10 days before physiological maturation. Since the cherry is an easily fermentable drupe; in order to avoid the development of mold or fungi affecting the viability of the embryo, it is important to manually or mechanically extract the seed immediately after harvest. After cleaning the seeds of pulp residue, they are sterilized by immerging them in a diluted solution of chlorine and bleach for a few minutes or as an alternative they can be treated by immerging them in a fungicide solution for 1 h. In either cases, afterward the seeds are carefully rinsed

with distilled water. During this phase, seeds without embryos and those that are not completely formed (which normally float) are culled.

Mature harvested sweet cherry seeds undergo a physiological and mechanical dormancy making immediate germination impossible. All parts of the seed (endocarp, endosperm, embryo) contribute to the dormancy. The seed will only germinate if the endocarp (or pit) is broken or removed and only after the embryo undergoes a physiological cold treatment (stratification). The seeds are stratified in refrigerated cells at −1 °C in sealed containers such as plastic bags or Petri dishes with bibula tissue paper that has been slightly moistened at the base with an anti-cryptogamic solution. For sweet cherry, the duration of this chilled storage varies from 2 to 3 months. Subsequently, the seeds are placed in air-conditioned rooms (e.g. greenhouses or phytotrons) to germinate in special seed boxes and then transplanted into small specially-shaped containers.

2.5.4 Embryo Culture

Cherry embryo culture is necessary when it is impossible to harvest mature seeds, due to an interruption in development, i.e. when the embryo inside the pit is aborted. In fact, very-early and early-maturing cultivars such as Burlat demonstrate insufficiency of embryonic growth and consequently the seeds will have a very low germination percentage. In these cases, the seed is extracted a few weeks before the fruit reaches full physiological ripeness, normally in the phenological period known as *veraison* (fruit changes color as it begins to ripen). In aseptic conditions under a hood, the extracted seed is deprived of its integuments and placed on an artificial agar-nutrient substrate to allow full embryo development and germination into a whole plantlet and acclimatization in the greenhouse.

2.5.5 Seedling Raising

The vegetative development and the survival rate of plantlets are optimized if they are not removed from stratification until the rootlet reaches a length of 0.5–1 cm. Because in this phase the young plantlets grow rapidly, they are transplanted within a week with a covering layer of a soil/vermiculite mixture in standard alveolar trays 6 cm in diameter and 25 cm deep. In this way, the root growth space is maximized and the plants are not too crowded, thus avoiding excess humidity that can quickly kill them. At the end of this phase (2–3 months), the plants are repotted into larger containers, normally a 9–12 cm caliber pot to then be transplanted at the end of the season into a nursery or directly outdoors. In general, seedlings can be moved outdoors by the end of the first year, when they will have sufficiently hardened off and the necessary lignification will have occurred. The planting density in the orchard plot is 1000–3000 trees per ha. Seedlings must receive intense care including

irrigation, fertilization, and preparatory pruning to favor rapid physiological maturity, to facilitate the necessary phase in which to begin the selection process.

2.6 Selection Phases

2.6.1 S1 Phase

The S1 phase is the evaluation of individual seedlings from the first propagation (which is also the first and only generation). According to the genotype, normally 3–5 years must pass before a cherry tree reaches the appropriate age to best manifest the characters of interest for the selection process. Once the juvenile phase has passed, with the aid of an appropriate parametric evaluation form, observations are made for at least 20 years on various characters, especially those that only appear when the sapling has passes the juvenile phase, i.e. a few years: phenology (e.g. bloom and ripeness period), morphology (vigor and growth habit), agronomy (fruiting habit and productivity) and pomology (size and shape of the fruit, skin color, flesh firmness, quality taste). Possible tolerances or sensibilities to biotic (e.g. pests and pathogens) or abiotic adversities (e.g. cracking of fruits or frost on flowers) will also be evaluated during this selection phase.

If we consider that the duration of the standard S1 process is not less than 6–8 years and that the selection can lead, at best, to a positive result of around 3–5% of the initial genotypes number (the remaining plants will eventually be culled), it would be necessary to start with the following prerequisites to set up an efficient and economically-sustainable breeding project: having an initial number of seedlings/year of no fewer than 1–2 thousand individuals; using populations of at least 150–200 individuals per crossing; reducing the selective screening time by using certain characteristics such as self-fertility, early assisted selection (conventional or molecular) and process-acceleration techniques to shorten the unproductive period of the plant. For example, various techniques that help cancel or shorten the seedlings' juvenile period include interruption of development by grafting or micrografting the seedlings onto dwarfing subjects (those rootstocks that generally reduce the unproductive period), the use of growth-retarding chemicals and not pruning or lightly pruning the plants during the first years.

2.6.2 S2 Phase

Phase S2 is the key stage in the selective process. The objectives and formulation of this phase are in many ways similar to what happens in the clonal and sanitary selection of other fruit tree species through agamic propagation, for example in grape vines. The operational procedure is as follows: the seedlings preselected in

the S1 phase are multiplied into a limited number of specimens and grafted onto one or more rootstocks, sometimes with repetitions. Usually, only a single vigorous rootstock is used, which can sometimes be coupled with a second dwarfing rootstock so as to allow the successive evaluation of the seedling according to two distinct selective processes: one that is accelerated, and one that follows the normal cycle. The pedoclimatic environment is also a determining factor in the correct and complete evaluation of the selections. In fact, it is not unusual that the experimental fields are sited in different locations so as to also evaluate the environmental adaptability of the new selections and possible genotype-environment interactions. In order to complete a homogenous and comparative evaluation in S2 fields, the trees of the selected genotype are compared to those of the most widespread commercial cultivar grown under the same conditions. A very important aspect during the passage of the genotypes from S1 to S2 regards the verification of their health, which can take place separately in a quarantined isolated environment where visual observations and diagnostic tests on the quarantined organisms and viral and bacterial agents can be carried out.

First of all, the verification conducted in S2 regards the genetic correspondence of the S1 propagated material: (i) phenotypic and comparative evaluation of the productive and pomological agronomic performance, (ii) fruit qualitative evaluation that also uses molecular analysis, (iii) verification of some characters that were not previously investigated, such as self-fertility or self-incompatibility (GSI) of the genotype. Instead, S3 goes on to carry out sensorial evaluations of qualitative fruit traits, the storage and shelf-life capability and their aptitude for possible technological transformation processes (for example, if somatic variations of an unspecified, epigenetic origin are suspected).

2.6.3 S3 Phase

Once the screening has been completed in S2, the genotypes become more interesting and exhibit valuable potential attributes that would make them superior to common commercially available cutivars. They are newly propagated to populate the level III selection fields or for prelicensing. Phase S3 evaluation is usually expanded to include various geographic areas that are climactically different through agreements made between public or private institutions in those regions or countries most suited to cherry cultivation.

In this phase, the activity normally carried out by a single breeder is now accompanied, integrated and sometimes substituted by the activity of other external subjects, figures and professional structures that are able to evaluate if the new genotypes possess the characteristics, and the commercial properties necessary to become a successfully marketed cultivars. At the same time, S3 genotypes are agronomically evaluated, under edaphic conditions that are very different from one another but with the same conditions and cultivation techniques of commercial orchards. At the same time, the aptitude of the fruits for modern processing and packaging systems can also

be used (e.g. cold chain, mechanical or electronic calibration, storability and shelf life) as well as the aptitude for other possible industrial uses.

Furthermore, the commercial potential of S3 specimens is evaluated through expert panels and consumer appreciation tests. If the genotypes confirm or exceed the initial expectations, then the new cultivar is licensed, but not before conducting a careful analysis of the development of the new cultivar through a combination of activities that, beginning with a market analysis, conclude with the nursery production and commercialization of the new product. Phase S3 requires 5–6 years to complete. Naturally, it is taken for granted that the national registration of the cultivar is completed, so as to make nursery multiplication possible, and, if propagation rights are desired, to the relative patent request in their home country as well as those where the new cultivar may be distributed.

2.6.4 Clonal Selection

Clonal selection as a breeding method is used in cherries to identify improved clones within the cultivar mostly belonging to local germplasm. With this technique, it is possible to identify those variants (mutant or sport) of tree or fruit behavior that are not easy to identify even though they are quite important, because they are able to influence complex characters such as productivity or tree vigor. It is no coincidence that clonal selection has become a fundamental instrument in the genetic improvement of rootstocks and also of the same cultivar, when the species should naturally be prone to mutations that might prove useful and thus will be *fixed* in a commercially-propagated clone that can also be patented if it is distinguishable in a uniform and permanent manner with respect to the mother variety (see the case of Pandy cv. sour cherry in Hungary; Schuster et al. 2017).

2.6.5 Mutagenesis

Mutagenesis induced by the application of physical agents has been used as a method of genetic improvement of the cherry in various programs in Canada (Webster 1996 and Kappel 2008) and Italy (Sansavini and Lugli 1996). The physical agents used were ionizing radiation such as ^{60}Co and gamma and X-rays. The principal objectives of these techniques are to obtain mutant clones and a compact growth habit (reduction of internode length) and/or spurs (greater density of flowering spurs). Furthermore, the application of physical agents allowed researchers to achieve floral self-compatibility of the cherry by de-activating the S locus (see the origin of cv. Stella). Further details are available at the Joint FAO/IAEA Mutant Variety Database (https://mvd.iaea.org/#!Home) where 15 officially released sweet cherry mutants are reported.

Limitations to mutagenesis include the appearance of recessive characters (minor fruiting, reduced tree vitality, reduced quality), regression of the compact phenotype towards the standard, low frequency of the positive mutations, long time period and elevated costs. In fact, the introduction of the dwarfing rootstocks to cherry cultivation has made this technique obsolete.

2.7 Sour Cherry Breeding

Prunus cerasus L. is a fruit species that extends up into the higher latitudes, thanks to its high resistance to cold. Thus, it is natural that the Nordic countries have developed sour cherry cultivation and have conducted genetic improvement prioritizing its resistance to winter temperatures below minus 20–30 °C.

Breeding programs were initiated during the first decades of the last century, and the countries most involved were Russia (then the Soviet Union), Ukraine, Belarus, Germany, Denmark, Poland, Hungary; followed by North America (Canada), with programs initiated in the 1940s (Schuster et al. 2017).

With respect to winter-frost resistance, there is a strong variability within *Prunus cerasus*, so much so that in many countries populations of local cultivars with noteworthy resistance to cold have been present since time immemorial (with clonal selection work having been carried out for decades in various countries, especially in Eastern Europe) and also due to the coexistence of a related species (*P. fruticosa* Pall.), which is intercompatible with *P. cerasus*, and also tetraploid, mainly used in Russia and Canada to create hybrids resistant to extreme temperatures. Schuster et al. (2017) credit the distinction made by Venjaminov in 1954 of three varietal groups of sour cherries based on resistance to cold. Most of all, the hybrids with *P. fruticosa* including the Russian cvs. Antonovska Kostychevskaya, Plodorodnaya Michurina, Ideal and Polevka, which survived years with record lows (during the Russo-German war) down to minus 50 °C unscathed. There is a second group of medium resistance down to minus 35–38 °C (including the Russian and European cvs. Ostheimer and Kentish) and finally a third group with a resistance down to minus 20–30 °C) with the European Schattenmorelle cv., which is still the most widespread today, Podbelskaya and others.

The most common breeding method is still that of the crossing, mostly drawing from the wide availability of germplasm, and taking care to safeguard self-compatibility, typical of most sour cherry cultivars, in order to have greater guarantees of plant productivity.

The resistances introduced with breeding also include those of plants able to tolerate cold springs or late freezes (frost damage), with a method of indirect prevention that is the use of late flowering parental lines (a widely expressed character in the *Prunus cerasus* germplasm). Another is tolerance to the fungi *Monilinia laxa* Honey and *Blumeriella jaapii* Arx., which severly damage flowers, fruits and buds, especially in areas with a mild and humid weather in spring. However, up until now no resistance genes have been identified against these two pathogenic agents, only

against blossom blight (the German cvs. Jade and Achat, and Hungarian cvs. Csengödi and Piramis) and to cherry leaf spot induced by *B. jaapii*, whose resistance is only unfortunately present in other species, *P. maackii* Rupr. and *P. canescens* Bois.

Regarding fruit quality, it is necessary to state that the variability of *Prunus cerasus* cherries is expressed by three types of fruit, largely linked to the geographical distribution area; the Amarelle or black cherries, spread throughout Europe and North America (e.g. the Montmorency cv. with clear translucent juice), the Morellos or Griottes or visciole cherries with dark red juice (northeastern Europe) and the Marasca type (e.g. cv. "Stevnbar") in Croatia, Serbia and Denmark (Schuster et al. 2017).

Compared to the sweet cherry, the capability of sour cherries to be transformed is considered more important than the quality of the fruit, in particular, the juice yield (depending on the dimensions of the pit) and how this corresponds to market tendencies. In countries with a fresh market demand for the sour cherry, cultivars have been developed (sometimes with the aid of hybrids from *P. cerasus* and *P. avium)* with characteristics such as size (20–24 mm), flesh consistency, lower content of organic acids (≅20–40 g/l) and greater content of sugars and dry material (>15 °Brix); by tradition the color of the juice must be clear in North America, but bright red in Europe. A qualitative sensory study on sour cherries was carried out by Poll et al. (2003), who also correlated anthocyanin content and aroma.

In Russia, breeding was carried out at various research stations (Orel, Moscow, Kazan and others). The breeder who obtained the greatest success was initially V. Michurin, beginning in the 1920s; he concentrated on interspecies hybridization with tetraploid species (*Prunus cerasus* L.), *P. fruticosa* Pall. (ground cherry) and *P. mackii* Rupr. (Manchurian cherry), in order to acquire resistance to cold and to diseases such as cherry leaf spot (*Blumeriella jaapii*). Other hybridizations with *P. avium* (diploid) were always conducted with the aim of acquiring cold resistance and making the fruit more pleasant tasting.

Initially, there was less interest in the quality of the fruit, except for a few characters (color and juice); among these the size (although the weight was just 3–4 g) because the cherries were and still are destined for industrial transformation, especially to extract juice (Shukov and Charitonova 1988).

More than a hundred cultivars have been produced in Russia (e.g. Lyubskaya, Pamyati Vavilova, Polevka, Turgenevka, Zhukovskaya) that have made it possible to extend the cultivation zone into Siberia and other locations subject to winter frost. In extreme places, however, hybrids of *P. cerasus* × *P. fruticosa* have been successful, as they are more resistant than *P. cerasus* to cold and various diseases as well as being less vigorous, earlier to fruit, and easier to handle, although the fruit is more sour, bitter and less pleasant tasting.

Meanwhile, in Kiev, Ukraine, the local institute of fruit and vegetable horticulture (IH NAAS) has undertaken breeding programs since 1920, led by N. V. Moisechenko and continued by V. I. Vasylenko. Recently, IH NAAS licensed the Bohuslavska cv. Other Ukrainian research stations (Melitopol and Opytne) have conducted collaborative programs for over 65 years (Taranenko 2004), licensing numerous sour cherry cultivars including Kseniia, Nochka, Chudo Vyshnia, Altruistka as well as others. The Alfa and Zhadana cvs. originate from another Pomology Institute in Mlijv.

Programs have also existed in Belarus since 1927. Several breeders have performed both interspecific hybridizations and crossbreeding selections with western cultivars, also making improvements in productivity, fruit quality and resistance to cold and disease. Recent cvs. include Griot Belorusskij, Lasukha, Konfitur and others obtained from the Samokhvalovichi Institute of Fruit Growing.

Starting in 1950, Hungary has certainly carried out the most interesting programs in Eastern Europe, at least three, with the aim of improving the cultivation and quality of sour cherries at the experiment stations of Érd and Újfehértó. Most of all, a clonal selection was conducted regarding the Hungarian cv. Pandy, which is quite appreciated by industrial processors although it is small and inconstantly productive due to its non-self-compatibility. Then, locally selected cultivars were introduced with the names Cigány 7, Cigány 59, Cigány 3 and Cigány C404, in order to recover their own very interesting traits for high productivity, self-compatibility, maturation period, and *dry* abscission of the peduncle from the fruit. Cultivars Újfehértoi Fürtös, Debreceni Bötermö, Eva and Petri (Szabó et al. 2008), were derived by crossing programs with landraces, while Apostol (2011), usually a breeder of sweet cherries, conducted a selection program between native sour cherries, which concluded with the licensing of cvs. Csengödi and Ducat and later Prima.

The crossbreeding programs (1968–2009) were also very prolific (chronologically the last one was that of S. Szügyi); some licensed cultivars include Meteor Korai, Favorit, Érdi Jubileum, Korai Pipacs, Érdi Bötermö and, lastly (1980) Érdi Korai, Érdi Kedves and Érdi Bíbor.

In Poland, breeding programs have been conducted since 1950 at the great research institute of Skierniewice. There were various breeders, here it is sufficient to name S. Zagaja, at the beginning and most recently Z. S. Grzyb and T. Jakubowski; the prioritized breeding objectives included positive taste traits, not just for industry, but also for direct consumption; some cultivars also had to be appropriate for mechanical harvesting or shaking. Until 1992, the Lucyna, Sabina, Wanda, Koral, Winer and Wilena cvs. were licensed, among others. The last part of the program conducted by E. Zurawicz and M. Szymajda since 2006 led to the licensing of Galena and Granda cvs.

Cherry breeding programs are present in Romania at the Cluj-Napoca Station (beginning in 1956), Pitesti, Focsani and Jasi in the northern of the Moldava region. Those started with a clonal selection on their landraces (cvs. Crişanac and Mocănesti), then, from crossings developed in Pitesti (Budan and Stoian 1996), numerous cvs. were licensed, including Amanda, Ilva, Timpurii de Cluj, Rival and Stelar. Other programs were aimed towards the introduction of resistances, which are in progress.

In Canada, in the extreme north for fruit cultivation, at the University of Saskatchewan (Saskatoon region) in the 1940s, L. Kerr began the first hybridization program between *Prunus fruticosa* and *P. cerasus*. The first cultivar was licensed in 1999. More recently licensed are cvs. Juliet, Romeo, Cupid, Valentine and Crimson Passion, which have a dwarf tree characteristic (Bors 2005).

Breeding has also been carried out in the northern USA, in Michigan, where winters are famously cold with recorded lows of minus 20–25 °C. At Michigan State University, East Lansing, a cooperation program was developed with a Hungarian

University and, furthermore, the Montmorency cv. was improved, the most popular in the United States introduced three centuries ago from France in order to have a high production capacity and a product with a firmer pulp and a bright red peel, as well as resistant to frost damage (Iezzoni 2005).

2.8 Improved Strategies: Genomics and New Selection Traits

Cherry breeding is passing through a transition period from programs based on phenotypic characters (where parental lines with the desired characteristics are identified) to a new and targeted cherry cultivation that is aided by precision biotechnology to achieve objectives imposed by the market of great agronomic, health, commercial and pomological interest. These have always had sustainability as their foundational aim, seeking health and environmental security, as well as financial security by making costs less burdensome.

But is it possible to expand on the objectives of the past, adding new objectives that were previously impossible? Some objectives have remained the same, principally those regarding the trees themselves. These include high and constant productivity, environmental adaptability and possible resistance to pathogens and pests. The greatest innovations in the fruits have become possible thanks to the identification of genes that were previously found empirically. For example, aesthetic appeal, bicoloredness, larger size, firmer pulp, reduced stone size, reduced peduncle length, resistance/tolerance to cracking and molding, storability and greater shelf-life to overcome distance between the area of cultivation and the market (3 weeks average for sea transportation).

It is probable that, for some of these characteristics and especially those regarding the fruit, it will be difficult to achieve further degrees of improvement (for example in shape, color, size-weight and firmness), notwithstanding the great variety of germplasm available in the various cultivation areas. Even crop yields per hectare will be difficult to surpass with the exception of changes in orchard layout or with new agricultural practices. However, with the help of new breeding technologies, new goals will certainly be achieved, and in a much shorter time period (largely avoiding the exhausting and expensive field assessments of seedlings that could even last from 10–15 years). With these technological approaches, it is possible to immediately cull the plantlets that lack the desired characteristics after seed germination. Then, within the first 2–3 years, the majority of the seedlings will be culled, and only those that have exceeded all of the biological tests will be planted in the field. In addition the remaining population will be verified with the available markers for robotized use, altogether with a very strong reduction in costs. It will soon finally be possible to select seedlings in advance for qualitative and polygenic fruit and plant traits distributed along different linkage groups (LGs). The majority of the

resistances to pest and pathogens, or to adverse climatic factors, are selected through similar procedures.

It will also be possible to create self-fertilizing cultivars, thanks to the ease of identifying and mapping the mutations of the GSI genes of floral self-incompatibility and that of modifying the elasticity index of the skin and its osmotic capacity to absorb less water and reduce cracking and waste, so as to reach a higher degree of enzymatic auto-equilibrium of flesh oxidation during fruit storage. This is a possible way to preserve freshness and quality longer during the various commercial phases.

2.9 Genetic Resources, Germplasm Biodiversity and Conservation

The European Prunus Database (EPDB), hosted by the ECPGR website, contains 5585 cherry accessions (3688 of *Prunus avium* and 1553 of *P. cerasus*) maintained by 42 institutions from 17 countries (a detailed list of Institutes and accessions is available at the following URL: http://www.bordeaux.inra.fr/eucherrydb/main/statsBySpecies). This ample database was implemented in 2005, with the aim of harmonizing EPDB passport descriptors and facilitating the transfer of data between databases such as EURISCO (the EURISCO web catalogue receives data from the European National Inventories and provides information about ex situ plant collections maintained in Europe).

Most of the institutions involved in cherry conservation (in situ and ex situ) are located in Europe (Belgium, Czech Republic, Germany, Spain, France, United Kingdom, Hungary, Italy, Ukraine and others) or Asia (mainly Israel and Turkey).

In the United States, the Agricultural Research Service (ARS) of the United States Department of Agriculture (USDA) maintains three ex situ germplasm collections (National Clonal Germplasm Repository, NCGR, Davis, California; Plant Genetic Resources Unit, PGRU, Geneva, New York; National Arboretum, Washington DC) under the coordination of the U.S. National Plant Germplasm System (NGPS; https://npgsweb.ars-grin.gov) which contains information on about 183 accessions of sweet, sour and ornamental cherries.

As expected, because of its importance in Japanese society and culture, another important database about cherry accession availability is the Japanese National Agriculture and Food Research Organization (NARO; https://www.gene.affrc.go.jp/databases-plant_search_en.php). This database contains the locations and data evaluation of 63 cherry accessions.

All of these collections are maintained in open fields so that they can be used for the characterization, evaluation and distribution of the accessions, but they are also exposed to the risk of pests, diseases and abiotic stresses (Iezzoni et al. 2017). A backup strategy of the available genetic material should be taken into account.

Table 2.2 Examples of cryopreservation approaches in cherries

Plant material	Genotype	Species	Method	References
In vitro shoot tip	Black Tartarian, Burbank, Early Rivers, Guigne de May, Schwarze Venus	*P. avium*	Vitrification	Niino et al. (1997)
In vitro shoot tip		*P. avium*	Vitrification	Shatnawi et al. (2007)
Dormant vegetative bud	Kentish, Meteor, Montmorency, North Star, Schattenmorelle	*P. cerasus*	Vitrification	Towill and Forsline (1999)
In vitro shoot tip	Montmorency and Schattenmorelle	*P. cerasus*	Vitrification	Barraco et al. (2012)
In vitro shoot tips	Gisela 5	(*P. cerasus* × *P. canescens*)	Vitrification	Ružić et al. (2014)

Cryopreservation (at −196 °C in liquid nitrogen) is a very important tool for the long-term storage of plant germplasm in a safe and cost-efficient manner (Engelmann 2004). Examples of cryopreserved cherry genotypes are reported in Table 2.2.

Genetic resources maintained in ex situ germplasm collections constitute the optimal plant material for evaluating genetic diversity and estimating population structure levels as well as identifying a *core collection* suitable for genetic association studies (Campoy et al. 2016).

2.9.1 Genetic Diversity and Phylogeny

Describing the genetic diversity among and within *Prunus* species has been a major issue since the first attempts at allele frequency analysis were made available more than 25 years ago. A preliminary isozyme survey of 34 *Prunus* species (subg. *Prunus*, *Amygdalus*, *Cerasus* and *Lithocerasus*) was able to detect 110 putative alleles at 11 isozyme loci (Mowrey and Werner 1990). Members of subg. *Cerasus* were closely associated, with allele frequencies that supported the origin of *P. cerasus* L. as derived from *P. avium* L. and *P. fruiticosa* Pall. (Olden and Nybom 1968) as well as of *P. gonduinii* Rehd. from *P. cerasus* L. and *P. avium* L. (Fogle 1975). Other members of this cluster are *P. campanulata* Maxim, *P. glandulosa* Thunb. and *P. japonica* Thunb. (Mowrey and Werner 1990). The analyses on cpDNA showed that species pairs such as *P. persica-P. dulcis*; *P. domestica-P. salicina*; and *P. cerasus-P. fruticosa* were completely monophyletic and that the *Cerasus* ancestors separated from the other *Prunus* species relatively early during the evolution of the genus (Badenes and Parfitt 1995). The analysis of genetic variation among flowering cherries (*Prunus*

subg. *Cerasus*), estimated by 25 SSRs on a panel of 144 individuals from 15 taxa, showed that most of the taxa were clustered in the sections to which they belong and that *P. maximowiczii* Rupr. and *P. pendula* Miq. were distant from the other Japanese taxa (Ohta et al. 2005).

A first description of the genetic diversity present within the *Prunus avium* species was performed by analyzing 76 sweet cherry genotypes with 24 SSRs developed in peaches. The UPGMA cluster analysis on the 68 unique genotypes divided the ancient cultivars into two fairly well-defined groups that reflect their geographic origin, the former with the southern European genotypes and the latter with the genotypes from northern Europe and North America (Wünsch and Hormaza 2002).

The same approach was performed by using AFLPs to characterize 38 sweet cherry accessions and to compare their genetic and phenotypic diversity after the assessment of several pomological traits (Struss et al. 2001).

Several recent studies describe genetic diversity within sweet cherry germplasm at the local level, mostly using SSR markers for genotyping. For example, the Spanish (Wünsch and Hormaza 2004), Latvian and Swedish (Lacis et al. 2009), Turkish (Ercisli et al. 2011), and French (plus accessions from other countries; Mariette et al. 2010) germplasm for sweet cherries as well as a few genotypes of Chinese *Prunus pseudocerasus* germplasm (Li et al. 2009) have been described. Altogether these papers provide a lot of information about the relationships between landraces and bred varieties. On the other hand, these SSR-based studies about genetic diversity showed how narrow the genetic base of modern cherry breeding is worldwide (Mariette et al. 2010; Tavaud et al. 2004).

A first integrated approach for the genetic diversity analysis that used both SNP (40) and SSR (7) markers screened a total of 114 sweet cherry germplasm accessions representing advanced selections, commercial cultivars and old cultivars imported from different parts of the world (Fernández i Martí et al. 2012). This study demonstrated that the SNP markers have the best potential to analyze sweet cherry diversity and are able to better distinguish accessions than SSRs.

Finally, Mariette et al. (2010) analyzed 210 accessions (landraces, early selections and varieties derived from modern breeding) from 16 countries (France, Spain, Italy, Greece, Romania, Germany, Switzerland, Hungary, Czech Republic, Poland, United Kingdom, USA, Canada, Argentina, Iran, Japan) and provided a very thorough description of the population structure in this broad genetic background as well as of the linkage disequilibrium (LD) in this species (Campoy et al. 2016). This paper is particularly innovative because of the use of the RosBREED cherry 6 K SNP array (Peace et al. 2012) for genotyping. This approach made it possible to define well two main groups through structure analysis mainly composed of modern cultivars and landraces, respectively. A nested approach identified 9 subgroups that, in several cases, corresponded to different geographic regions. As expected, because of the relatively high level of heterozygosity of this species, the LD of sweet cherry was found to be lower than that of the peach. The availability of a large and genotyped collection and the information about LD in this context are very important pre-requisites for the future development of association studies aimed to identify genes (and the allelic variants of genes) controlling important agronomic and pomological traits and

to develop related markers for marker assisted selection (MAS). The authors also defined a *core collection* of 156 accessions representative of the INRA sweet cherry collection to be used in breeding programs, germplasm management and genetic association studies (Campoy et al. 2016).

2.10 Molecular Breeding Tools and Approaches

The development of efficient protocols for marker assisted selection to support breeding programs requires knowledge of the linkages between marker loci and agronomic traits. Since many traits (most of those related to fruit quality) are polygenic, a linkage mapping approach is fundamental to identifying the quantitative trait loci (QTLs) in which to look for the genes controlling those traits. Next generation sequencing techniques, SNP arrays (for the analysis of thousands of markers in a single experimental step) as well as the availability of the whole genome sequence of fruit tree species are producing a new revolution regarding strategies for genotyping and genome scanning. Most of these innovative tools and approaches are already available for the genomic analyses in cherry species.

2.10.1 Linkage Maps, Markers and QTLs

Map construction makes it possible to localize genes responsible for both qualitative and quantitative traits with the aim of improving and shortening breeding selection (Tanksley et al. 1989). In the case of cherries, several maps have been made available by using segregating progenies (Table 2.3).

The first available maps were constructed by using the few isozymes available in plants and two progenies from interspecific crosses: Emperor Francis (*Prunus avium* L.) × E621 (*P. incisa* Thunb.) and Napoleon (*P. avium* L.) × F1292 (*P. nipponica* Thunb.) were analyzed (Boskovic and Tobutt 1998; upgraded by Clarke et al. 2009). This latter progeny was implemented by using 160 SSRs and 6 gene-specific markers to map the S locus in the LG 6 (Clarke et al. 2009).

In the sour cherry, linkage maps were constructed from the cross of cv. Rheinische Schattenmorelle × cv. Erdi Botermo by using RFLPs (Wang et al. 1998). These maps were used to identify QTLs for flowering (LG1) and ripening (LG4 and 6) times as well as fruit quality traits such as fruit weight (LGs 2 and 4), and soluble solids concentration (LGs 6 and 7; Wang et al. 2000).

Cultivar Emperor Francis was also crossed with the wild forest cherry New York 54 to construct linkage maps with SSRs, AFLPs, and SRAPs as well as gene-derived markers such as CAPS and INDEL based on *Prunus* expressed sequence tags and gene-derived markers for a vacuolar invertase, a gene controlling fruit size with two sorbitol transporters (Olmstead et al. 2008). The NY 54 × Emperor Francis maps

Table 2.3 Linkage maps available in sweet, sour and wild cherry

Population	Size	cM	Markers	References	Note
Emperor Francis × E621 (*Prunus incisa*)	63		Isoenzymes	Boskovic and Tobutt (1998)	
Napoleon × F1292 (*P. nipponica*)	47		Isoenzymes	Boskovic and Tobutt (1998)	
Rheinische Schattenmorelle × Erdi Botermo	86	461, 279	126, 95 RFLPs	Wang et al. (1998)	
Napoleon × F1292 (*P. nipponica*)	94	680	160 SSRs and 6 genes	Clarke et al. (2009)	
NY 54 × Emperor Francis	190	711, 565	102 SSRs, 61 AFLP, 7 SRAP, 27 gene-derived markers	Olmstead et al. (2008), Zhang et al. (2010)	
Regina × Lapins	133	585.5, 369.0	94 SSRs	Dirlewanger et al. (2008)	
Regina × Lapins	121	640	687 SNPs	Klagges et al. 2013)	6K SNP chip
Black Tartarian × Kordia	89	753	723 SNPs		
Regina × Garnet	117	657, 823	723 SNPs	Castède et al. (2014)	6K SNP chip
Namati × F5-18-167, Namati O.P.	72		SNPs and SSRs	Stegmeir et al. (2014)	6K SNP chip
Rainier × Rivedel	166	549, 582	8476 SNPs	Guajardo et al. (2015)	Genotyping-by-sequencing (GBS)
Beniyutaka × Benikirari [Benishuho × (Benisayaka × Rainer)] × Benikirari Nanyo × Benisayaka	94 84 384	660. 825 745, 926 969, 1223	1524 (dd-RAD and SSRs) 1044 (dd-RAD and SSRs) 1264 (dd-RAD and SSRs)	Shirasawa et al. (2017)	dd-RAD seq

located 3 functional markers (gene controlling fruit size on LG 5 and 2 sorbitol transporters both on LG 8) and confirmed the position of the S locus on LG 6.

Another SSR-based map used 133 seedlings from the cross cv. Regina × cv. Lapins (Dirlewanger et al. 2008). Seedlings segregated for fruit cracking resistance, self-compatibility, blooming and maturity dates, peduncle length, fruit color, weight, firmness, titratable acidity and sugar content.

The NY 54 × Emperor Francis and Regina × Lapins maps have also been used to identify QTLs for fruit size on LG 2 and 6 as well as to describe the role of cell number regulator (CNR) genes in the control of this trait (De Franceschi et al.

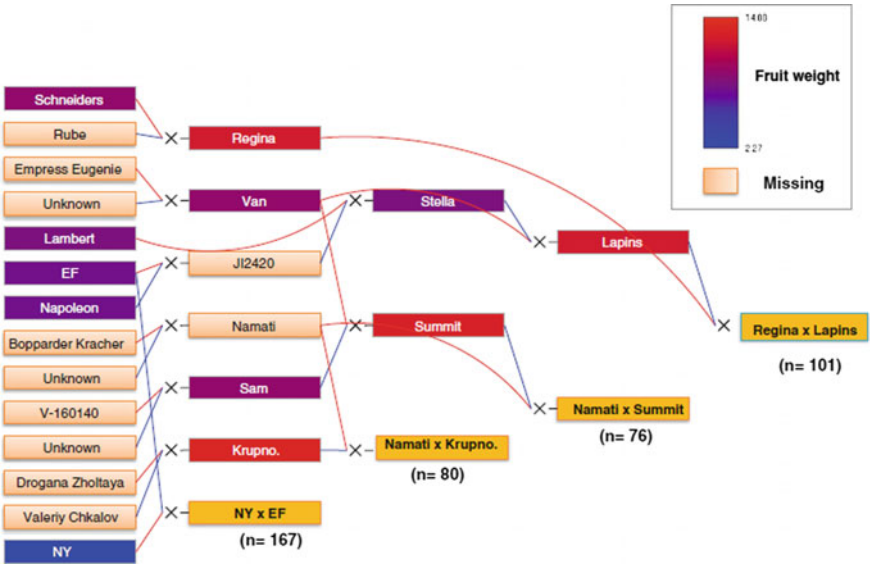

Fig. 2.1 Pedigree of the mapping populations used for the pedigree-based approach (Rosyara et al. 2013). A color scale indicates the phenotype for fruit weight (from the lowest in blue to the highest in red) of the analyzed pedigree members (except for the mapping populations)

2013). Blooming and maturity dates were placed on the LG 4 of Regina × Lapins (Dirlewanger et al. 2012).

A consensus cherry map was constructed by using RosCOS SNPs and SSRs on 4 segregating progenies: NY 54 × Emperor Francis, Regina × Lapins, Namati × Summit and Namati × Krupnoplodnaya (Cabrera et al. 2012). This paper offered the base for a very detailed study about the genetic control of fruit size in the sweet cherry by applying a pedigree-based approach.

As for other traits related to fruit quality in fruit tree species, fruit size is a quantitative trait in sweet cherries. A number of 6 quantitative trait loci (QTL) for this trait (LG1, 3 in LG 2, LG 3 and LG6) have been identified by using phenotypic and molecular data (78 SNPs and 86 SSRs) from multiple families and their pedigreed ancestors. Data was collected on 23 founders and parents and more than 400 seedlings from 4 full-sib families and analyzed through a Bayesian approach developed using FlexQTL^{TM} software (Fig. 2.1; Rosyara et al. 2013). This paper confirmed previous data collected in the NY 54 × Emperor Francis progeny (Zhang et al. 2010) and 2 additional QTLs (LG1 and LG3) were detected for the first time thanks to the advantages offered by analyzing multiple populations with a pedigree-based approach. Since the sweet cherry is highly heterozygous, it should be feasible to increase fruit size by pyramiding the positive alleles for these QTLs (Rosyara et al. 2013). QTLs on LG 2 and 6 co-localize with the position of CNR genes. The role of these genes as candidates for fruit weight control has been described by De Franceschi et al. (2013).

The Regina × Lapins map was upgraded by using the 6K RosBREED cherry SNP array (Peace et al. 2012) and data was compared with that obtained in a second segregating progeny derived from a cross between cvs. Black Tartarian and Kordia (Klagges et al. 2013).

The Regina × Lapins and the Regina × Garnet maps made it possible to co-localize the QTLs for flowering time and chilling requirement on LG 4 (Castède et al. 2014).

The same SNP array was used to genotype a *Prunus canescens* accession and 72 seedlings derived from the cross Namati × F5-18-167 and from open pollinated Namati and a major QTL linked to cherry leaf spot (caused by the fungus *Blumeriella jaapii*) resistance was found in LG5 (Stegmeir et al. 2014). The presence of this QTL (named CLS-4) was validated by using 2 sour cherry progenies derived from crosses with UF and Montmorency (susceptible parents) with a *P. canescens*-derived parent (23-23-13) that was resistant (Stegmeir et al. 2014). In this case, the FlexQTL™ approach for QTL analysis was also used.

Finally, genotyping-by-sequencing (GBS), a new methodology based on high-throughput sequencing, was used to develop and map 8476 SNPs on a 166 seedlings progeny derived from the Rainier × Rivedel cross. High synteny and co-linearity was observed between obtained maps and with Peach v1.0. These new high-density linkage maps provide valuable information on the sweet cherry genome and represent a solid tool for identification of QTLs and genes relevant for cherry breeding in the coming years (Guajardo et al. 2015).

During the sweet cherry genome assembly, the three F1-mapping progenies derived from the cross of cvs. Beniyutaka × Benikirari (n = 94), [Benishuho × (Benisayaka × Rainer)] × Benikirari (n = 84) and Nanyo × Benisayaka (n = 384) were genotyped by double-digest restriction site-associated DNA sequencing (ddRAD-Seq) and SSRs to construct genetic linkage maps (Shirasawa et al. 2017). The 6 linkage maps were integrated into a consensus linkage map consisting of 2317 SNPs and 65 SSRs, covering a total of 1165 cM (Fig. 2.2.). This huge work of anchorage to genetic maps is fundamental to order and orientate the thousands of contigs generated by high throughput sequencing of cv. Satonishiki used to produce the cherry genome (Shirasawa et al. 2017).

Despite the huge quantity of molecular information about sweet cherry, literature describing marker assisted selection applications is quite scanty. Unfortunately, many of the important traits that have been positioned on the cherry maps are polygenic and the development of efficient markers might be easier, at first, only for QTLs with major effects: the favorable alleles should be easy to distinguish from the others with negative effects for each trait.

To date, successful applications of available markers for marker assisted selection in the sweet cherry have been reported for self-compatibility (Pav-S4-indel, see next section) and fruit size (LG2) on more than 2200 seedlings derived from the Pacific Northwest (PNW) sweet cherry breeding program (Ru et al. 2015). It is reasonable to assume that the availability of powerful tools such as the 6K SNP chip for the cherry (Peace et al. 2012) and of the sweet cherry genome will highly support the development of markers linked to important traits to improve this approach in the coming years.

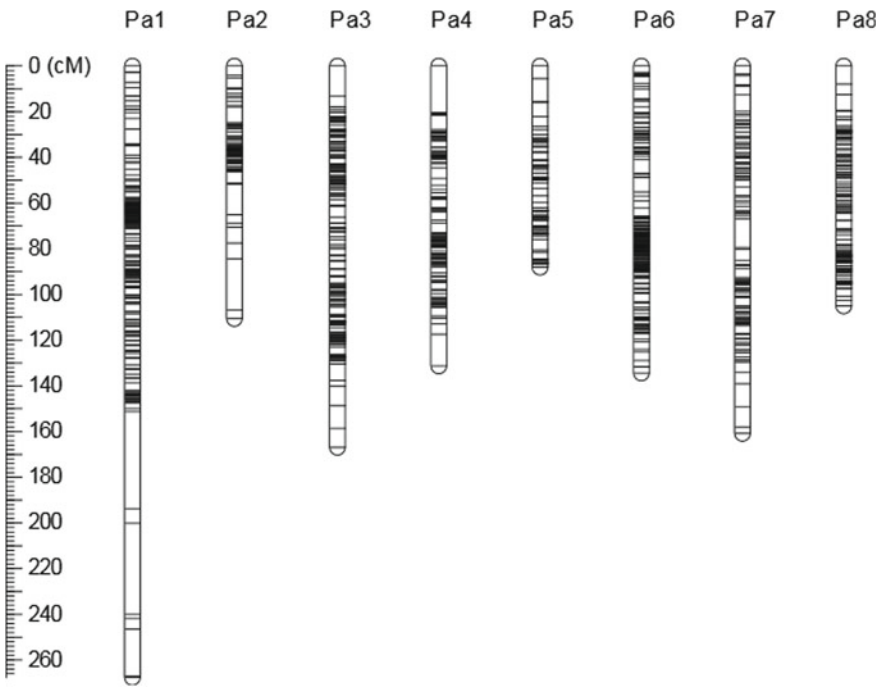

Fig. 2.2 Integrated consensus map used for the cherry cv. Satonishiki genome assembly (Shirasawa et al. 2017). The 8 chromosomes are indicated (PA1–PA8). Bars indicate the marker density

2.11 Genes and Functional Genomics

The large number of articles about genetics of sweet and sour cherries provided the base for a thorough study about the identification of genes that play very important roles in traits such as self-incompatibility, fruit size and color.

2.11.1 Self-incompatibility

The sweet cherry, like other Rosaceae species, exhibits a self-incompatibility system that has naturally evolved to promote cross-fertilization by preventing the fertilization of flowers of a single genotype by its own pollen (De Nettancourt 2001). As a consequence, commercial fruit set in this species requires the presence of other compatible genotypes (to be used as pollinators) or of self-compatible cultivars.

Sweet and sour cherries present the type of self-incompatibility known as gametophytic self-incompatibility (GSI), which is typical of species in the Rosaceae, Solanaceae and Plantaginaceae families (De Nettancourt 2001). This mechanism is

controlled by the interaction of genes belonging to the S locus (S from *sterility*): a ribonuclease called S-RNase is the female determinant [by RNase specific staining, Boskovic and Tobutt (1996); by PCR, Tao et al. (1999)] while the pollen determinant is an F-box gene called SFB (Yamane et al. 2003). All the variants of the S locus are known as S-haplotypes (Tao and Iezzoni 2010).

The two determinants interact in an allele-specific manner to confer the self-incompatibility reaction: if pollen is carrying an allele in common with those of the pistil, its growth in this specific pistil is inhibited. Fertilization can occur if the SFB allele of the pollen belongs to a different S-haplotype in respect to the S-RNases expressed by the pistil. Then, other modifier genes are required to put into practice this mechanism (Tao and Iezzoni 2010). The identification of an F-box protein as the pollen S determinant (probably involved in proteolysis) suggests that the cytotoxic effect of the S-RNases could be prevented by the S-RNase degradation rather than by the inhibition of its activity (Tao and Iezzoni 2010).

On the bases of the S-allele combination of the cultivars, cross-incompatibility groups of sweet cherry cultivars have been defined (with varieties sharing the same S-allele combinations included in the same self-incompatible group).

The availability of S-RNAses sequences have made it possible to develop molecular techniques for S-genotyping that are able to determine the S alleles in sweet cherries and to assign them to incompatibility groups. A recent review reported the S-genotype of 734 sweet cherry accessions and the existence of 47 incompatibility groups (on the basis of the combinations of 18 S alleles) as well as a 0 group of unique S-genotypes (15 cultivars or universal pollen donors) and of a group of 44 self-compatible cultivars (Schuster 2012). The analyses of another 73 Spanish cultivars could raise the number of self-compatible varieties to 48 and those included in the group 0–17 (most of the available results are reported in Table 2.3). Additionally, another 6 S-alleles have been described that are only in the wild cherry; these are S27 to S32 (De Cuyper et al. 2005; Vaughan et al. 2008).

To date, 31 S-haplotypes have been identified in sweet and sour cherries with numbers from S1 to S38 with some of them (S8, S11, S15, S23, S24, S25) being synonymous of others (S3, S5, S7, S14, S22 and S21, respectively) Herrero et al. (2017).

The self-compatible cultivars are often preferred to the self-incompatible ones in order to avoid the necessity of pollinators in the orchards and ensure an optimal fruit set. As reported in Table 6.3, more than 40 self-compatible cultivars have been made available by modern breeding. From a molecular point of view, it is known that mutations to S-RNAse and SFB genes resulted in self-incompatibility breakdown (Tao and Iezzoni 2010).

The first two sources of self-compatibility have been obtained by crossing cv. Emperor Francis with X-irradiated pollen of cv. Napoleon (Lewis and Crowe 1954). Molecular characterization of the mutated S-haplotypes of Napoleon showed: (i) the existence of an S4′ allele derived from a deletion in the SFB4 gene (with a consequent frame-shift in the downstream sequence; Sonneveld et al. 2005); (ii) a complete deletion of SFB3 (Sonneveld et al. 2005). Several self-compatible cultivars derived from these two mutational events such as Stella, Lapins, Skeena, Blaze Star

and others all carrying the S4′ allele while the S3′ is present in cultivars such as Axel (Table 2.4; Schuster 2012).

Another source of self-compatibility is represented by the deletion of the SFB5 gene (S5′-haplotype) that confers self-compatibility to cv. Kronio. In this case, the mutational event was not artificially induced since Kronio is a Sicilian cultivar that was reported to be naturally self-compatible (Marchese et al. 2007).

Interestingly, not all of the mutations that result in self-compatible cherry varieties occur in genes located in the S locus. A bulked segregant analysis performed on four progenies with the Spanish cv. Cristobalina (a self-compatible Spanish cultivar) used as parent, showed that the mutational event occurred in the LG3 (instead of LG6, as previously expected; Cachi and Wünsch 2011) and that this mutation could also affect the growth rate of the pollen during self-pollination (Cachi et al. 2014).

The identification of self-compatibility sources is also important in the sour cherry, even if a large number of sour cherry cultivars are self-compatible (Sebolt et al. 2017). Analogously to the sweet cherry, even in this case, the self-incompatible cultivars require a pollinator to achieve fruit set (Herrero et al. 2017).

In sour cherry, 12 functional S-haplotypes (S1, S4, S6, S9, S12, S13, S14, S16, S26, S33, S34, and S35) have been described as well as the presence of 9 non-functional ones (S1′, S6 m, S6m2, S13 m, S13′, S36a, S36b, S36b2 and S36b3 where Sm indicates stylar part mutations and S′ pollen part mutations) Sebolt et al. (2017).

In the sour cherry, a pollen grain carrying at least one functional S-haplotype enabled to match a functional S-haplotype in the style, will be rejected (Hauck et al. 2006). Therefore, in several cases, S-genotyping in this species is not limited to the identification of the S-haplotypes but it is important to determine if the S-haplotype is a pollen-part or stylar-part mutant (Sebolt et al. 2017). These authors presented the results of S genotyping in 21 sour cherry cultivars representative of the Michigan State University breeding program as well as a strategy for S-genotype determination and mutated S-haplotypes identification with the aim of maximizing the number of seedlings selected because of their self-compatibility before planting them outdoors.

2.11.2 Fruit Size and Color

Modern cultivars are distinguished from their smaller-fruited wild progenitors by a substantial increase in fruit size. The first genes able to control fruit weight were identified in tomatoes (the FW2.2 gene) and maize (named CNR, cell number regulator). These are members of a family of genes that play a role in regulating cell number and organ size. This family provides an excellent source of candidates for fruit size genes in other domesticated species, such as the sweet cherry (De Franceschi et al. 2013).

The high expression of these genes inhibits cell division in the early stages of fruit development. Two of these CNRs were located within confidence intervals of major quantitative trait loci (QTL) previously discovered on LG 2 and 6, named

Table 2.4 Incompatibility groups of inter-compatible varieties and finally the group of self-compatible varieties

Incompatibility group (S-allele)	Cultivars
I (S1S2)	Agris, Amfurter Kirsche, Basler Langstieler, Baumanns May A, Bedford Prolific A, Black Downton, Black Eagle (Eagle), Black Tartarian, Canada Giant (Sumgita), Carnation C, Coe, Early Rivers, Emperor Francis B, Ferdouce, Ficktaler Rotstieler, Kaštanka (Early Rivers), Knight's Early Black A, Luciens Kirsche, Nanni, Rivan, Ronald's Heart, Roundel Heart, Sparkle, Starking Hardy Giant, Summit, Ursula Rivers
II (S1S3)	Abundance, Ambrunes Rabo, Belle Agathe, Bigarreau Nior de Winkler, Bigarreau de Schrecken (Schreckens Kirsche), Black Elton (Elton), Black Star, Braune Prinzer (Prinzenkirsche), Caroon B, Cristalina (Sumnue), Early Ruby, Early Van Compact, Erika, Frogmore Early, Fromms, Frühe Rosmarin, Garrns Bunte, Giant Red (Giant Ruby), Gil Peck, Giorgia, Hamid, Heidi, Helga, Hongdeng, Hongyan, Huldra, Jubilee, Karešova, Kasandra, Késői vadcseresznye, Kouka-Nishiki, Ladeho Pozdni, LaLa Star, Lamida, La Pinta (Barrado), Merton Bigarreau, Merton Bounty, Merton Crane, Merton Favorite, Na 19, Oktavia, Olympus, Pedro Merino, Prime Giant, Puntalazzese, Rebekka, Regina, Ruby, Samba (Sumste), Satin (Sumele), Schubacks Frühe Schwarze, Sir Douglas, Sodus, Sonnet, Sumbigo, Sumbola, Sweet September, Szeptember, Tigré, Troprichterova (Troprichters Schwarze Knorpelkirsche), Ulrich, Valeska, Van, Venus, Vera, Victor Black A, Waterloo, Wesselhöfts, Windsor, Žemaičių rožine, Zimmermänner, Zu Pippinu
III (S3S4)	Abardeh, Altenburger Melonenkirsche (Büttners Späte Rote Knorpelkirsche), Ampfurter Knorpelkirsche, Angela, Augusztus elején érő (0949), Badacsoner, Belge, Bigarreau Esperen (Esperens Knorpelkirsche), Bing, Blancal, Botond, Brigitte, Büttners Späte Rote Knorpelkirsche, Castañera, Cemal, Csákvári korai, Czarna pozna, Czersnia-pozna ciemnoczerwona, De la Piedra, Didi, Disznódi fűszeres, Eisenbraune, Elifli, Frühe von der Weid, Ghermez Rezayieh, Große Prinzessin (Napoleon/Lauermannskirsche), Haj yousefi, Hamedan, Hedelfingeni Orias, Heinrichs Riesen, Hongfeng, Jacinta, János cseresznye, Jueze, Kaiser Franz (Emperor Francis, Kaiser Franz Josef), Karina, Kavics, Késői fekete nagy, Késői helyi fekete, Kristin, Lambert, Langstieler B33, Late Maria, Marmotte, Mojtahedi, Müncheberger Frühe (Munchebergi Korai), Namare, Namosa, Napoleon (Royal Ann), Ochsenherzkirsche, Pflugwirtle B10, Querfurter Königskirsche, Seda, Sefid Rezayieh, Shoa al Saltaneh, Solymari Gömbölyü, Solymari Scinscersznye, Somerset, Späte von Wedler, Solymári gömbölyű, Star, Sultan Hisar, Szőlősi pollenadó, Sweet Lorenz (PA2 Unibo), Sweet Valina (PA4 Unibo), Tarka cseresznye, Teickners Schwarze Herzkirsche, Transportabelnaja, Turkey Heart B, Ulrichs Braune, Ulster, Vernon, Werdersche Braune, Yellow Spanish, Zard Daneshkadeh
IV (S2S3)	Aci Kara 0922, Allmän Gulröd, Aydin Siyahi, Bigalise (Enjidel), Blaietes, Blankenburger, Cavalier, Coralise (Gardel), Dame Nancy, Edime, Früheste der Mark (Küppers Frühkirsche), Kara 0908, Kassins Frühe, Kentish Bigarreau, Knights Bigarreau, Late Amber (Amber Gean), Ludwig's Bigarreau, Merton Premier, Naresa, Newmoon (Sumini), Nimba, Pollux, Quarantana, Sari, Sue, Újszászi cseresznye, Vega, Velvet, Victor, Vit Spansk, Viva, Vogue, White Spanish, Zeyitali 0960

(continued)

Table 2.4 (continued)

Incompatibility group (S-allele)	Cultivars
V (S4S5)	Althof (Graf), Carmen, Kati, Late Black Bigarreau, Maisenbühler, Májusi korai cseresznye, Sauerhäner, Schwarze Bohlsbacher Weg, Spraul (Früh), Turkey Heart
VI (S3S6)	Ambrunesa, Anita, Artvin 4, Badacsony, Benedetta, Bigarrå fröplanta från E. Petterss, Brjanskaja Rozovaja, Cappuccia Castelbuono, Coloradilla, De la Casa, Del Gordo, Dönissens Gelbe (Gold, Stark's Gold), Durone Nero 3 (Duroni 3), Early Amber, Early Magyar, Elton Heart EM, Fehér cseresznye, Fehér ropogós pollenadó, Fehércseresznye VK, Ferdiva, Fertard, Fertille, Gårdebo, Garganteña, Giniusa, Governor Wood (Wood), Gran Catalunya, Grossa di Pistoia, Hartland, Hofkirsche, Hongmi, Jarandilla, Julia, Kecskecsöcsű, Kordia, Maiolina Castelbuono, Maiolina Messina, Merton Heart, Merton Marvel, Mindauge, Mollar, Muddisa, Nafrina, Nanyo, Napoleona Precoce, Ohio Beauty, Ovale Frühe Herzkirsche, Pákozdi fehér, Pat, Pflugwirtle B11, Pico Limon Colorado, Pico Limon Negro, Pico Negro, Pico Negro Barrado, Pretera, Rival, Sasha, Saslonovskaja, Sato-Nishiki, Schlesische Braune, Schüttler vom Albtrauf, Tápiósági cseresznye, Těchlovan, Toscana, Turkish Black, Venancio, Vinka, Viola, Vytenų rožine, Wölflisteiner DE, Yabani 0889, Zolotaja Lashitskaja
VII (S3S5)	Augusztusi fehér, Badeborner, Bigarreau Gaucher (Gauchers Knorpelkirsche), Black Eagle A, Bleyhls Braune, Bradbourne Black, Braune Herzkirsche, Cserkeszőlő 7, Early Burlat, Farnstädter Schwarze, Frühe Luxburger, Gamma, Gaucher, Geisenheimer Braune, Große Schwarze Knorpelkirsche, Hedelfinger, Hookers Black, Maiolina a Rappu, Meda, Moreau NY, Nadino, Schmalfelds Schwarze, Spansche Knorpel, Tünde, Weiße Herzkirsche, Witzenhausener Frühe
VIII (S2S5)	Balzams, Malling Black Eagle, Mona BC, Vista
IX (S1S4)	Bada, Black Giant, Black Republican, Changbahong, Chinook, Dawson, Dollenseppler B5, Dolls Langstieler, Dunabogdányi szív alakú cseresznye, Durone Tardivo de Valstaffora, Early Lyons BC, Fiesenhofer, Frisco, Garnet, Giant, Hudson, Karls Große, Késői tarka, King, Krächerle, Langstieler B34, Meckenheimer Frühe, Merton Late, Merton Reward, Namati, Naremi, Rainier, Republican (Black Republican), Rube, Salmo, Summer Jewel, Sweet Gabriel (PA3 Unibo), Sylvia, Viscount, Winterbacher
X (S6S9)	Abdullah, Black Tartarian E, Cserkeszőlő 2, Dányi cseresznye, Del Pollo, Early Lyons NY (Jaboulay), Ferobi, Folfer, Jaboulay (Bigarreau Jaboulay), Jurgita, Kadi 0878, Maiolina Etna, Napoleona Virifica, Penny, Planera, Planera Menuda, Ramon Oliva, Tilau, Turfanda
XI (S2S7)	Cryall's Seedling, Early Purple (Hinode), Guigne d'Annonay
XII (S6S13)	Buholzer, Cserkeszőlő 4, Durona di Vignola (Nero I di Vignola), Fertődi csüngő, Noble EM, San Pritisa B, Tasziló, Turca
XIII (S2S4)	Corum, Deacon, Feria, Generalskaja, Kaiser Franz, Kasberger Schwarze, Kronprinz von Hannover, Leipziger Lotkirsche, Maibigarreau, Maisenbühler Schlapper, Namada, Noire de Schmidt (Schmidt), Ord, Patricia, Peggy Rivers, Remstäler, Royalton, Ruefler, Sam, Schmidt (Große Schwarze Knorpel), Spalding, Szomolai Fekete, Tragana d'Edessa, Türkine (Flamentiner), Vic, Vitaby

(continued)

Table 2.4 (continued)

Incompatibility group (S-allele)	Cultivars
XIV (S1S5)	Alma, Annabella, Basler Adlerkirsche, Beta, Bianca, Drogans Gelbe, Dura Succosa, Grolls Schwarze Knorpelkirsche, Heidegger, Pflugwirtle B13, Schauenburger, Sekunda, Seneca, Sture Holmberg, Valera, Yalanci Napolyon
XV (S5S6)	Colney, Delta, Early Lyons, Elfrida, Erianne, Ermstaler II, Segliņu, Skedes, Talsu 1, Trusenszkaja 2, Zweitfrühe
XVI (S3S9)	Beata, Bigarreau de Mezel, Burlat, Chelan, Durone Nero PILL, Furistera Napoleona, Garonowskaja, Hongdeng, Jueze, Korai ropogós 1062, Mona MI, Moreau (Big. Moreau, Souvenir des Charmes), Moretta, Nabigos, Nalina, Napoleona Tardiva, Napoletana Castelbuono, Naprumi, Navalinda, Précoce Bernard, Şekerpare, Souvenir des Charmes (St. Charmes, Big. Moreau), Tieton, Winklerova Rana (Winklers Frühe Schwarze), Yuzhou, Zaohongbaoshi
XVII (S4S6)	Beni-Shuho, Elton Heart BC, Hedemora, Heidi, Hemmiker, Jiahong, Kracher, Larian, Meelika, Merton Glory, Nutberry Black, Schöne von Einigen, Torbágyi késői fekete
XVIII (S1S9)	Bellise (Bedel), Brooks, Earlise (Rivedel), Early Bigi (Bigisol), Early Red (Early Garnet), Firm Red, Marvin 4-70, Norbury's Early Black, Qihao, Smoky Dun, Sumertime, Sweet Early (Panaro 1), Tamara, Valerij Tschkalov, Vytenų juodoji, Zoe
XIX (S3S13)	Agostina, Blaser, Cognac, Péceli, Reverchon, Sir Tom, Wellington A
XX (S1S6)	Alfa, Almore, Augusztus elején érő (0644), Baschimeiri, Beni-Sayaka, Bowery Heart (Bowyer Early Heart), Corniola, Dazi, Debanka, Erstfrühe, Fernier, Ferpact, Guillaume, Halil Efendi, Henriette, Hertford, Karl, Lauerzer, Lijana, Louis, Merla, Mermat, Noble NY, Pavesi, Pomázi középérésű, Rockport Bigarreau (Taksago), Talsu Sārtais, Temprana Negra, Vanda, Wölflisteiner CH, Zopf
XXI (S4S9)	Agila, Cashmere, Cowiche, Fengjin, Germa, Inge, Juhong, Kiona, Merchant, Merpet, Navon, Sujan, Summer Sun, Vasare, Youyi
XXII (S3S12)	Akşehir Napolyonu, Allahdiyen, Dicke Braune, Farmosi cseresznye, Ferrovia, Germersdorfi 1, Germersdorfi 3, Karakiraz, Kazancioğlu, Korai ropogós 1107, Kozerska, Linda, Móri K cseresznye, Nadal, Nasenkirsche, Noire de Meched, Nordwunder, Princess (Prinzesskirsche), Rekord, Ropogós cseresznye 0834, Rubin, Schneiders Späte Knorpelkirsche, Senyoreta Tardana, Uluborlu, Valenta, Ziraat 0900,
XXIII (S3S16)	Minnulare, Napoletana Etna (Durona), Rodmersham Seedling, San Pritisa A, Strawberry Heart, Žemaičių geltonoji
XXIV (S6S12)	Aida, Flamentiner, Segorbina
XXV (S2S6)	Early Korvik, Fercer (Arcina), Fryksås, Great Black Delicious EM, Große Braune, Kaman Cayiri 0895, Kirdar, Knauffs Schwarze (Knauffskirsche), Luke, Mahagony från Näsum, Rekordiska

(continued)

Table 2.4 (continued)

Incompatibility group (S-allele)	Cultivars
XXVI (S5S13)	Ferbolus (Vernel), Goodnestone Black, Schwarze der Lobenroth, Unterländer
XXVI (S4S12)	Katalin, Kavics, Margit, Ropogós cseresznye 0628
XXVIII (S1S7)	Benjaminler, Charger, Dollenseppler, Gumpp Runz, Könningerle, Önsbacher, Ortenauer, Paulis, Polenkirsche
XXIX (S4S7)	Hartwälder, Niğde, Ritterkirsche, Stolzer, Sultan
XXX (S6S7)	Brennkirsche Doll, Schwarze Schüttler
XXXI (S4S14)	Binam, Feuerkirsche, Rote Herrbach, Rotstieler, Siah Ghazvin, Sorati Hamedan, Stöckemer Rote
XXXII (S1S12)	Castor, Johanna
XXXIII (S1S14)	Adriana, Femina
XXXIV (S3S14)	Carlotta, Durone di Verona (Mora di Cazzano), Vittoria
XXXV (S5S14)	Dikkelon, Herzkirschen, Melitopolskaja rannaja
XXXVI (S7S19)	Pflugwirtle B12, Rote Kirsche
XXXVII (S5S9)	Cavallaro, Fekete helyi cseresznye, Krupnoplodnaja
XXXVIII (S3S22)	Carrubbedda, Cherovina Tardana, Niura dell"Etna, Telegal, Toscanella
XXXIX (S6S16)	Anta, Cappuccia Imperiale, Forma di Cuore, Jurga
XL (S6S22)	Cappuccia Bivona, Cappuccia Doppia, Reginal
XLI (S12S22)	Cherovina Primerenca, Nadal Tendre
XLII (S2S10)	Bademil 0898, Tabanli
XLIII (S2S9)	Ferprime (Primulat), Kara Turani 0888, Narana, Norta
XLIV (S3S7)	Aci Bursa, Augustkirsche, Hermesberg
XLV (S4S13)	Lampnästler, Marquardter Frühe
XLVI (S3S24)	Del Cardito, Garrafal

(continued)

PavCNR12 and PavCNR20, respectively. In particular, the less efficient allelic variants of PavCNR12 (with polymorphisms in the promoter region) are linked with a consistent increase of fruit size (Fig. 2.3).

Table 2.4 (continued)

Incompatibility group (S-allele)	Cultivars
XLVII (S6S24)	Pico Colorado, Virgo Juliana Group 0 Caddusa (S13S22), Chispas (S12S21), Ciliegio Selvarico (S21S22), Danners Späte B17 (S1S19), Danners Späte B18 (S4S22), Donn' Antoni (S16S22), Ermstaler I (S7S14), Garrafal de Lérida (S9S13), Grosszweiete (S6S14), Halbhaslacher (S5S7), Hrebnickio1 (S12S16), Kiechlingsberger Kracher (S1S22), Moscatella (S17S21), Nucigliara (S14S16), Orleans 171 (S7S10), Rita (S5S22), Žemaičių juodoji (S1S13) Self-compatible cultivars Axel (Alex) (S3S3′), Blackgold (S4′S6), Big Star (S1S4'), Blaze Star (S4′S6), Celeste (Sumpaca) (S1S4′), Columbia (Benton) (S4′S9), Compact Stella (S3S4′), Cristobalina (S3S6), Dame Roma (S4′S13), Early Star (S4′S9), Endiablà (S3S3), Glacier (S4′S9), Grace Star (S4′S9), Habunt (S3S4′), Index (S3S4′), Kronio (S5′S6), Lapins (S1S4′), Marysa (PA6 Unibo) (S3S4') New Star (S3S4′), Pál (S4′S9), Paulus (S4′S9), Petrus (syn. Péter) (S3S4′), Ripoll (S3S6), Runar (S3S3), Sandor (S4′S9), Sandra Rose (S3S4′), Santina (S1S4′), Selah (Liberty Bell) (S3S4′), Senyoreta Primerenca (S3S6), Sir Don (S4′S13), Sir Hans (S2S4′), Skeena (S1S4′), Sonata (Sumleta) (S3S4′), Son Llobet (S3S6), Staccato (S3S4′), Stardust (S1S4′), Starkrimson (S3S4′), Stella (S3S4′), Sumesi (S3S4′), Sunburst (S3S4′), Sweetheart (Sumtare) (S3S4′), Sweet Aryana (PA1 Unibo) (S3S4'), Sweet Saretta (PA5 Unibo) (S3S4'), Sweet Stephany (PA7 Unibo) (S3S4'), Symphony (S1S4′), Talegal Ahim (S6S22), Tehranivee (S3S4′), Temprana de Sot (S3S6), Vandalay (S3S4′), Waiyin No.7 (S3S4′)

Source Modified after Cachi and Wünsch (2014), Iezzoni et al. (2017), Schuster (2012) and this chapter

For sour cherry, the difficulties found in CNR gene sequencing led researchers to develop markers in close proximity to the CNR12 and CNR20 (G2SSR1566 18 kb downstream of CNR12 and G6SSR2208 13 kb downstream of CNR20) genes by using the peach genome sequence and several putative alleles with a significant effect on fruit size were identified (De Franceschi et al. 2013).

Regarding fruit color, a major QTL for skin and flesh color co-localize in the NY 54 × Emperor Francis progeny with a MYB transcription factor (PavMYB10; Sooriyapathirana et al. 2010), known to play a major role in anthocyanin accumulation in other fruit tree species such as apple (Espley et al. 2007) and pear (Pierantoni et al. 2010). A SNP marker in position 120 of this gene linked to red color was developed (Sooriyapathirana et al. 2010).

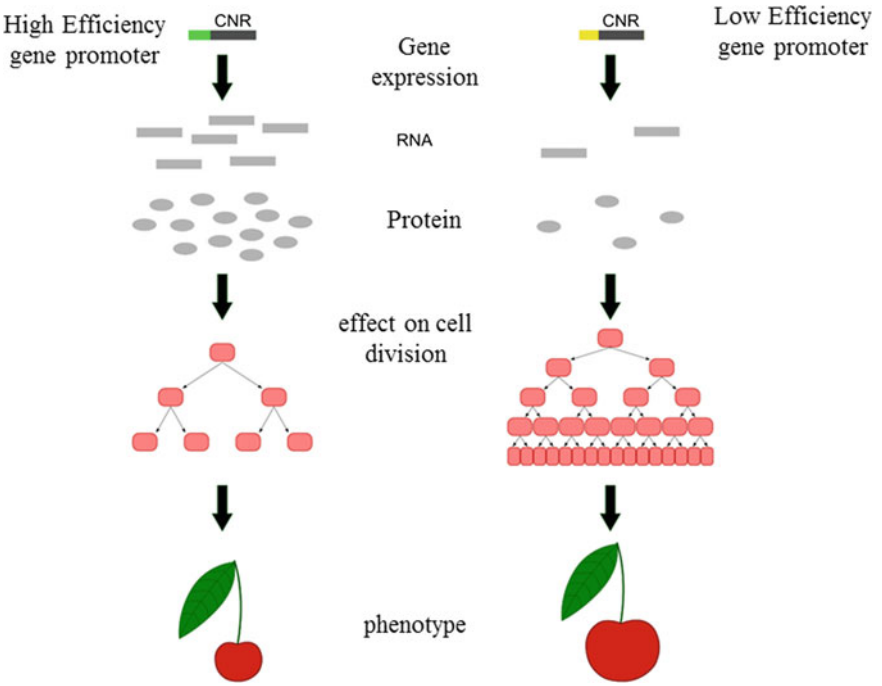

Fig. 2.3 Proposed mechanism of CNR genes in fruit size determination (De Franceschi et al. 2014). The increased fruit size is controlled by a low efficient promoter of the CNR gene

2.12 Cherry Genomics

The availability of next-generation sequencing approaches made the development of an SNP-based molecular tool for genome scanning possible at a very low cost.

In sweet and sour cherries, a very powerful tool was developed by the RosBREED community to facilitate marker-assisted breeding. More in detail, a 6K SNP array for use by the Illumina Infinium® system was designed with SNPs selected from the re-sequencing data of 16 sweet and 8 sour cherry accessions (Fig. 2.4) well distributed along the cherry genomes and tested on a panel of sweet and sour cherry breeding accessions (Peace et al. 2012).

The cherry SNP array is commercially available from Illumina and SNP markers included in this tool are available at the Genome Database for Rosaceae (GDR; http://www.rosaceae.org).

The sweet cherry genome (Shirasawa et al. 2017) is the last member of the wide group of the sequenced genomes of species belonging to the Rosaeae family. This group includes apple (*Malus* × *domestica*, Daccord et al. 2017; Velasco et al. 2010), strawberry (*Fragaria vesca*, Shulaev et al. 2011), Japanese apricot (*Prunus mume*, Zhang et al. 2012), peach (*Prunus persica*, Verde et al. 2013, 2017), Chinese pear

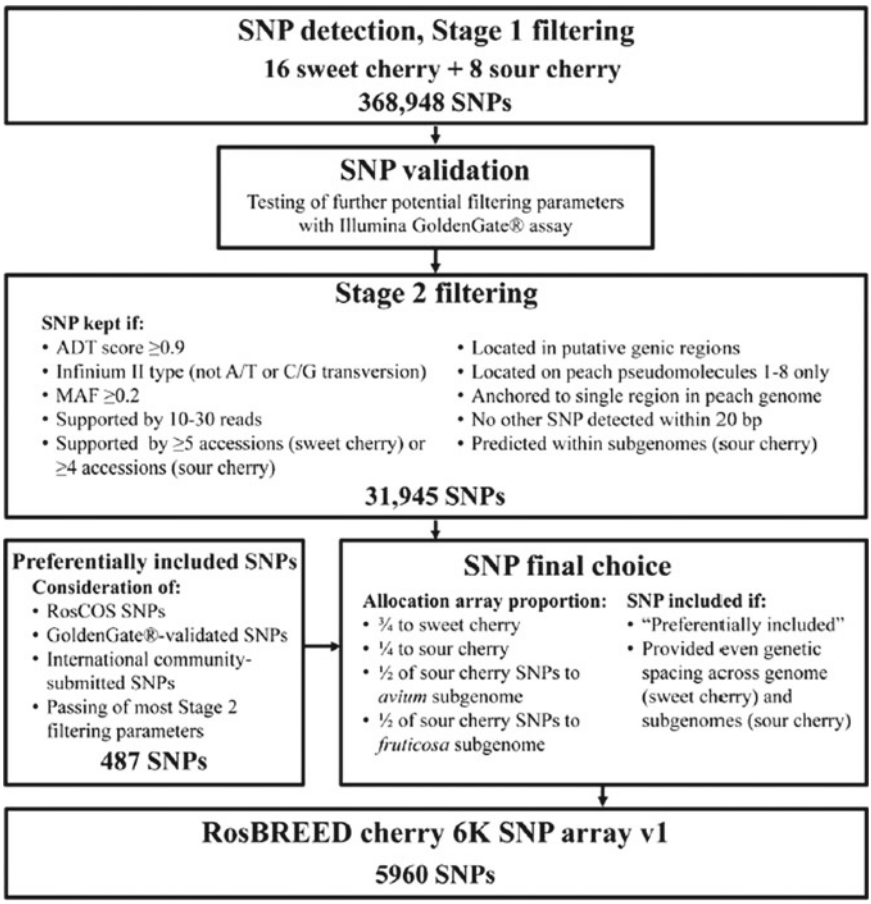

Fig. 2.4 Workflow for the 6K SNP array design (modified from Peace et al. 2012)

(*Pyrus*× *bretschneideri*, Wu et al. 2013), European pear (*Pyrus communis*, Chagné et al. 2014) and black raspberry (*Rubus occidentalis*, Van Buren et al. 2016).

This sequencing project could use all of the information about synteny between Rosaceae that has been made available since Dirlewnger et al. (2004). This paper first displayed the positions of major genes and QTLs for agronomically-important traits such as disease resistances and fruit quality traits in the frame of the 8 *Prunus* chromosomes. For several years, before the availability of the 6K SNP chip (Peace et al. 2012), genomic information for cherry breeding was transferred from other related species, mainly from peaches. As expected, the cherry sequence showed a very high co-linearity with the peach genome even if the genome sizes are different: researchers estimated a cherry genome size of 352.9 Mb (length that is higher than the 225 Mb of the peach, but compatible with the value of 338 Mb measured by flow cytometry) and a number of 43,349 genes were predicted (Shirasawa et al. 2017).

Annotation was performed on the basis of the sequencing results of 8 transcriptomes from different plant tissues: leaf, root, flower, fruit (at different developmental stages such as stone hardening, ripening, harvesting or challenged by brown rot) and callus.

As reported, all the genome assembly data, annotations, gene models, genetic maps and DNA polymorphism are available at the cherry database (DB cherry, http://cherry.kazusa.or.jp/) and linked with the Genome Database for Rosaceae (GDR) at the following URL (https://www.rosaceae.org/species/prunus_avium/genome_v1.0.a1).

The genomic information available today will speed up both genetic analysis and breeding programs in sweet cherry, similar to what has occurred in the past few years for other fruit tree species.

2.12.1 Genetic Engineering

The availability of gene information and knowledge of their related functions offers a valid support for the use of genetic transformation in fruit tree species as an alternative to conventional breeding techniques. It should be particularly useful in cherry development because of the heterozygosity of the cultivars, the presence of different ploidy levels and the length of field trials. In spite of this, only a few studies are available describing the genetic transformation approach in this species.

Employing the standard *Agrobacterium* spp. (and less frequently the biolistic approach), clones of transformed Colt cv. rootstock (*Prunus avium* × *P. pseudocerasus*) carrying the rice phytochrome A to induce more compact internodes have been produced (Fig. 2.5; Negri et al. 1998) as well as lines of the cvs. Damil and Inmil rootstocks by transformation using GUS and *rol* genes via *Agrobacterium* (Druart et al. 1998). The Colt cv. rootstock was also transformed by *Agrobacterium rhizogenes* with the non-disarmed pRi1855 T-DNA. The transformed plants presented an enhanced rooting capacity, shortened internodes and wrinkled leaves (Gutièrrez-Pesce et al. 1998).

Then, a successful stable transformation of the sour cherry scion cv. Montmorency and of the dwarfing rootstock cv. Gisela 6 (*P. cerasus* × *P. canescens*) was described by using *Agrobacterium tumefaciens* (Song and Sink 2006).

More recently, an RNA interference (RNAi) vector (containing an inverted repeat region of Prunus necrotic ring spot virus) was introduced into two cherry rootstocks, cvs. Gisela 6 and Gisela 7. This approach enhanced their resistance levels to the virus (Song et al. 2013).

Genetically modified cv. Gisela 6 rootstocks transformed with the construct for RNAi were able to transfer the Prunus necrotic ring spot virus resistance to a non-transformed scion of the cv. Emperor Francis. More in detail, small interfering RNAs (siRNAs) derived from the RNAi vector, and produced in the transgenic rootstocks, are transferred to the non-transgenic scions (Zhao and Song 2014). This study demonstrates the potential of using transgenic rootstocks by grafting improved products of non-transgenic scions.

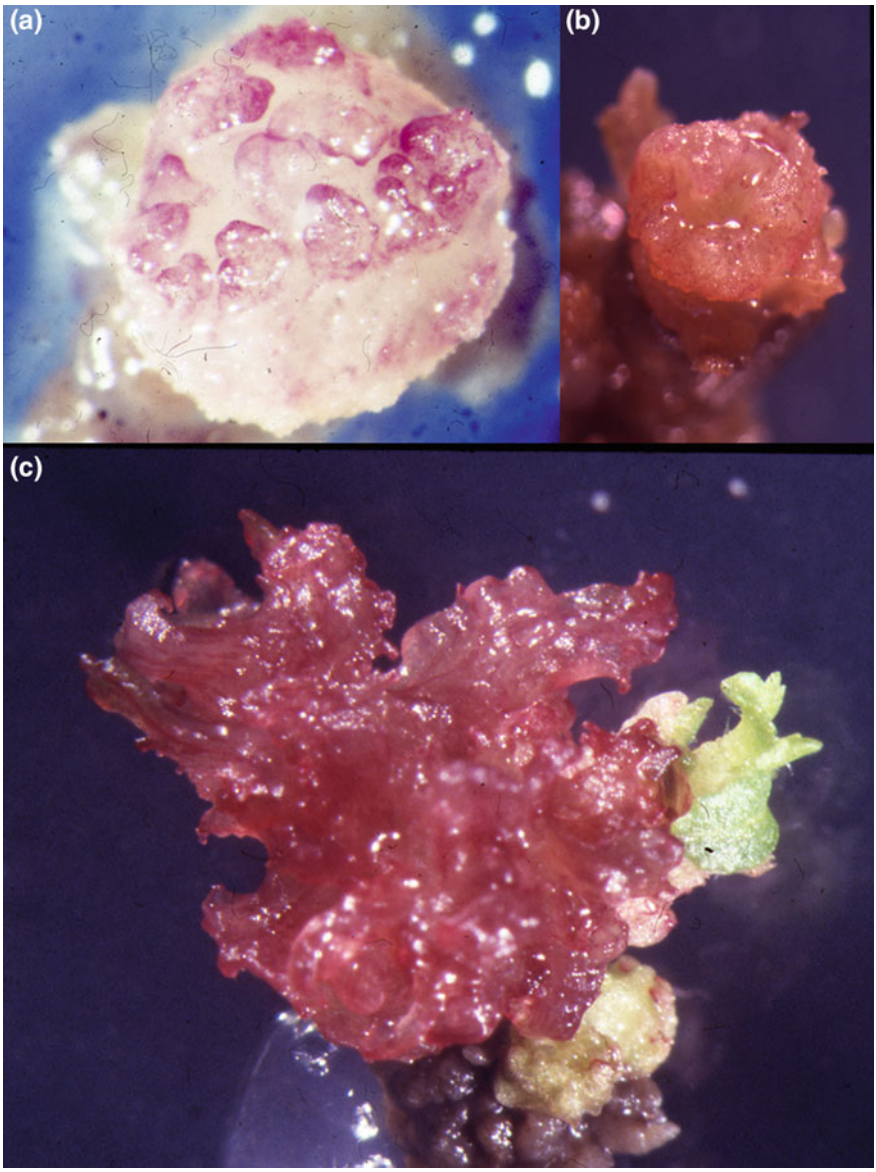

Fig. 2.5 Regeneration of Colt cv. rootstock transformed with rice phytochrome A (Negri et al. 1998). **a** and **b** early stages of shoot regeneration. **c** Development of mature green tissues from an anthocyanic cluster of early differentiating shoots

2.13 Conclusions and Prospects

Currently, no temperate tree species has as many opportunities as the cherry for crop renewal in its already large worldwide areas of cultivation. In fact, the biodiversity present in the germplasm and the genetics of the species make it possible to extend cultivation to new areas, to the northernmost limits of the temperate areas, and into Mediterranean climatic zones.

Thanks to the new molecular technologies and sequenced genome that identified the position and function of the genes that determine characters of interest, it is possible to design targeted breeding programs, with faster and less burdensome selective processes. There are many cherry breeding programs in the world, some already converted to these new technologies. Today, many new varieties are available on a commercial scale (see Appendix).

Moreover, the knowledge concerning the variability at the S locus makes it possible to plant orchards with interfertile cultivars and with higher production and quality yields.

In terms of cultivation, new genetic material and relative techniques make it possible to create new cherry orchards that are radically different from those of the past, thanks to the availability of dwarf and semi-dwarf rootstocks, widely experimental growing forms and the use of covered and protected plantings to anticipate the harvest calendars. We are now setting up medium and even high-density orchards, which bear fruit of better quality (size, appeal and taste) much faster and with much lower management costs, making the cherry tree as highly specialized and sustainable a crop as other pip and stone fruits.

Acknowledgements The authors thank Mrs. Clementina Forconi for her valuable assistance.

Appendix

List of the main sweet cherry varieties available for new orchards. The source of each profile is indicated by the number reported at the bottom as follow: [1] The Brooks & Olmo Register of Fruit & Nut Varieties. ASHS Press; October 1, 1997, 3rd ed. and Register of New Fruit and Nut Cultivars (List 34–48); [2] Reworked by Quero-Garcia J, Schuster M, Lopez-Ortega G, Charlot G (2017) Sweet cherry varieties and improvement. In: Quero-Garcia J, Iezzoni A, Pulawska J, Lang G (eds.) Cherries: botany, production and uses, 60–94.

An asterisk following the variety name signifies "patented name" while ® signifies "registered trade mark name".

Benton® PC7146-8*	
Dark colored, large, shiny cherry with good taste and firmness. Origin Prosser/Washington State University (WSU), USA. Parents: Stella × Beaulieu (1971). Ripening time cherry week 4–5; ca. 19 days after Burlat; about 5 days for Kordia; 2 days before Bing. Fruit characteristics very good fruit quality; large, firm fruits with a deep red color and light colored flesh. Crack sensitivity. Flowering & Pollination Blooms 7–10 days after Burlat; about the same as Kordia. S-Allelen S4'S9. Productivity: Benton® is self fertile. Despite this, the production on strong rootstocks may leave something to be desired. On the other hand, with weaker rootstocks like cvs. Gisela 5 or 6 the production is good to very good with a risk of excessive fruit bearing and therefore smaller fruits. Growth: Benton® is growing fairly strongly; branches well and has a somewhat upright growth habit [1]	
Black Star*	
Mid-season, dark-red skinned, pink-fleshed cherry. Origin: University of Bologna, Bologna, Italy, by S. Sansavini and S. Lugli. Lapins × Burlat; crossed 1985; selected 1992; tested as DCA BO 85.723.002 (D2); introd. 2001. EU PVR 19,951; 2007. Fruit: large; cordate; symmetrical; very firm flesh; very sweet; clingstone; ripens early- to mid-season, 16–18 d after Burlat; medium-long, thick stem; almost fully resistant to rain-induced splitting. Tree: self-fertile; medium-high vigor; upright; spreading; early bloom, 4 days before Burlat; very productive [1]	
Brooks*	
Origin at Wolfskill Ranch, Univ. of California, Davis, by P.E. Hansche, W. Beres, J. Doyle, and W.C. Micke. Introd. in 1988. Rainier × Early Burlat. Plant pat. 6676, 14 Mar. 1989; assigned to Regents of Univ. of California. Fruit: large; skin dark burgundy red, occasionally some streaking and mottling over basal shoulders; flesh color variable with rays and streaks of red and pink, firm; numerous fibers; nearly freestone without air space; susceptible to rain cracking. Flavor exceptional, sweet, well-balanced, rich; not aromatic; overall quality outstanding. Ripens midway between Early Burlat and Bing; fruit hangs well on tree. Tree slightly smaller than most sweet cherries; upright to upright spreading; hardy in San Joaquin Valley; very productive; annual bearing. Bloom date between Early Burlat and Bing [1]	

Carmen*	
Orig. at Fruitculture Research Institute, NARIC, Hungary by J. Apostol. Parentage: Yellow Dragan (Dogans Gelbe) × H-303 (Germersforfer × open pollination (o.p.)). Tree growth: semi-upright, moderate vigor. S-alleles: S4S5. Productivity: medium to good. Blooming time: early/mid-season. Ripening time: 10–12 days after Burlat. Fruit characteristics: flattened, round, very large, firm, deep red. Resistances/specifics: susceptible to cracking [2]	
Celeste® Sumpaca*	
High-quality cherry similar to Van but earlier. Origin: in Summerland, British Columbia, by K.O. Lapins and H. Schmidt, Pacific Agri-Food Research Centre. Introd. in 1993. Canadian Plant Breeders' Rights (#0316) granted in 1997 and USPPAF; assigned to Okanogan Plant Improvement Co. Van × Newstar; cross made in 1974, selected in 1981; tested as 13S-24-28. Registered in Europe in 1995 as Sumpaca. Fruit: moderately large, resembling Van with short stems and slightly *blocky* shape; flesh is dark, firm, sweet; very good flavor; good tolerance to rain-splitting, somewhat prone to pitting; early ripening, ≈5–7 days before Van. Tree: self-fertile, begins bloom 4 days after Van; semi-compact growth habit; moderate productivity [1]	
Chelan*	
Early-ripening dark red cherry, resistant to powdery mildew. Origin: in Prosser, Wash., by Thomas Toyama and Ed Proebsting, Washington State Univ. Introduced in 1991. USPP 8545; assigned to Washington State Univ. Research Foundation. Stella x Beaulieu; cross made in 1971, sel. in 1978; tested as PC 7146-23. Fruit: medium-large to large, smaller when overcropped; round to broadly cordate, with medium long slender stems; glossy, dark mahogany red skin; exceptionally firm, dark red flesh, juicy with good flavor at full ripeness; less than average susceptibility to rain-induced splitting; ripens 10–12 days before Van. Tree: self-infertile (S3S9), incompatible with Tieton and Burlat); blooms early, 3 days before Van; vigorous, upright-spreading growth habit; very productive; resistant to powdery mildew. Cristalina (Sumnue) [1]	

Columbia*	
Self-fertile midseason dark red cherry. Origin: in Prosser, Wash., by Thomas Toyama, Ed Proebsting, and Gregory Lang, Washington State Univ. Introd. in 2000. USPPAF; assigned to Washington State Univ. Research Foundation. Stella × Beaulieu; cross made in 1971, sel. in 1977; tested as PC 7146-8. Fruit: large, heart-shaped, with moderate length stems; dark red skin; firm, dark red flesh, with an excellent sweet-tart flavor; less than average susceptibility to rain-induced splitting; ripens midseason, with Van. Tree: self-fertile, blooms late, 3–5 days after Van; vigorous, upright growth habit; good productivity [1]	
Cowiche® PC7903-2*	
Dark red fruit, large, with very firm flesh, midseason. Origin: Washington State University by T. Toyama, E. Proebsting, G. Lang and M. Whiting. PC7147-4 (Stella × Early Burlat) PC7146-11 (Stella × Beaulieu); tested as PC7903-2; USPP 21,073; 22 June 2010. Fruit: large, cordate; flesh very firm, dark red; skin red-purple; flavor sweet-tart; stem length medium, 40 mm. Tree: vigorous, upright; self-incompatible (S5S9); blooms midseason, just after Bing; ripens midseason, with Bing; very productive [1]	
Cristalina® Sumnue*	
Early-ripening Bing type. Origin: in Summerland, British Columbia, by K.O. Lapins and H. Schmidt, Pacific Agri-Food Research Centre. Introd. in 1996. Canadian Plant Breeder's Rights (#0318) granted in 1997 and USPPAF; assigned to Okanogan Plant Improvement Co. Star × Van; cross made in 1962, selected in 1968; tested as 2C-61-22. Registered in Europe in 1995 as Sumnue. Fruit: moderately large, resembling Bing (heart-shaped); flesh and skin are dark red; firm, moderately sweet; good tolerance to rain-splitting; can be picked stemless; early-to-midseason ripening, ≈5 days before Van. Tree: self-sterile, begins bloom 1 day after Van; spreading branch habit; very productive [1]	

Earlise® Rivedel* (syn. Early Lory®)	
Origin: selected by Pierre Argot, France. Parentage: Starking Hardy Giant × Burlat. Tree growth: semi-upright, medium to strong vigor. S-alleles: S1S9. Productivity: very good. Blooming time: early. Ripening time: 0–4 days before Burlat. Fruit characteristics: reniform round, large, low firmness, red. Resistances/specifics: very susceptible to cracking, not susceptible to mild winters [2]	
Fertard*	
Origin at INRA Grand Ferrade, France. Parentage: Sunburst × o.p. Tree growth: main branches upright, laterals spreading, medium to strong vigor. S-alleles: S3S6. Productivity: medium to good. Blooming time: late to very late. Ripening time: 31–39 days after Burlat. Fruit characteristics: elongated to heart-shaped, large to very large, very firm, dark red. Resistances/specifics: susceptible to cracking, high chilling requirements, sensitive to mild winters [2]	
Folfer*	
Origin at INRA Grand Ferrade, France. Parentage: Arcina® Fercer × o.p. Tree growth: semi-upright, medium to strong vigor. S-alleles: S6S9. Productivity: very good. Blooming time: very early. Ripening time: 7–13 days after Burlat. Fruit characteristics: round, large to very large, very firm, red. Resistances/specifics: susceptible to cracking, double fruit and mild winters [2]	
Frisco*	
Origin at SDR Fruit LLC, California, USA. Parentage: unknown. Tree growth: semi-upright to upright, low vigor. S-alleles: S1S4. Productivity: good. Blooming time: early. Ripening time: 4–7 days after Burlat. Fruit characteristics: reniform, very large, very firm, red to dark red. Resistances/specifics: susceptible to cherry leaf spot [2]	

Giant Red* Giant Ruby®	
Very large, red cherry well-adapted to warmer climates. Origin: in Lodi, Calif., by Marvin Nies. Introd. in 1995. USPP 9659; Large Red × Ruby; cross made in 1976, sel. in 1982; tested as T 8-22-4. Fruit: very large, globose with prominent shoulders, with moderate length stems; shiny dark red skin; firm, dark red flesh, with excellent mild sweet flavor; tolerates high temperatures that cause fruit doubles/spurs; average susceptibility to rain-splitting; early-ripening, 8 days before Van. Tree: self-infertile (S alleles unknown), blooms a week before Van; moderately vigorous, open growth habit with wide-angled, pendulous branching; very good precocity [1]	
Grace Star*	
Mid early season, dark-red skinned, pink-fleshed cherry. Origin: University of Bologna, Bologna, Italy, by S. Sansavini and S. Lugli. Burlat× o.p.; crossed 1984; selected 1992; tested as DCA BO 84.703.003 (F23); introd. 2001. EU PVR 20,804; 2007. Fruit: large; cordate; symmetrical; semi-firm flesh; sweet; semi-clingstone; ripens mid-early season, 12 days after Burlat; long stem; medium resistance to rain-induced splitting. Tree: self-fertile; medium-high vigor; semi-upright; mid-season bloom, 2 days after Burlat; very productive [1]	
Kiona® PC8007-2*	
Dark red fruit, large, early season. Origin: Washington State University by T. Toyama, E. Proebsting, G. Lang and M. Whiting. PC7144-7 (Stella × Early Burlat) PC7144-3 (Stella × Early Burlat); tested as PC8007-2; USPP 20,526; 8 Dec. 2009. Fruit: large, round; flesh firm, red-purple; skin red-purple; flavor excellent; stem medium-long, 43 mm. Tree: semi-vigorous, spreading; self-incompatible (S4S9); blooms late, 6 days after Bing; ripens early, 6–10 days before Bing productive [1]	

Kordia	
As far as fruit characteristics are concerned, Kordia is unmatched so far. The large, dark colored and firm fruits have a very good taste. Due to the high sensitivity to low temperatures in and around the flowering, production is sometimes disappointing. Origin: Found as random seedling in the Czech Republic. Synonyms Techlovicka II, Attika. Ripening in time Cherry week 6. Fruit characteristics: large (Ø 27–29 mm, Ø 10–12 g), heart-shaped, shiny, violet-dark red to black fruits. Very attractive appearance. Firm meat firm, crunchy, very juicy and sweet (15–17 °Brix). Very good taste. Ctifl color: 5–7. Long stems. Crack sensitivity little crack sensitive. Flowering & Pollination: not self-fertile, flowering time: mid-late, S-Allele S3S6. Productivity: early entering production. Production level is strongly influenced by growth level, weather conditions, plot location, pollination etc. Growth with lots of hanging fruit. Tends to scale. Good branching. Particulars sensitive to low temperatures before and during flowering. [1]	
Korvik*	
Dark red fruit, large, late season. Origin: Horice, Czech Republic by V. Ludvik. Kordia × Vic; USPP 22,245; 15 Nov. 2011. Fruit: large, rounded cordate; medium firm; flesh and skin gray purple; sweet, 18 °Brix; stem long, 52 mm. Tree: vigor medium, upright; blooms moderately late, fourth week of April in Horice; ripens late, second week of July; yields very good on dwarfing rootstocks; tolerant to brown rot and cherry leaf spot (*Blumeriella jaapii*), resistant to rain-induced cracking [2]	
Pacific Red*	
Origin at SMS Unlimited, California, USA. Parentage: unknown. Tree growth: shape semi-open, vigor medium-high. S-alelles: S4" S9. Productivity: very high. Blomming time: early. Ripening time: 6 days after Burlat. Fruit characteristics: large (28–32 mm), round-shaped, stem medium, very firm, dark red. Resistances/specifics: relatively tolerant to cracking [2]	
Regina	
Late-season dark red cherry, with excellent tolerance to rain-induced fruit splitting. Origin: in Jork (Hannover), Germany, by Karl-Heinz Tiemann at the Fruit Experiment Station, Jork. Introd. in 1998. USPP 11530; assigned to Jork Fruit Research Station. Schneiders Spate Knorpelkirsche × Rube; tested as Jork 57/201. Fruit: large, slightly heart-shaped, with moderate length stems; attractive dark red skin; very firm, dark red flesh with very good sweet-tart flavor; excellent tolerance to rain-induced splitting; late ripening, 12–16 days after Van. Tree: self-infertile (S1S3), incompatible with Van and Olympus, blooms late, 4–6 days after Van; vigorous, upright growth habit; moderate precocity and productivity [1]	

Rita*	
Orig. at Fruitculture Research Institute, NARIC, Hungary by J. Apostol. Parentage: Trusenskaja 2 × H-2 (Germersdorfer × o.p.). Tree growth: little pendula, medium vigor. S-alleles: S5S22. Productivity: very good. Blooming time: early. Ripening time: 7–14 days before Burlat. Fruit characteristics: flat to round, medium size, firm, deep red. Resistances/specifics: highly susceptible to cracking, susceptible to *Leucostoma* canker [2]	
Rocket*	
Orig. at SMS Unlimited, California, USA. Parentage: unknown. Tree growth: semi-upright to upright, low vigor. S-alleles: S1S9. Productivity: medium to good. Blooming time: early. Ripening time: 4–7 days after Burlat. Fruit characteristics: cordiform, large to very large, very firm, red. Resistances/specifics: susceptible to cherry leaf spot [2]	
Royal Bailey*	
Bright red fruit, large, with firm flesh, early season. Origin: Modesto, CA by G.N. Zaiger, L.M. Gardner and G.G. Zaiger. 22ZB383 (Royal Dawn·Minnie Royal) o.p.; USPP 21,835; 5 Apr. 2011. Fruit: large, cordate; flesh firm, red; skin bright red; sweet,18 °Brix; flavor very good; stem length medium-long, 45 mm. Tree: vigorous, upright; self-incompatible; blooms early midseason, early March in Modesto; ripens early, second week of May, 6 days after Burlat; productive, chilling requirement about 750 h [1]	

Royal Edie*	
Red fruit, very large, with firm flesh, late season. Origin: Modesto, CA by G.N. Zaiger, L.M. Gardner and G.G. Zaiger. 92LB341 (Bing O.P. × Royal Dawn) O.P.; USPP 19,365; 21 Oct. 2008. Fruit: very large; round; flesh very firm, red; skin red; sweet, 19 °Brix; flavor very good; stem medium-long, 47 mm; ripens late, first week of June, 10 days after Bing, in Modesto. Tree: vigorous; upright; self-incompatible; productive; blooms late midseason, third week of March in Modesto; chilling requirement 750 h [1]	
Royal Hazel*	
Red fruit, large, with very firm flesh, low chilling requirement, early season. Origin: Modesto, CA by G.N. Zaiger, L.M. Gardner and G.G.Zaiger. 25Z116 (Bing O.P. ×·Bing O.P.) o.p.; USPP 19,920; 14 Apr. 2009. Fruit: large, round to slightly oblate; flesh firm, red; skin red; sweet, 16 °Brix; flavor very good; stem medium-short, 30 mm; ripens early, second week of May, 12 days before Bing, in Modesto. Tree: vigorous, upright; very productive; self-incompatible; blooms midseason, second week of March in Modesto, 10 day before Bing; chilling requirement 500 h [1]	
Royal Helen*	
Red fruit, very large, very sweet, late midseason. Origin: Modesto, CA by G.N. Zaiger, L.M. Gardner and G.G. Zaiger. 92LB341 (Bing O.P. × Royal Dawn) o.p.; USPP 19,595; 23 Dec. 2008. Fruit: very large, round; flesh firm, red; skin red; very sweet, 22 °Brix; flavor very good; stem long, 53 mm; ripens late midseason, first week of June, 8 days after Bing, in Modesto. Tree: vigorous, upright; self-incompatible; productive; blooms late midseason, third week of March in Modesto; chilling requirement 750 h [1]	

Royal Tioga*	
Red fruit, medium to large, with firm flesh, low chilling requirement, very early season. Origin: Modesto, CA by G.N. Zaiger, L.M. Gardner and G.G. Zaiger. 25Z134 (Bing O.P. × Royal Lee) 6GM73 (Bing O.P. × Minnie Royal USPP 22,779; 12 June 2012. Fruit: medium to large, round; skin red; flesh very firm, red; sweet, 16 °Brix; flavor good; stem length medium, 40 mm; ripens very early, late April/early May, 24 days before Brooks, in Modesto. Tree: vigorous, upright; productive; self-compatible; blooms early, late February/early March in Modesto, 10 days before Bing; chilling requirement 500 h [1]	
Samba® Sumste*	
Midseason, dark red cherry that is self-fertile. Origin: in Summerland, British Columbia, by David Lane, Pacific Agri-Food Research Centre. Introd. in 1996. Canadian Plant Breeders' Rights and USPPAF; assigned to Okanogan Plant Improvement Company. 2S-84-10 × Stella; tested as 13S-36-18. Registered in Europe in 1995 as Sumste. Fruit: large, bright and attractive dark red skin; firm, moderately sweet, with good flavor; good tolerance to rain-splitting; ripens midseason, ≈2 days after Van. Tree: self-fertile, begins bloom 3 days before Van; spurry growth habit; not precocious [1]	
Santina*	
Self-fertile black-skinned cherry. Origin: in Summerland, British Columbia, by K.O. Lapins and H. Schmidt, Pacific Agri-Food Research Centre. Introd. in 1996. Canadian Plant Breeders' Rights and USPPAF; assigned to Okanogan Plant Improvement Company. Stella × Summit; cross made in 1973, selected in 1981; tested as 13S-5-22. Fruit: moderately large, oval with a medium-long stem; black skin; firm flesh, moderately sweet; moderate tolerance to rain-splitting; early-ripening, ≈8 days before Van. Tree: self-fertile, begins bloom 1 day after Van; moderate productivity [1]	

Sequoia® Glenoia*	
Early midseason, dark red-skinned, red fleshed cherry. Origin: Le Grand, Calif., by L. Glen Bradford. Unnamed seedling open pollinated; seed collected in 1993; selected in 1997; introd. in 2003. USPP 15,157. Fruit: large; oblate; dark red skin; red flesh; dark red juice; firm; clingstone; sweet flavor; ripens early midseason (6 days after Brooks); some resistance to rain-splitting (not as resistant as Tulare). Tree: self-infertile (S alleles unknown; Tulare and Glenred recommended as pollinizer); very early bloom (3 and 5 days before Tulare and Brooks, respectively); medium vigor; upright; productive [1]	
Skeena*	
Very firm, late-ripening cherry. Origin: in Summerland, British Columbia, by David Lane, Pacific Agri-Food Research Centre. Introd. in 1997. Canadian Plant Breeders' Rights (#0319) granted in 1998 and USPPAF; assigned to Okanogan Plant Improvement Co. 2N60-07 × 2 N-38-32; tested as 13S-42-48. Fruit: large, round, with thick stems; very attractive dark red to black skin; very firm, moderately sweet; good tolerance to rain-splitting; late-ripening, ≈12–16 days after Van. Tree: self-fertile, blooms with Van; spreading growth habit; precocious and very productive [1]	
Sonata® Sumleta*	
Self-fertile, black-skinned cherry. Origin: Summerland, British Columbia, by David Lane, Pacific AgriFood Research Centre. Introd. in 1996. Canadian Plant Breeders' Rights (#0317) granted in 1997 and USPPAF; assigned to Okanogan Plant Improvement Company. Lapins × 2N-39-5; tested as 13N-6-59. Registered in Europe in 1995 as Sumleta. Fruit: very large, with long and thick stems; attractive black, lustrous skin; very firm, moderately sweet; susceptible to rain-splitting; mid-to-late ripening, ≈7 days after Van. Tree: self-fertile, begins bloom 1 day after Van; good productivity [1]	

Staccato® 13S2009*	
Very late-season, red to dark red-skinned, self-fertile cherry. Origin: Summerland, British Columbia, by David Lane and Frank Kappel. Sweetheart open pollinated; seed collected 1982; selected in 1991; tested as 13S-20-09; introd. in 2000. USPP pending; protected in Canada. Fruit: large to very large; flattened cordate; long stems; shiny red to dark red skin; dark red flesh; red juice; very firm; clingstone; sweet flavor; ripens very late season (27 days after Bing); resistant to rain-induced splitting. Tree: self-fertile (S3S4); midseason bloom (same as Bing); moderately vigorous; upright to spreading; precocious; very productive [1]	
Sweetheart® Sumtare*	
Self-fertile, red-skinned cherry. Origin: in Summerland, British Columbia, by David Lane, Pacific Agri-Food Research Centre. Introd. in 1994. Canadian Plant Breeders' Rights and USPPAF; assigned to Okanogan Plant Improvement Company. Van × Newstar; cross made in 1975; tested as 13S-22-8. Fruit: moderately large, round; red skin and flesh; very firm, moderately sweet with fair to good flavor; moderate tolerance to rain-splitting; may be susceptible to sunscald under very hot conditions; late-ripening, ≈19–22 days after Van. Tree: self-fertile, begins bloom 1 day before Van; spreading growth habit; precocious and very productive [1]	
Sweet Aryana® PA1UNIBO*	
Shiny dark red fruit, large, with firm flesh, early season. Origin: University of Bologna, Italy by S. Lugli, R. Correale and M. Grandi. Parentage unknown; tested as DCABO A1A1; USPP 25,996; 20 Oct. 2015. Fruit: large, rounded cordate; flesh firm, dark red; skin shiny, very dark red; sweet, 18°Brix; stem medium-short, 35 mm. Tree: vigorous, spreading, drooping; self-compatible (S3S4), blooms second to third week of April in Vignola, Italy; ripens 14 days before Bing and 4 days after Burlat; very productive. [1]	
Sweet Gabriel® PA3UNIBO*	
Bright red fruit, very large, with firm flesh, early midseason. Origin: University of Bologna, Italy by S. Lugli, R. Correale and M. Grandi. Parentage unknown; tested as DCABO A1C40; USPP 25,997; 20 Oct. 2015. Fruit: very large, cordate; flesh firm, rose; skin bright purplish-red; sweet, 17°Brix; stem medium short, 34 mm. Tree: vigor moderate to high; habit spreading; self-incompatible (S1S4), blooms second to third week of April in Vignola, Italy; ripens 4 days before Bing; very productive [1]	

Symphony® Selina*	
Self-fertile, late-ripening cherry. Origin: in Summerland, British Columbia, by David Lane, Pacific Agri-Food Research Centre. Introd. in 1997. Canadian Plant Breeders' Rights (#0321) granted in 1998 and USPPAF; assigned to Okanogan Plant Improvement Company. Lapins × Bing; tested as 13S-25-25. Fruit: moderately large; firm, moderately sweet with a pleasant flavor; good tolerance to rain-splitting; late-ripening, ≈20 days after Van. Tree: self-fertile, begins bloom 1 day after Van [1]	
Sweet Lorenz®PA2UNIBO*	
Shiny dark red fruit, very large, with firm flesh, moderately early season. Origin: University of Bologna, Italy by S. Lugli, R. Correale and M. Grandi. Parentage unknown; tested as DCABO A1C27; USPP 25,982; 13 Oct. 2015. Fruit: very large, cordate; flesh firm, rose; skin shiny, very dark red; sweet, 18°Brix; stem length medium, 37 mm. Tree: vigorous; growth habit semi-spreading, branching; self-incompatible (S3S4), blooms second to third week of April in Vignola, Italy; ripens 8 days before Bing; very productive [1]	
Sweet Saretta® PA5UNIBO*	
Bright red fruit, very large, with firm flesh, late midseason. Origin: University of Bologna, Italy by S. Lugli, R. Correale and M. Grandi. Parentage unknown; tested as DCABO B5A87; USPP 25,972; 6 Oct. 2015. Fruit: very large, cordate; flesh firm, red; skin bright red; sweet, 18–19°Brix; stem length medium, 37 mm. Tree: vigorous, spreading; self-compatible (S3S4); blooms second week of April in Vignola, Italy; ripens 5 days after Bing; very productive [1]	

Sweet Valina® PA4UNIBO*	
Bright red fruit, very large, with firm flesh, midseason. Origin: University of Bologna, Italy by S. Lugli, R. Correale and M. Grandi. Parentage unknown; tested as DCABO B5D23; USPP 25,981; 13 Oct. 2015. Fruit: very large, cordate; flesh firm, rose; skin bright red; very sweet, 21°Brix; stem length medium, 40 mm. Tree: vigorous, semi-spreading; self-incompatible (S3S4); blooms second to third week of April in Vignola, Italy; ripens 1 day after Bing; very productive [1]	
Tamara*	
Orig. at Research and Breeding Institute of Pomology, Holovousy, Czech Republic. Parentage: Krupnoplodnaja × Van. S-alleles: S1S9. Productivity: very good. Blooming time: medium late. Ripening time: 28 days after Burlat. Fruit characteristics: very large, wide heart-shaped form, dark to black red, firm, crunchy flesh. Resistances/specifics: relatively tolerant to cracking [2]	

Tehranivee*	
Self-fertile, upright cherry ripening in midseason. Origin: in Vineland Station, Ontario, by Ghassem Tehrani, Horticultural Research Institute of Ontario. Introd. in 1996. Canadian Plant Breeders' Rights (#0327) granted in 1997. Van × Stella; cross made in 1969, selected in 1982; tested as V690620. Fruit: large, flat-round shaped and symmetrical, with a long stem; dark red skin and flesh; moderately firm, juicy, very sweet; good tolerance to rain-splitting; mid-to-late ripening, ≈5 days after Van. Tree: self-fertile, blooms late; upright growth habit; precocious and very productive. Prepared for release under a different name by G. Tehrani and renamed by his colleagues upon his untimely death [2]	
Tieton*	
Very large, glossy, early-ripening cherry. Origin: in Prosser, Wash., by Thomas Toyama, Washington State Univ. Introd. in 1998. USPPAF; assigned to Northwest Nursery Improvement Institute. Stella × Early Burlat; cross made in 1971, selected in 1977; tested as PC 7144-6. Fruit: very large, somewhat blocky heart shape, thick stems; very attractive, glossy dark red skin; firm, moderately sweet with a mild flavor; average susceptibility to rain-splitting; early ripening, 7–9 days before Van. Tree: self-sterile, blooms with Van; upright growth habit; light to moderate productivity [1]	
Vandalay*	
Very sweet, midseason cherry. Origin: in Vineland Station, Ontario, by Ghassem Tehrani, Horticultural Research Institute of Ontario. Introd. in 1996. Canadian Plant Breeders' Rights (#0326) granted in 1997. Van × Stella; cross made in 1969, selected in 1980; tested as V690618. Fruit: large, kidney-shaped and symmetrical, with a medium stem; wine-red skin and dark-red flesh; firm, moderately juicy, very sweet; good tolerance to rain-splitting; early to-mid ripening season, ≈5 days before Van. Tree: self-fertile, blooms early; spreading growth habit; precocious and very productive [1]	

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Chapter 3
Mulberry (*Morus* spp.) Breeding for Higher Fruit Production

Kunjupillai Vijayan, Gopalapillai Ravikumar and Amalendu Tikader

Abstract Mulberry (*Morus* L.; Moraceae), is a fast-growing deciduous woody perennial tree widely present in China, Japan, India, Bangladesh, Pakistan and several other Asian countries. Taxonomically, mulberry belongs to the genus *Morus*. It is believed that mulberry originated in the northern hemisphere, particularly in the Himalayan foothills, and later spread into the tropics of the southern hemisphere. Today, it is present in all regions between 50°N Lat. and 10°S Lat. from sea level to elevations as high as 4000 m. Mulberry has tremendous economic importance as its leaf is used to rear silkworms, to feed animals and fishes and its fruit is highly delicious and contains a number of health-promoting compounds such as sugar, carbohydrate, alkaloids, vitamins, fats, minerals, amino acids, carotenoids, flavonoids, antioxidants etc. Furthermore, the root and stem bark of mulberry has purgative, anthelmintic, and astringent properties and is also a rich source of phenolic compounds such as maclurin, rutin, isoquercitrin, resveratrol and morin. Mulberry is cultivated as a tree, high bush and low bush under rain fed and irrigated conditions. It is propagated by seeds, seedlings, stem cuttings, saplings and grafting. Considering the great impact of industrialization, urbanization and global warming, mulberry, like any other crops, faces challenges from various quarters. In order to sustain mulberry cultivation to provide continuous income to rural people of several countries,

K. Vijayan (✉)
Central Silk Board, BTM Layout, Madiwala, Bangalore 560068, Karnataka, India
e-mail: kvijayan01@yahoo.com

G. Ravikumar
Seri-Biotech Research Laboratory, Central Silk Board, Kodathi, Carmelram Post, Bangalore 560035, Karnataka, India
e-mail: ravikumarpillai@gmail.com

A. Tikader
203 Aranya Enclave, 11th Main, 3rd Cross, Kakappa Layout Begur Main Road, Hongasandra, Bangalore 560068, Karnataka, India
e-mail: atikader_csgrc@yahoo.co.in

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including India and China, it is necessary to develop new mulberry varieties with enhanced productivity and adaptability by employing modern biotechnological tools. In this chapter, we present an overview of the origin, distribution, taxonomic position, genetic resource characterization and conservation, crop cultivation practices and recent developments on biotechnology, and molecular biology and their application for crop improvement in association with conventional breeding methods of mulberry.

Keywords Anthocyanin · Breeding · Fruit · *Morus* · Mulberry · Sericulture

3.1 Introduction

Mulberry (*Morus* L.; Moraceae), is a fast-growing deciduous woody perennial tree widely present in China, Japan, India, Bangladesh, Pakistan and several other Asian countries, in different forms depending on the cultural and training practices (Fig. 3.1). Taxonomically, mulberry belong to the genus *Morus* (Linnaeus 1753), tribe Moreae, family Moraceae and order Urticales (Takhatajan 1980). However, the interspecific relationship of the genus *Morus* is yet to be resolved due to its wider geographical distribution, morphological plasticity, ease of hybridization among species, long history of domestication, and introduction and naturalization of species (Burgess et al. 2005; Vijayan et al. 2011a). Therefore, various studies have recognized different numbers of species (Zeng et al. 2015). Nonetheless, according to the widely-accepted classification of Koidzumi (1917), the genus *Morus* is divided into two sections: *Dolichostylae* (long style) and *Macromorus* (short style); each section is again divided into two groups namely Papillosae and Pubescentae, based on the nature of stigmatic hairs. A total of 24 species are recognized in this classification (Table 3.1). So far, more than 150 species of mulberry have been cited in the Index Kewensis, but most of them have been treated either as synonyms or as varieties (mostly of *M. alba*) rather than species, and some have been transferred to allied genera. For instance, *M. tinctoria* L. and *M. zanthoxylon* L. have been transferred to the genus *Maclura* Nuttal. It is remarkable that *Morus* is the only genus of the Moraceae that has not yet been revised (Berg 2001). Nonetheless, 68 species of mulberry have now been widely recognized and the majority of them are found in Asia, especially in China, Japan, Korea and India (Datta 2000). However, it is important to note that most of these species undergo natural cross-hybridization and produce fertile hybrids (Tikader and Dandin 2007). This high success rate of cross-species reproduction suggests that these species have relatively closer genetic relationships and thus, individual species need further investigation (Wang and Tanksley 1989).

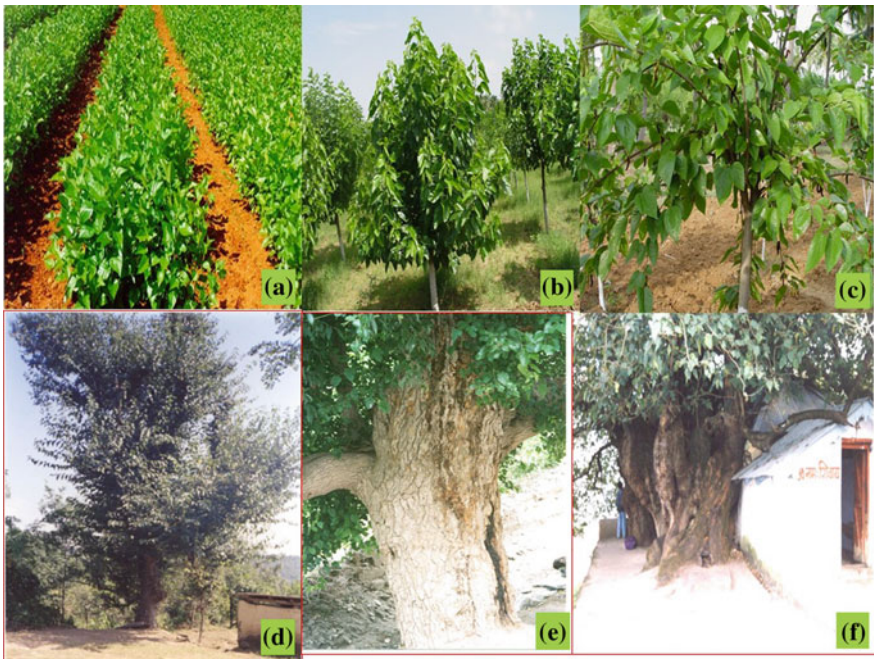

Fig. 3.1 Mulberry exhibits different growth forms depending on the cultural and training practices. **a** *M. alba* in low-bush form. **b** *M. alba* in medium-bush form. **c** *M. laevigata* in high-bush form. **d** *M. laevigata* in tree form. **e** *M. alba* in high-bush form. **f** *M. serrata* in tree form

Table 3.1 Mulberry species recognized by Koidzumi (1917)

Species name	Species name
M. bombycis Koidz.	*M. latifolia* Poir.
M. alba L.	*M. acidosa* Griff.
M. indica L.	*M. rotunbiloba* Koidz.
M. kagayamae Koidz.	*M. notabilis* C. K. Schn.
M. boninensis Koidz.	*M. nigriformis* Koidz.
M. atropurpurea Roxb.	*M. serrata* Roxb.
M. laevigata Wall.	*M. nigra* L.
M. formosensis Hotta	*M. rubra* L.
M. mesozygia Stapf.	*M. celtidifolia* Kunth
M. cathayana Hemsl.	*M. tiliaefolia* Makino
M. microphylla Bickl.	*M. macroura* Miq.
M. rabica Koidz.	*M. multicaulis* Perr.

3.1.1 Origin and Distribution

It is believed that mulberry originated in the northern hemisphere, particularly in the Himalayan foothills, and later spread into the tropics of the southern hemisphere (Benavides et al. 1994). Recent excavations of early tertiary Moraceae fossils also strengthen the hypothesis that mulberry originated in the northern hemisphere (Collinson 1989). Molecular biological studies also support this view of an early diversification of Moraceae in Eurasia and subsequent migration into the southern hemisphere (Zerega et al. 2005). The temperate origin of mulberry is further evident from the nature of the growth as at the end of each growing season the apical meristems of the long shoots abort and abscise. Today, mulberry is present in all regions between 50°N Lat. and 10°S Lat. (Yokoyama 1962), from sea level to elevations of 4000 m (Machii et al. 1999a), which include Asia, Europe, North and South America, and Africa (Fig. 3.2). Continental America has four species viz., *Morus insignis*, *M. celtidifolia*, *M. corylifolia* Kunth and *M. mexicana* Benth. In India, there are many species, of which *M. alba* and *M. indica* are fully domesticated while *M. serrata* and *M. laevigata* grow wild in the Himalayas. China has 24 species but only four species viz., *M. alba*, *M. multicaulis*, *M. atropurpurea* and *M. mizuho* Hotta are largely cultivated for sericulture purposes and the remaining are considered wild species. In Japan, out of its 19 species only *M. alba*, *M. bombycis* and *M. latifoila* are cultivated. In Africa, *M. mesozygia* has been reported to occur in humid, sub-humid and semiarid areas. According to Sharma et al. (2000) white mulberry is native to China but has spread into several other countries as shown in Fig. 3.3. Similarly, *M. nigra* is called *black mulberry* is native to Iran and is cultivated for its fruits in Southern Europe, Southwest Asia and is the most important species in the Mediterranean countries (Tutin 1996). *Morus rubra* is called *red mulberry* and is native to North America, and it has been cultivated in America since colonial times. Similarly, *M. tartarica* L. is called as *Russian mulberry*, *M. serrata* as *Himalayan mulberry*, *M. mesozygia* as *African mulberry*, *M. celtidifolia* as *Mexican mulberry*, *M. microphylla* as *Texas mulberry* and *M. australis* Poir. as *Chinese mulberry*.

3.1.2 Economic Importance

Mulberry has tremendous economic importance as is used for various purposes. In most Asian countries, the main purpose for growing mulberry is to provide leaves for rearing silkworms to produce lustrous silk fiber by caterpillars of the silk-producing insect *Bombyx mori* L., which only feeds on mulberry leaves (Vijayan et al. 1997). Mulberry leaves are also used as animal fodder because they are highly nutritious, palatable and digestible (70–90%) to herbivorous animals (Sanchez 2000a, b) and is used as supplementary feed for cows to increase milk output considerably (Uribe and Sanchez 2001). Mulberry is an ideal tree species for city landscaping due to its excellent features in tree form, leaf color, growth vigor, tenacity and resistance. As a

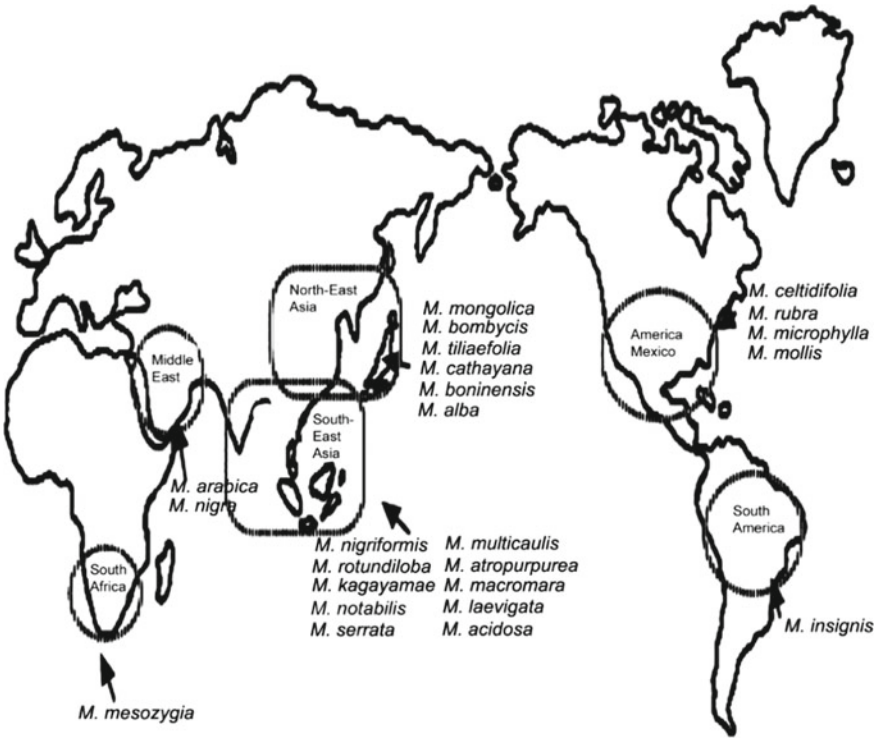

Fig. 3.2 Geographical distribution of *Morus* spp. showing their great diversity in North and South Asia

deciduous arbor species, mulberry is resistant to drought and flood. It can be planted along riversides, at field edges, on slopes, at garden corners, along roadsides, in public parks and other recreational places (Qin et al. 2012). Mulberry is also equally useful in water conservation and prevention of soil erosion as it has a very strong greatly tangled densely networked root system capable of withstanding sandstorms and conserving water and soil (Qin et al. 2012). However, one of the most promising potential areas of the use of mulberry is use of its fruit which is highly delicious and contains a number of health-promoting compounds. These include sugar, carbohydrate, alkaloids, vitamins, fats, minerals, amino acids, carotenoids, flavonoids, antioxidants etc. (Tables 3.2, 3.3, 3.4 and 3.5; Asano et al. 2001; Ercisli and Orhan 2007; Hassimotto et al. 2007; Singhal et al. 2009, 2010; Yang et al. 2010; Yigit et al. 2010). Mulberry fruit is a sorosis composed of a collection of individual fruits (achens). The ripe fruit is succulent, fat and full of juice. The color of the ripe fruit varies greatly from white to black with different color shades (Fig. 3.4). White mulberry (*Morus alba*) fruits are generally very sweet, red mulberry (*M. rubra*) fruits are sweet and usually deep red or almost black. Black mulberry (*M. nigra*) fruits are attractive, large and juicy; with a good balance of sweetness and tartness making them the best-flavored fruits

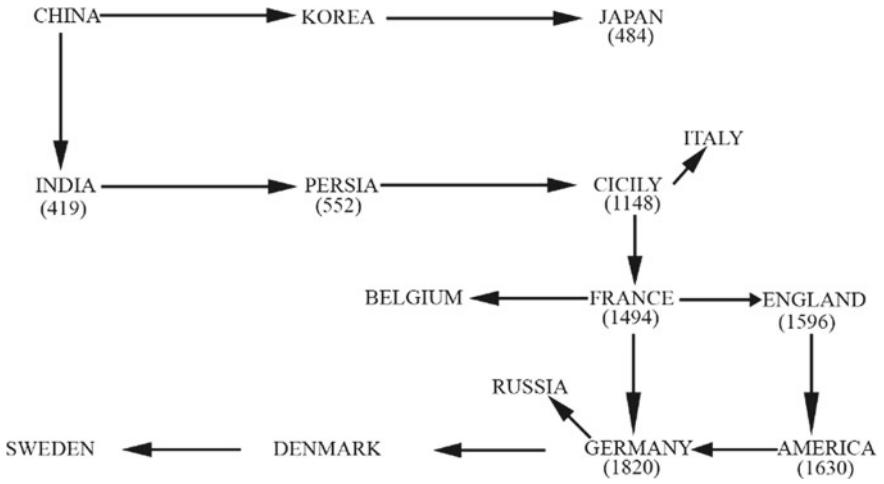

Fig. 3.3 A line diagram showing the domestication of the most widely grown mulberry species, *M. alba*, a native of China and its spread to India, Europe and the Americas

in mulberry. The ripe fruits of all mulberry species are very perishable and must be handled carefully while harvesting and subsequent processing, transportation and marketing. Fresh mulberry fruits contains nearly 20 mg/100 g ascorbic acid (Chu et al. 2001). The antioxidant compounds such as anthocyanins, resveratrol, zeaxanthin, litein and alpha and beta carotiensare, apigenin, luteolin, quercetin, morin, caffeic acid, gallic acid, rutin, umbelliferone, chlorogenic acid and kaempferol are also present in the fruit (Arfan et al. 2012). Anthocyanins have antineoplastic, radiation-protective, vasotonic, vasoprotective, anti-inflammatory, chemopreventive and hepato-protective properties (Krishnan et al. 2015). Mulberry fruit also contains essential fatty acids like palmitic, oleic and linoleic acids which are important for cell membrane formation, proper development and functioning of the nervous system, production of eicosanoids and many anti-inflammatory responses (Ercisli and Orhan 2007).

The alkaloid contents of mulberry fruits range from (390 ± 3.22) mg/100 g FW in *Morus laevigata* to (660 ± 5.25) mg/100 g FW in *M. alba* (Table 3.3; Imran et al. 2010). It is estimated that 1 L of mulberry fruit juice contains 148–2725 mg of anthocyanins; the content depending on climate, area of cultivation, and is particularly higher in sunny climates (Kumar and Chauhan 2011).The total carotenoids content of mulberry fruits collected from India varied between 0.006 and 0.010 mg/gm (Table 3.5; Kumar and Chauhan 2011). Mulberry fruits have been used medicinally as a worming agent, as a remedy for dysentery, and as a laxative, hypoglycaemic, expectorant, anthelmintic, odontalgic and emetic (Chen et al. 2006; Kang et al. 2006). Hong et al. (2004) found that mulberry fruit strengthens the antioxidative defense system and reduces damaging oxidative substances in the erythrocytes of diabetes. According to Kang et al. (2006) the cyanidin-3-O-β-D-glucopyranoside present in

Table 3.2 Chemical composition of mulberry (*M. indica*) fruit

Chemical constituents	Quantity
Carbohydrates	7.8–9.0%
Protein	0.5–1.4%
Fatty acids (linoleic, stearic and oleic acids in seeds	0.3–0.5%
Free acid (mainly malic acid)	1.1–1.8%
Fiber	0.9–1.3%
Ash	0.8–1.0%
Moisture	85–88%
Calcium	0.17–0.39%
Potassium	1.00–1.49%
Magnesium	0.09–0.10%
Sodium	0.01–0.02%
Phosphorus	0.18–0.21%
Sulfur	0.05–0.06%
Iron	0.17–0.19%
Carotene	0.16–0.17%
Ascorbic acid	11.0–12.5 mg/100 g
Nicotinic acid	0.7–0.8 mg/100 g
Thiamine	7.0–9.0 μg/100 g
Riboflavin	165–179 μg/100 g

Source Imran et al. (2010)

the fruits of mulberry prevents the neuronal cell damage. To keeping these properties of mulberry fruit, it is consumed fresh, used for making juice, jelly, sauce, cakes, tea, fruit powder, beverages like Mouro, wine, etc. Recently, mulberry juice has gained an important position in the Korean soft drink market (Bae and Suh 2007).

Furthermore, the bark of mulberry roots and stems have purgative, anthelmintic, and astringent properties, and is also a rich source of phenolic compounds such as maclurin, rutin, isoquercitrin, resveratrol and morin (Chang et al. 2011). Leaves of mulberry are considered disphoretic and emollient. A decoction of mulberry leaves can also be used as a medicine for throat inflammation (Reed 1976). Mulberry leaves have long been used in Chinese medicine for the prevention and treatment of diabetes; they contain some compounds which suppress high blood sugar levels (Andallu et al. 2001). The root bark contains calcium malate; the bark of the branches contains tannins, phlobaphens, a sugar and phytosterol, ceryl alcohol, fatty acids and phosphoric acid (Wu et al. 2010). An alkaloid, deoxynojirimycin (DNJ) extracted from the root bark of the wild mulberry *Morus nigra* interferes with the synthesis of sugar chains and is supposed to be the *magical* drug for diabetes (Hansawasdi and Kawabata 2006). Mulberry is also used for landscaping in Europe and the United States (Tipton 1994).

Table 3.3 Chemical composition in the fruits of a few *Morus* species

Species	Total sugar (g/100 gFW)	Reducing sugar (g/100 gFW)	Non reducing sugar (g/100 gFW)	Riboflavin (g/100 gFW)	Niacin (g/100 gFW)	Ascorbic acid (g/100 gFW)	Total phenols (g/100 gFW)	Alkaloid (g/100 gFW)
M. alba	7.55 ± 1.01	5.90 ± 0.92	1.65 ± 0.11	0.088 ± 0.001	3.10 ± 0.06	15.20 ± 1.25	1650 ± 12.25	660 ± 5.25
M. nigra	6.64 ± 1.2	4.94 ± 0.73	1.70 ± 0.15	0.040 ± 0.000	1.60 ± 0.10	15.37 ± 0.89	880 ± 7.20	630 ± 5.93
M. laevigata (white fruit)	10.89 ± 1.91	8.11 ± 1.10	1.78 ± 1.21	0.055 ± 0.002	0.40 ± 0.03	16.35 ± 1.80	1300 ± 8.53	390 ± 3.22
M. laevigata (black fruit)	8.18 ± 1.83	6.62 ± 0.97	1.56 ± 0.17	0.072 ± 0.003	0.85 ± 0.11	17.03 ± 1.71	1100 ± 9.65	630 ± 4.95

Source Imran et al. (2010)

Table 3.4 Mineral composition in the fruits of a few *Morus* species

Species	K (g/100 g)	Ca (g/100 g)	Na (g/100 g)	Mg (g/100 g)	Fe (g/100 g)	Zn (g/100 g)	Ni (g/100 g)
M. alba	1731 ± 11.50	576 ± 7.37	280 ± 3.50	240 ± 3.90	73.0 ± 2.60	50.20 ± 1.93	50.20 ± 1.93
M. nigra	120 ± 9.36	470 ± 6.95	272 ± 5.32	240 ± 3.51	77.6 ± 1.98	59.20 ± 2.25	1.60 ± 0.11
M. laevigata (white fruit)	1650 ± 8.86	440 ± 3.21	260 ± 3.86	360 ± 4.20	48.6 ± 2.32	53.40 ± 2.43	1.20 ± 0.05
M. laevigata (black fruit)	1644 ± 13.10	576 ± 4.85	264 ± 3.37	240 ± 2.90	63.6 ± 1.96	50.80 ± 1.22	1.80 ± 0.09

Source Imran et al. (2010)

Table 3.5 Biochemical constituents in the fruits of a few Indian mulberry varieties

Sl. no	Mulberry varieties	Protein (mg/gm)	Carbohydrate (mg/gm)	Carotenoid (mg/gm)	Anthocyanin (mg/gm)	Lipids (mg/gm)
1	AR-12	0.237	0.521	0.010	102.413	0.920
2	S-54	0.185	0.582	0.007	90.550	0.627
3	S-13	0.246	0.415	0.006	21.887	1.110
4	S-36	0.281	0.655	0.009	93.387	0.567
5	Br-2	0.227	0.373	0.009	98.653	0.783
6	S-146	0.166	0.443	0.008	102.763	0.670
7	S-1	0.128	0.478	0.007	19.943	0.863

Source Kumar and Chauhan (2011)

Fig. 3.4 Interspecific and intraspecific variability in the size and color of fruits in a few mulberry species. **a** *M. indica*. **b** *M. indica*. **c** *M. alba*. **d** *M. laevigata*. **e** *M. laevigata*. **f** *M. serrata*

Mulberry wood has also been used for manufacturing sports articles, turnery items, house construction, agricultural implements, furniture, spokes, poles, shafts and bent parts of carriage and carts; it is comparable to teak as regards shock resistance ability, strength, hardness etc. The wood is also found suitable for low-grade plywood and for paneling, carving, tea boxes and toys. Since all parts of the mulberry is useful to humankind. It is known in India as "KalpaVruksha" which means a divine tree providing everything one wants (Sharma et al. 2013).

3.1.3 Domestication, Selection and Early Improvements

Domestication of mulberry is believed to have taken place in China sometime before 2200 BC (FAO 1990) and later expanded to other countries and currently is grown in several Asian countries. However, only a small number of the *Morus* species are used for commercial purposes; most are still wild plants. Due to the economic importance of mulberry, China has made efforts to develop high-yielding mulberry varieties from among the more than 1000 cultivated varieties known. Most of them originate from the four main species: lu mulberry (*M. multicaulis*), white mulberry (*M. alba*), mountain mulberry (*M. bombycis*) and Guangdong mulberry (*M. atropurpurea*). In India, there are several species, of which *M. alba*, *M. indica*. *M*. *serrata* and *M. laevigata* grow wild in the Himalayas. Several varieties have been introduced including *M. multicaulis*, *M. nigra*, *M. sinensis* and *M. latifolia.*

Thus, it is seen that mulberry is a multipurpose tree being grown extensively in several countries. Although, the major purpose for mulberry growing is to use the leaf for silkworm rearing, mulberry fruit has considerable potential which is yet to be realized. This chapter provides an overview of the methods for improvement of mulberry by traditional and modern biological means with special emphasis on enhancing fruit production.

3.2 Cultivation and Traditional Breeding

3.2.1 Current Cultivation Practices

Mulberry is propagated by seeds, seedlings, stem cuttings, saplings and grafting. Although, seed is the least expensive and most rapid mode of propagation, it is seldom used in most of countries due to the lack of inbred lines and the high heterozygosity of the parents. Thus, the most widely used method is through stem cuttings; in this manner the desirable characteristics of the parent plants are perpetuated without alteration. Stem cuttings of 22–25 cm length and ~50 mm diameter with 5–6 healthy buds from 8–10 months old plants are ideal for planting. Generally, three cuttings are planted in a pit, in a triangular form with spacing of 15 cm, leaving only one bud exposed above the soil surface. The success of rooting from stem cuttings depends on the genotype, environmental factors and the physiological state of the cuttings (Narayan et al. 1989). In general, 60–80% success can be obtained if appropriate cultural practices are employed. Saplings are commonly used in areas where irrigation facilities are limited. Saplings from stem cuttings of above-mentioned size and age are raised in nurseries with adequate care. Six month to one-year-old saplings are ideal for planting. In a single pit, a single sapling is planted. Since most of the temperate mulberry species are hard to root from stem cuttings, grafting is adopted for their propagation. Sprig budding is the most common method where a T-shaped cut is made in the rootstock and a smooth, sloping cut made on the lower end of

the scion. The scion is then inserted into the T, wrapped, and sealed. The successful graft union depends on the close matching of the callus producing tissues near the cambium layers and the compatibility of the materials used as stock.

3.2.2 Current Agricultural Problems and Challenges

The impact of the recent economic boom, industrialization and urbanization and the resulting unavoidable reduction of productive agricultural lands are affecting mulberry cultivation considerably as it has to compete with other agricultural crops for the available arable land. Furthermore, global warming and consequent drought, the appearance of new pests and insects and their changing host preferences put extra pressure on new varieties to overcome such stress conditions. Keeping this in view, efforts have been made to develop new varieties suitable for wider cultivation.

3.2.3 Improvement of Strategies

Major strategies for improving mulberry have so far been confined to a well-defined set of activities related to leaf quality and include collection and evaluation of germplasm, selection of suitable parents, hybridization between selected parents, collection of seed and raising of seedlings in the nursery, and transplantation to progeny row-trail plots for initial screening and selection. Since almost all mulberry genotypes are highly heterozygous, and have a long gestation period, traditional breeding methodologies have mostly relied on the production of F1 hybrids (Das 1984). Hybrids with desirable traits, identified through progeny row trials, are further evaluated in primary yield trials (PYT) for important agronomic, biochemical and silkworm feeding qualities. From the PYT, the top 5–10% of hybrids are selected for detailed assessment in a final yield trial (FYT) using 3–5 replications and 25–49 plants per replication. Here, the plants are subjected to thorough assessment for leaf yield, leaf quality, adaptation, susceptibility to pest and diseases, rooting ability, response to agronomic practices, and silkworm feeding qualities. The best hybrid is selected and mass multiplied vegetatively for further testing at different regions, multi-location trials (MLT). Usually 8–9 hybrids are used for MLTs and those hybrids which perform consistently well in all the seasons, locations and years are selected. In general more than 15 years are required to develop a mulberry variety (Fig. 3.5). Since most of the phenotypic traits used for selection are highly influenced by the environment and growth stages of the plant, recently efforts were made to employ more stable, robust and environmentally-less influenced genotypic traits for selection, especially for stress-related traits.

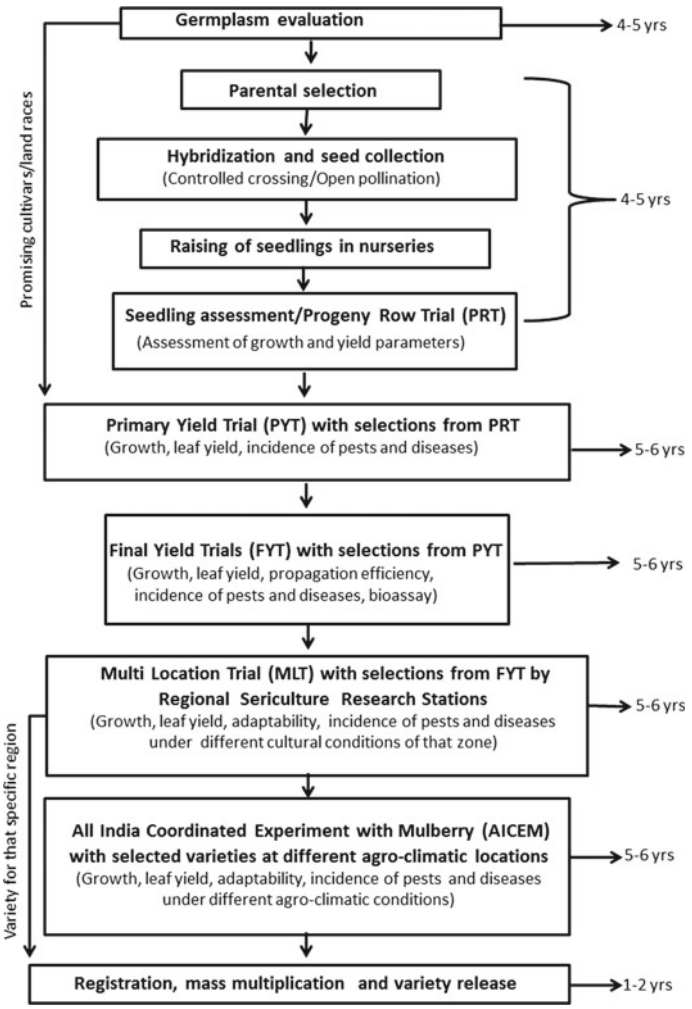

Fig. 3.5 Schematic representation of mulberry variety development in the context of Indian sericulture

3.2.4 Traditional Breeding Methodologies and Limitations

Mulberry breeding has traditionally focused on improvement of polygenic traits, as most of the agronomically important traits are polygenic. Since the leaf is the primary product of mulberry in most of the Asian countries, leaf productivity has been the principal trait targeted by most breeding schemes (Tikader et al. 2009; Vijayan et al. 1997). However, efforts were also made to identify high fruit yielding mulberry varieties (Table 3.6). The other important traits that have been targeted by the mulberry breeders are adaptability, resistance to pests and diseases, tolerance to abiotic

Table 3.6 Mulberry accessions identified in Central Sericulture Germplasm and Research Centre (CSGRC) Hosur, India that have high fruit-yielding potential

Sl. no	Accession no of CSGRC Hosur	Color	Taste	Avg. branch/ plant	Inter nodal distance (cm)	Avg. fruit length (cm)	Wt. of single fruit (g)	Wt. of 10 fruits (g)
1	MI-0020	Black	Sour	38	4.8	1.5		
2	MI-0056	Purple	Sweet	26	6.3	1.5	0.82	6.32
3	MI-0062	Black	Sour	17	6.3	2.3	1.82	13.48
4	MI-0118	Purple	Sweet	33	4.8	3.0	1.42	14.30
5	MI-0155	Black	Tasteless	26	4.8	3.0	1.33	13.00
6	MI-0171	Black	Sweet	10	3.8	5.0	3.14	26.00
7	MI-0188	Black	Sweet	16	5.5	3.0	3.02	21.00
8	MI-0189	Black	Sweet	18	6.3	3.0	3.12	31.00
9	MI-0209	Black	Sour	31	5.7	2.5	2.20	17.00
10	MI-0249	White	Sweet	30	05.1	3.0	1.40	13.90
11	MI-0266	Black	Sour	33	3.1	2.3	1.20	13.00
12	MI-0300	Black	Sweet	22	4.3	5.6	3.12	31.90
13	MI-0380	Pink	Sweet	15	7.3	7.0	4.40	38.40
14	MI-0420	Black	Sweet	17	6.7	3.4	2.50	24.50
15	MI-0427	Black	Sweet	10	7.6	7.0	2.90	28.54
16	MI-0495	Black	Sour	32	5.3	4.0	2.30	18.00
17	MI-0496	Black	Sour	37	5.2	3.8	1.60	17.00
18	MI-0497	Black	Sweet	34	6.0	2.2	1.40	13.90
19	MI-0508	Black	Sour	26	5.6	4.1	3.70	31.00
20	MI-0512	White	Sweet	46	3.8	2.5	1.50	14.00
21	MI-0516	Black	Sweet	39	3.5	2.5	1.50	14.20
22	M-0518	Black	Sour	29	5.5	4.0	2.00	20.50
23	MI-0632	Black	Sweet	19	5.5	2.5	0.80	6.50
24	MI-0777	Black	Sweet	23	5.8	5.0	3.24	25.80
25	MI-0789	Black	Sweet	28	5.8	5.0	3.50	27.70

stresses like drought, salinity and cold, higher vegetative propagation ability, better leaf quality, and better coppicing ability. However, a lack of adequate information on genetic control of the traits, long juvenile period, predominantly out-crossing breeding system, high inbreeding depression and high heterozygosity of the plant remain as major bottlenecks for the development suitable mulberry varieties (Vijayan et al. 2012).

3.2.5 Role of Biotechnology

Biotechnological tools have been applied effectively and efficiently to enhance the pace of crop improvement in mulberry. Significant contributions have been made to enhance the yield potential, resistance to pests and diseases, tolerance to abiotic stresses and resistance to herbicides of the crop. Some of the important contributions of biotechnology in mulberry include micropropagation of hard-to-root genotypes (reviewed by Vijayan et al. 2011b), plant regeneration from young leaves (Chitra and Padmaja 2005), cotyledons and hypocotyl regions (Bhatnagar et al. 2001) and stem segments (Lalitha et al. 2013). Tissue culture has also been applied to screen genotypes for stress tolerance (Vijayan et al. 2003). In vitro techniques have been utilized to develop tetraploids through repeated use of colchicine (Chakraborti et al. 1998). Furthermore, molecular markers have been used for germplasm characterization in mulberry as detailed elsewhere in this chapter.

3.3 Germplasm Diversity and Conservation

Germplasm is the raw material plant breeders use to develop new varieties and it consists of diverse types of collections such as primitive cultivars, natural hybrids, wild and weedy relatives, wild species, obsolete varieties, elite lines, breeding lines, mutants and polyploids, interspecific and intergeneric hybrids developed systematically (Haussmann et al. 2004).

3.3.1 Germplasm Diversity

A proper understanding of genetic variation within and between species and populations, as well as distribution of genetic diversity helps germplasm curators and breeders to devise better conservation strategies and efficient utilization of the valuable plant genetic resources. Using morphological traits, including fruit characters, genetic divergence in different *Morus* species and accessions have been analyzed (Tikader and Rao 2002; Tikader et al. 1995). It has been found that the inflorescence of *M. laevigata* and *M. serrata* are larger than other cultivated species in India and the genetic distance between *M. serrata* and *M. indica* (0.465) was the highest and that of *M. serrata* and *M. alba* (0.457), and *M. serrata* and *M. macroura* (0.401), were the second and third highest, respectively. The interspecific distance between *M. indica* and *M. alba* (0.168) was the least. Similarly, Machii et al. (1999b) recorded the fruit characters of 260 accessions of mulberry in Japan and found that a wide range of variation exists among the mulberry accessions and genotypes such as Okaraguwa and Kataneo, which have excellent traits as fruit trees. Since morphological traits are often misleading due to excessive environmental influence, stable and easily

observable markers are essential to characterize a large number of germplasm accessions. One of the convenient methods of assessing genetic diversity in plant population is the utilization of genes and gene products such as isozymes. Rao et al. (2011), using nine isozymes viz. peroxidase (PRX), α and β esterase (EST), amylase (AMY), acid phosphatase (ACP), malate dehyrogenase (MDH), superoxide dismutase (SOD), polyphenol oxidase (PPO) and diaphorase (DIA), studied the genetic diversity among 14 mulberry species (Fig. 3.6). Similarly, DNA markers such as random amplified of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeats (ISSR) and simple sequence repeats (SSR) were used for assessing the genetic diversity among mulberry genotypes (Table 3.7). The phylogenetic tree constructed with internal transcribed spacer (ITS) of rDNA showed that the genus *Morus* should be classified into eight species, including *M. alba*, *M. nigra*, *M. notabilis*, *M. serrata*, *M. celtidifolia*, *M. insignis*, *M. rubra* and *M. mesozygia* (Zeng et al. 2015). Furthermore, recent advances in sequencing and annotation have made it possible to sequence the genome of *M. notabilis* (He et al. 2015) to open new vistas in mulberry research. *Morus notabilis* has a 330-Mb genome containing 29,338 genes and 128 Mb repetitive sequences. Mulberry gene sequences appear to evolve ~3 times faster than other Rosales, perhaps facilitating the species' spread worldwide. Furthermore, the chloroplast genome of mulberry has also been sequenced (Ravi et al. 2008). The sequence information generated from the genomic/chloroplast data will help to characterize the resources of mulberry for better conservation and utilization.

3.3.2 Genetic Resources Conservation Approaches

Conservation of mulberry genetic resources is becoming more important than ever because of the increasing threat from urbanization, climatic changes, outbreak of new diseases and pests, and the frequent occurrence of natural calamity increases due to global warming and rapid industrial growth. Conservation strategies for vegetatively-propagated plants are different from those adopted for conserving seed crops. Being propagated mainly through stem cuttings, the methods used for conservation of vegetatively-propagated plants are equally applicable to mulberry (Fig. 3.7). The advantages and disadvantages of these methods are given in Table 3.8.

3.3.2.1 In Situ Conservation

Wild mulberry species and weedy relatives are conserved and maintained in their original habitats, in situ conservation, as natural areas are rich in genetic diversity (Tikader and Thangavelu 2002). The major categories of in situ conservation approaches are habitat conservation, and establishment of biosphere reserves, national parks and gene sanctuaries. Examples of such conservation strategies in India are the Namdapha Biosphere Reserve of India in Arunachal Pradesh for the

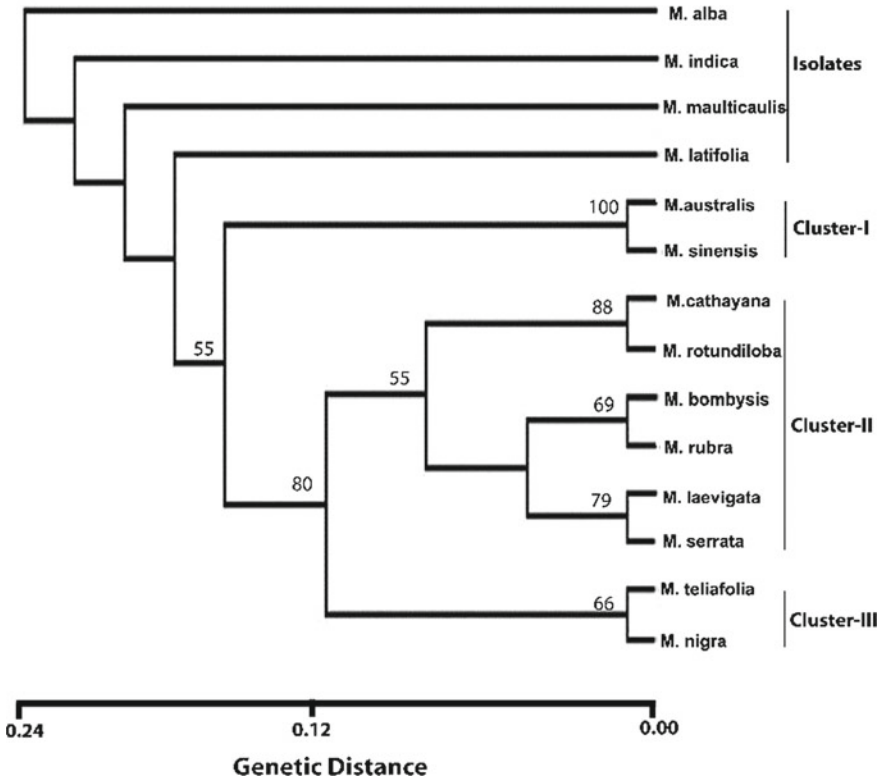

Fig. 3.6 Genotypic relations elucidated with isozymes in mulberry

medicinal herb *Coptis teeta*; Demabeyang Valley in North Sikkim for *Panax pseudoginseng* and Nanda Devi Biosphere for Rhododendron, pine etc. including *Morus serrata* (Tikader et al. 2000).

3.3.2.2 Ex Situ Conservation

The conservation of genetic resources, away from their natural habitat, in botanical gardens, research institutes, experiment stations, nurseries or home gardens and seed gene banks is called ex situ conservation (Tikader et al. 2009). Ex situ field gene banks of mulberry are developed through planting of stem cuttings/saplings or by grafting the buds on appropriate rootstocks (Tikader and Thangavelu 2006).

Table 3.7 Molecular markers used for genetic characterization in *Morus* spp.

Sl. no	Marker type	Purpose	Country	References
1	ISSR	Cultivar analysis for crop improvement	India	Vijayan and Chatterjee (2003)
2	ISSR, RAPD	Genetic diversity in mulberry (genus *Morus*)	India	Awasthi et al. (2004)
3	ISSR, RAPD	Genetic relationship of Indian and Japan accessions	India	Vijayan (2004)
4	ISSR, RAPD	Phylogentic relationship among 5 species	India	Vijayan et al. (2004a)
5	ISSR, RAPD	Genetic diversity among wild populations of mulberry	India	Vijayan et al. (2004b)
6	ISSR, RAPD	Genetic diversity among indigenous to India	India	Vijayan et al. (2005)
7	ISSR	Genetic relatedness among cultivated and wild mulberry	China	Zhao et al. (2006)
8	ISSR	Molecular characterization and identification of markers	India	Vijayan et al. (2006a)
9	ISSR, RAPD	Genetic relationships between wild and cultivated mulberry (*Morus*) species	India	Vijayan et al. (2006b)
10	ISSR, RAPD	Relationship among wild and domesticated species	India	Vijayan et al. (2006c)
11	RAPD	Genetic variability and phylogenetic relationship among 15 white mulberry genotypes	Turkey	Orhan et al. (2007)
12	ISSR	Genetic diversity among 66 local varieties belonging to 8 populations	China	Zhao et al. (2007a)
13	ISSR and SSR	Genetic diversity among 27 mulberry accessions including 19 cultivated and 8 wild accessions	China	Zhao et al. (2007b)
14	ISSR	Phylogenetic relationship among 18 germplasm collections and association with biochemical parameters	India	Kar et al. (2008)
15	AFLP	Genetic variability among 43 accessions belonging to *M. alba*, *M. nigra* and *M. rubra*	Turkey	Kafkas et al. (2008)
16	ISSR	Genetic diversity among ecotypes	China	Zhao et al. (2008)

(continued)

Table 3.7 (continued)

Sl. no	Marker type	Purpose	Country	References
17	RAPD	Molecular characterization of interspecific and intraspecific hybrids	India	Tikader and Dandin (2008)
18	AFLP	DNA fingerprinting of ten cultivars	China	Huang et al. (2009)
19	ISSR and RAPD	Association with sprouting and sex expression traits	India	Vijayan et al. (2009b)
20	SRAP	Genetic diversity among 23 mulberry germplasm accessions from China	S Korea	Zhao et al. (2009)
21	RAPD	Phylogenetic relationship among 47 genotypes	Turkey	Ozrenk et al. (2010)
22	ISSR	Genetic diversity among 73 local mulberry varieties for development of core collection	China	Lin et al. (2011)
23	RAPD	Genetic variability among control and ethyl methane sulfonate (EMS) treated clones of mulberry genotype RFS135	India	Anil Kumar et al. (2012)
24	RAPD and ISSR	Genetic diversity and phylogenetic relatedness among 20 mulberry varieties	India	Chikkaswamy and Prasad (2012)
25	RAPD and ISSR	Genetic diversity among 20 mulberry varieties	India	Chikkaswamy et al. (2012)
26	RAPD and ISSR	Genetic diversity among 21 mulberry genotypes collected from 4 geographic regions of Turkey	Turkey	Ipek et al. (2012)
27	RAPD	Genetic diversity among 36 genotypes collected from South India	India	Naik et al. (2002)
28	RAPD, ISSR and SSR	Standardization of novel and efficient DNA extraction protocol	India	Anuradha et al. (2013)
29	RAPD and ISSR	Genetic stability of cryo-preserved dormant buds of different *Morus* species belonging to indigenous and exotic collection	India	Choudhary et al. (2013)

(continued)

Table 3.7 (continued)

Sl. no	Marker type	Purpose	Country	References
30	SSR	Genetic diversity among 36 mulberry genotypes ('breeders' collections)	India	Krishnan et al. (2013)
31	RAPD	Genetic diversity among nine mulberry genotypes with contrasting traits for water use efficiency (WUE)	India	Mishra et al. (2013)
32	SSR	Genetic diversity among ten accessions belonging to *M. alba* and *M. indica*	Kenya	Wangari et al. (2013)
33	SSR	Phylogenetic relatedness among 17 mulberry genotypes	India	Wani et al. (2013)
34	SSR	Assessed the hybrid nature of two high yielding mulberry varieties	India	Arora et al. (2014)
35	RAPD, ISSR and SSR	Genetic diversity among 850 germplasm accessions collected from 23 countries for development of core collection of diverse accessions	India	Guruprasad Krishnan et al. (2014)
36	SSR	Genetic diversity in wild mulberry species of India	India	Naik et al. (2015)
37	ISSR, RAPD	Genetic diversity and relationship of mulberry		Banerjee et al. (2016)
38	RAPD and ISSR	Genetic fidelity of in vitro regenerated mulberry plants (cv. S1)	India	Saha et al. (2016)

Source Adopted from Sarkar et al. (2017)

3.3.2.3 In Vitro Conservation

Under in vitro conservation, plant parts are conserved using tissue culture. In general, two types of in vitro conservation methods are usually employed for conserving mulberry genetic resources. They are the slow growth method and the cryopreservation method. In the slow growth method, shoot tips or axillary buds are cultured under conditions which keep the plant alive but retard the growth significantly. Under cryopreservation, the plant materials are stored at −196 °C in liquid nitrogen. At this temperature, cell division and metabolic activities remain suspended and the material can be stored without any genetic changes for long periods. Two different techniques are used for cryopreservation of plant materials: the classical method and the vitrification method (Engelmann 2000). Classical cryopreservation techniques involve slow cooling at a controlled rate (usually 0.1–4 °C/min) down to about −40 °C,

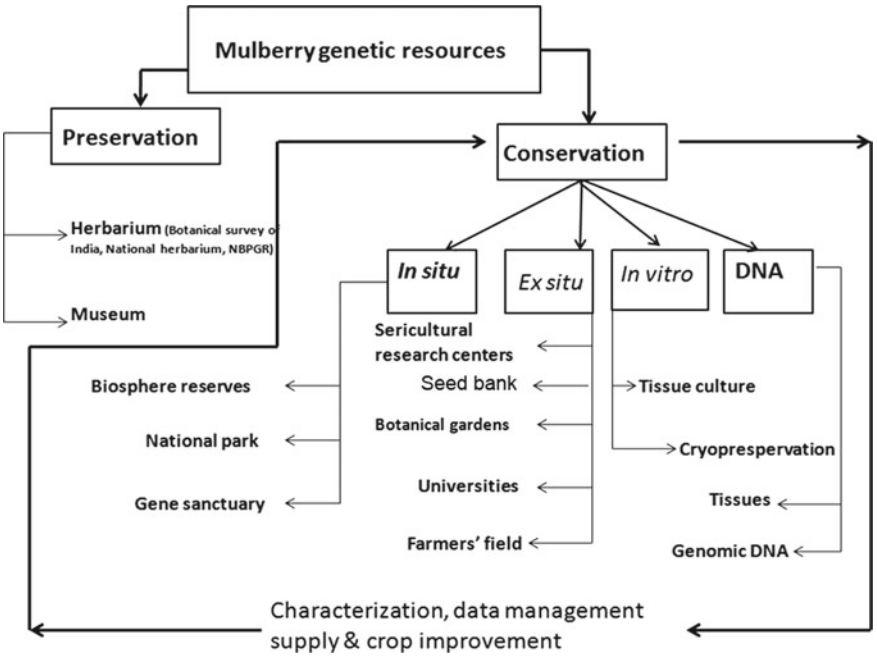

Fig. 3.7 Strategies for **c**onservation of mulberry genetic resources

followed by rapid immersion of samples in liquid nitrogen. In this method, the plant materials are treated with cryoprotective chemicals like dimethyl sulfoxide (DMSO), ethylene glycol, polyethylene glycol, mannitol, sorbitol or sucrose, either alone or in combinations, to prevent intracellular crystallization of water and subsequent cell damage. Under vitrification, the materials are vitrified first by increasing the viscosity of the solution via reducing the intracellular and intercellular freezable water by either exposing it to physical desiccation or through the help of highly concentrated cryoprotective mixtures followed by rapid cooling by immersion into liquid nitrogen (Withers and Engelmann 1997). Different combinations of DMSO, glycerol, ethylene glycol, polyethylene glycol, amino acids and sugars are used as cryoprotectans (Sakai et al. 1991). Using the vitrification method, 908 mulberry germplasm accessions have been cryopreserved in India (Rao et al. 2007). About 450 germplasm accessions have been preserved in Japan (Kazutoshi et al. 2004). For the recovery of shoot apices after cryopreservation, they are immersed quickly in water at 35–40 °C and cultured on MS medium (Murashige and Skoog 1962).

3.3.2.4 DNA Banks

The genetic information encoded in the genome of a plant is another source for long-term preservation of plant genetic resources. Isolation and preservation of DNA

Table 3.8 Advantages and disadvantages of different methods used for conserving mulberry genetic resources

In situ	Ex situ	In vitro	DNA banking
Apt for forest species and wild crop relatives	Only option for the asexually reproducing plants	Suitable for both sexually and asexually reproducing plants	Suitable for both sexually and asexually reproducing plants
Field oriented, laborious and expensive	Field oriented, laborious and expensive	Laboratory oriented minimum space and less laborious	Laboratory oriented, minimum space and less laborious
Allows evolution to continue	Evolution restricted	No chance of evolution	No chance of evolution
Increases genetic diversity	Less prone to genetic variability	No genetic variation	No genetic variation
Vulnerable to disease and other natural calamities	Vulnerable to disease and other natural calamities	Well protected against disease and other natural calamities	Well protected against disease and other natural calamities
Strengthens the link between conservationists and local people who traditionally maintain the plant	Minimum interactions	No interactions	No interactions
Exchange of materials is difficult	Exchange of materials possible but needs extra care	Easy exchange of materials	Easy exchange of materials

enable efficient preservation of the genetic information from endangered plant species and even from fossilized plant specimens (Roger and Bendich 1988). Plant genomic DNA can be extracted using different routine techniques depending on the nature of the materials and species (Vijayan 2004). Pure DNA can be stored in several ways. The most common method is by dissolving the DNA in TE buffer (10 mM Tris-HCl and 1 M EDTA) and storing at −80 °C. The purified DNA can also be stored in alcohol indefinitely as DNA is highly stable; is estimated that fully hydrated DNA can be kept at room temperature taking about 10,000 years to depolymerise into small fragments (Mandal 1999). A list of important DNA banks for plant genetic resources conservation around the world is given in Table 3.9.

3.3.3 Cytogenetics

Mulberry is most commonly a diploid (2x, 2n = 28); *Morus alba*, *M. indica*, *M. latifolia*, *M. multicaulis*, *M. australis* all are diploids. Others in nature are triploids (3x, 3n = 42) e.g., *M. bombysis*; tetraploids (4x, 4n = 56) e.g., *M. laevigata*, *M. cathayana* and *M. boninensis*; hexaploids (6x, 6n = 84) e.g., *M. serrata* and *M. tiliaefolia*; and

Table 3.9 List of important DNA banks for plant genetic resources conservation around the world

DNA bank	Website
Australian Plant DNA Bank (APDB), Centre for Plant Conservation Genetics, Southern Cross University, Lismore, NSW, Australia	http://www.dnabank.com.au
Botanic Garden and Botanic Museum (BGBM) DNA Bank, Berlin, Germany	http://www.bgbm.org/bgbm/research/dna/
DNA Bank Brazilian Flora Species, Rio de Janeiro Botanic Garden, Brazil	http://www.jbrj.gov.br/pesquisa/div_molecular/bancodna/index.htm
DNA Bank at Kirstenbosch, South African National Biodiversity Institute, Kirstenbosch, South Africa	http://www.nbi.ac.za/research/dnabank.htm
International Rice Research Institute (IRRI), DNA Bank, Philippines	http://www.irri.org/GRC/GRChome/Home.htm
Missouri Botanic Garden DNA Bank, (MBGDB) St Louis, MO, USA	http://www.mobot.org/MOBOT/research/diversity/dna_banking.htm
National Bureau of Plant Genetic Resources (NBPGR). New Delhi, India	http://www.nbpgr.ernet.in/
National Herbarium Netherlands DNA Bank (NHNDB), The Netherlands	http://www.nationalherbarioum.nl/taskforcemolecular/dna_bank.htm
National Institute of Agrobiological Science (NIAS) DNA Bank,, Tsukuba, Ibaraki, Japan	http://www.dna.affre.go.jp/
Plant DNA Bank Korea (PDBK), Graduate School of Biotechnology, Korea University, Seoul, Korea	http://www.pdbk.korea.ac.kr/index.asp
Royal Botanic Garden Edinburgh DNA Bank, Edinburgh, Scotland	http://www.rbge.org.uk/rbge/web/science/research/
Royal Botanic Garden Kew DNA bank, Richmond, England	http://www.rbgkew.org.uk/data/dnaBank/
TCD DNA Bank, Department of Botany, School of Natural Sciences, Trinity College, Ireland	http://www.dnabank.bot.ted.ie
Tropical Plant DNA Bank, Fairchild Tropical Botanical Garden and Florida International University, FL, USA	http://www.ftg.org/research

octaploids (8x, 8n = 112) e.g., *M. cathayana* (Basavaiah et al. 1989). The highest ploidy among phanerogams of 308 chromosomes (22x, 22n = 308) was reported in *M. nigra*. A natural haploid with 14 chromosomes was also reported in mulberry (Maode et al. 1996).

Table 3.10 Molecular linkage map developed in mulberry

Sl. no.	Molecular map	Pedigree of mapping population	Markers	Hereditary nature of marker	Agronomic trait targeted	References
1	Genetic linkage	S36 × V1	RAPD, ISSR, and SSR	Dominant, co-dominant	No	Venkateswarlu et al. (2006)
2	QTL map	V1 × Mysore Local	RAPD and ISSR	Dominant	Yield traits	Naik et al. (2014)
3	QTL map	Himachal Local × MS3	RAPD and ISSR	Dominant	Water use efficiency	Mishra (2014)
4	QTL map	Dudia White × UP	RAPD and ISSR	Dominant	Root traits	Mishra (2014)
5	Genetic linkage	Dudia White 9 UP105	SSR	Co-dominant	No	Thumilan et al. (2016)

Source Sarkar et al. (2017)

3.4 Molecular Breeding

3.4.1 Molecular Marker-Assisted Breeding

Mulberry breeding thus far has focused on improvement of leaf and fruit yield through selection of phenotypes. This selection method has achieved a certain level of genetic gain as evidenced from the higher leaf yield presently obtained from improved cultivars (Vijayan 2010). Further improvement of the rate of genetic gain, particularly for the complex traits such as growth rate, fruit yield, propagation efficiency, resprouting and survival, can be achieved through indirect selection on trait-linked molecular markers. In mulberry, five molecular linkage maps have been constructed with RAPD, ISSR and SSR markers (Table 3.10) using the pseudo-testcross method. However, in these linkage maps the markers are sparsely distributed and parental dependent, hence, they can only be used for specific crosses until validation in different genetic backgrounds is done with more markers to saturate them. Furthermore, as no QTL-trait association has been validated for any trait, marker assisted selection breeding is yet to start in mulberry.

3.4.2 Functional Genomics

Functional genomics in mulberry is still in its infancy; little work has been carried out in this area, except for a few attempts to develop expression sequence tags (ESTs) for stress tolerance (Checker et al. 2012a). Development of ESTs are essential steps for genome annotation to provide information about gene structure, splicing and level of expression. ESTs have been developed from leaf (Gulyani and Khurana 2011) and root (Checker et al. 2012b) of *Morus indica*. *M. laevigata* and *M. serrata* (Saeed et al. 2015). Genes like remorins and chalcone synthase have been functionally characterized from mulberry (Checker and Khurana 2013; Wang et al. 2017). Other gene

families such as NAC and WRKY transcription factors were also analyzed to understand their role in biotic and abiotic stress responses (Baranwal and Khurana 2016; Baranwal et al. 2016). The chloroplast genome of mulberry was also sequenced to help rapid, accurate and automated identification of species (Ravi et al. 2008). It is a 158,484 bp circular double-stranded DNA comprised of two identical inverted repeats of 25,678 bp each, separating a large and a small single copy region of 87,386 bp and 19,742 bp, In order to elucidate genomics of fruit ripening in mulberry, quantitative real-time polymerase chain reaction was performed with 17 genes participating in ABA signal transduction including five *MnPYL*, six *MnPP2C*, and six *MnSnRK2* genes, isolated from the *M. notabilis* genome database in *M. atropurpurea* cv. Jialing No. 40 and found that the transcripts of *MaPYL4*, *MaPP2C5* and *MaSnRK2.6* were expressed at a relatively higher level during the entire development process. Similarly, the transcript levels of *MaPYL1/3/5*, *MaPP2C2/4/5/6* and *MaSnRK2.3/2.6* were lower during the early-maturation stage and higher during the post-maturation stage, suggesting that they may play a vital role in regulating mulberry fruit ripening (Zhu et al. 2017).

3.4.3 Bioinformatics

The bioinformatics of mulberry has not been developed as it has in many other agriculture crops, but there are several data banks which are of use. Important among them are MorusDB (http://morus.swu.edu.cn/morusdb) which provides genomic data including gene structures, functional annotation of genes, transcriptome data, and EST of *M. notabilis*; (http://btismysore.in/mulsatdb/)and MulSatDB give information on mulberry genome and microsatellites in mulberry respective (Krishnan et al. 2014; Li et al. 2014) and NCBI (http://www.ncbi.nlm.nih.gov/) can be used for all bioinformatics purposes.

3.5 Genetic Engineering

Since the development of mulberry varieties with specific characters takes a long time due to the long juvenile period, lack of inbreds and dioecy of the plant (Vijayan 2010), it is necessary to resort to genetic engineering to develop varieties with specific traits. The two different methods viz., bombardment and the *Agrobacterium tumefaciens* mediated gene delivery system, have widely been used to insert foreign genes into the mulberry genome. Machii et al. (1996) first used particle bombardment and later Sugimura et al. (1999) reported delivery of the reporter gene GUS into intact leaf tissues and suspension-cultured cells. Bhatnagar et al. (2002) also successfully incorporated the GUS reporter gene into *Morus indica* cv. K2 using hypocotyl, cotyledon, leaf and leaf callus explants. However, due to low transformation efficiency of particle bombardment (Travella et al. 2005), *A. tumefaciens*-mediated transformation has

Table 3.11 Transgenesis in mulberry for abiotic stress tolerance

Gene	Expression profile	References
*WAP21**	Cold tolerance	Ukaji et al. (1999)
COR	Cold tolerance	Ukaji et al. (2001)
AlaBlb	Salinity tolerance	Wang et al. (2003)
OC	Insect resistance	Wang et al. (2003)
SHN 1	Drought tolerance	Sajeevan et al. (2017)
HVA1	Drought and salinity stress	Lal et al. (2008)
bch1	Drought and salinity stress	Saeed et al. (2015)
Osmotin	Drought and salinity stress	Das et al. (2011)

**AlaBlb* Soybean glycine gene; *bch-1* inhibitor 2-aminobicyclo-(2, 2, 1)-heptane-2-carboxylic acid; *COR*—cold on regulation; *HVA1—Hevea braziliensis* abiotic stress gene; *OC*—osteocalcin *Osmotin*-osmotic stress induced gene; *SHN 1*—schnurri from *Drosophila melanogaster*; *WAP21*—water allocation plan
Adopted from Vijayan et al. (2011d)

been adopted extensively in mulberry (Checker et al. 2012b). Using this method, several transgenic mulberry with desired traits such as tolerance to drought and salinity, high temperature, and moisture retention have already been developed (Table 3.11; Saeed et al. 2015; Sajeevan et al. 2017).

3.6 Mutation Breeding

Mutation is the process by which genes are permanently altered, naturalally or induced, and it can occur at the chromosome or gene level. Owing to mutation, a character may be lost or gained, hence, breeders use the mutation technique in plant breeding to develop new varieties with specific traits. Mutations can be induced through chemical or physical agents. The most commonly used mutation agents are physical mutagens such as ultra-violet (UV) light, X-rays and neutrons (Koornneef 2002). Chemical mutagens such as nitrous acid, ethyl methane sulfonate (EMS), methyl methane sulfonate (MMS) and diethyl sulfate (DES), have also been used to induce variations in plants. Compared to radiological methods, chemical mutagens tend to cause single base-pair (bp) changes, or single-nucleotide polymorphisms (SNPs) rather than deletions and translocations. Of the abovementioned chemical mutagens, EMS is today the most widely used as it selectively alkylates guanine bases causing the DNA-polymerase to favor placing a thymine residue over a cytosine residue opposite the O-6-ethyl guanine during DNA replication, which results in a random point mutation. A majority of the changes (70–99%) in EMS-mutated populations are GC to AT base pair transitions (Till et al. 2004, 2007).

3.6.1 Conventional Mutagenesis

Since mulberry is highly heterozygous, seed propagation is not widely practiced. The common propagule is stem cuttings and it has been used extensively for induction of mutation. In mulberry, mutation breeding was first initiated at the Sericultural Experiment Station, Ministry of Agriculture and Forestry, Japan in 1957 using gamma radiation (Nakajima 1972). Later, it was tried in many countries and with many employing different mutagens (Dandin et al. 1996; Katagiri 1970; Ramesh et al. 2013; Rao et al. 1984; Sastry et al. 1974; Sugiyama and Tojyo 1962). For instance, Fujita and Wada (1982) used chronic and acute irradiation to induce mutation in mulberry cv. Ichinose and found that the five lobed leaf was converted into an entire leaf and produced high rooting ability in some mutants. Similarly, exposure of juvenile cuttings of different mulberry varieties to different doses of gamma rays (1–10 kR) showed varietal and dose dependent effect on growth and rooting parameters (Deshpande et al. 2010; Ramesh et al. 2013). The chemical mutagen EMS was used to induce several beneficial mutations in mulberry (Anil Kumar et al. 2012, 2013; Sastry et al. 1974).

3.6.2 In Vitro Mutagenesis and Selection

Very little work has been done in mulberry to induce point mutations; however, in vitro mutagenesis was used to develop tetraploids from diploids in an efficient and cost effective manner (Chakraborti et al. 1998) and also to isolate beneficial somaclonal variants (Narayan et al. 1989).

3.6.3 Molecular Analysis

PCR analysis of mutants obtained from EMS treatment with RAPD primers revealed genetic variations between control and EMS induced clones. Mutants developed through 0.1 and 0.3% EMS showed higher levels of genetic variations and more unique alleles compared to the control (Anil Kumar et al. 2012). Protein profiles of leaf from gamma irradiated plants revealed a significant decrease in the quantity of 55 kDa protein in dosage from 7 to 10 kR. A 6 kR dose was found to be the optimum limit of gamma irradiation (Reddy and Munirajappa 2013).

3.6.4 Enhanced Traits and Improved Cultivars

Treatment with EMS, resulted in the development of a few popular mulberry varieties such as S13, S34, S36 with enhanced drought tolerance (Sastry et al. 1974).

Induction of mutations with EMS in the genotype RFS135 resulted in isolation of varieties with wide economic importance in sericulture (Anil Kumar et al. 2012, 2013); it was found that 0.1 and 0.3% EMS treatment were effective in altering the morphometric characters, biomass yield and phytochemical constituents such as proteins, reducing sugars, minerals and moisture content. Similarly, a somaclonal variant (SV1) developed from a popular variety S1 (*Morus alba*) showed increased branching, tolerance to drought and higher leaf yield (Chakraborti et al. 1999). Similarly, Susheelamma et al. (1996) isolated another somaclonal variant from plantlets developed through callus culture of var. S-14. The somaclonal variant showed beneficial traits like shorter internodal distance, thicker leaves, higher chlorophyll content, higher moisture content and better moisture retention capacity.

3.7 Hybridization

3.7.1 Conventional Hybridization

Since, mulberry is a plant with a long juvenile period and high heterozygosity, traditional breeding has mostly relied on the production of F1 hybrids and their subsequent screening and selection (Vijayan et al. 2012). Desirable parents are selected, crossing effected during the regular flowering seasons and seeds collected after the fruit ripens. The seeds collected are sown in the nurseries and the seedlings then transplanted to the field for further screening and selection as stated elsewhere. The hybrid with desirable traits is multiplied clonally to maintain the unique combinations of traits captured. Using this method a number of varieties were developed in different countries (Vijayan et al. 2009a, 2011c).

3.7.2 Somatic Cell Hybridization

Protoplast isolation and fusion is one of the techniques used to create hybrids that cannot be developed otherwise due to incompatibility resulting from non-synchronization of flowering, or other reproductive barriers (Vijayan et al. 2011b). Protoplasts isolated from mesophyll cells using a combination of 2.0% cellulase, 1.0% macerozyme and 0.5% macerase were fused with different chemical fusogens; polyethylene glycol (PEG) was found to be best for mulberry (Umate 2010). However, application of an electric field was found more preferable due to its simplicity, less toxicity, less physical damage to the protoplasts, larger fusion volume and fine control of the fusion process (Cheng and Saunders 1995).

3.7.3 Hybrid Cultivars

Using various breeding methods, countries like China and India have developed a number of mulberry cultivars suitable for different agroclimatic conditions (Appendix 2, Tables A, B). Most of these mulberry varieties were developed for higher leaf yield and are mostly from a few species such as *Morus alba*, *M. atropupurea*, *M. bombycis*, *M. indica*, *M. latifolia* and *M. multicaulis*. Attempts have been made to identify fruit yielding mulberry varieties in several countries including India and China. By screening the germplasm of 1269 accessions, a few mulberry cultivars with high fruit yielding potential have been identified at CSGRC, Hosur, India (Table 3.6). These genotypes can be used for breeding high-yielding mulberry varieties.

3.8 Conclusion and Prospects

3.8.1 An Overview of the Current Status

Considering the great economic significance of mulberry due to its association with the silk industry, fruit industry, pharmaceuticals and landscaping, a good amount of research has already been carried out across the globe in general and especially in Asian countries like China, Japan and India. Since mulberry is a highly heterozygous tree with a long juvenile period, developing new varieties/cultivars through conventional breeding techniques is laborious and time consuming. Often breaking of the linkage between two traits makes it nearly impossible to utilize the better one in the breeding process. Nonetheless, a number of varieties suitable to different climatic conditions have been developed and are being used at a commercial scale. However, most of these mulberry cultivars/varieties have been developed from only a few mulberry species leaving behind a great number of species with specific traits such as stress resistance. Thus, it is necessary to develop new methods to utilize those species.

3.8.2 Current Research Initiatives to Combat Global Climate Change

Global warming is one of the most significant climatic changes attracting considerable world attention. It has resulted in an array of changes in the world starting from rising of temperature, inundation of low-lying areas due to rising of sea level, changes in the pattern of seasonal rains to the appearance/predominance of new pests and diseases to migration/disappearance of species of organisms. The major causes of global warming are the increase of pollutants from the burning of fossil fuels, greenhouse gases emanated from modern equipment and agricultural crops,

and from industrial effluents. In order to minimize the harmful effects of global warming, countries across the world have taken a number of measures; important among them are the adoption of green technologies. Carbon dioxide is one of the most important greenhouse gases increasing the earth's temperature by absorbing energy from the sun and causing climate changes across the world. Thus, it is important to reduce the rate of accumulation of CO_2 in the atmosphere. Carbon sequestration is one of the means by which plants are able to capture CO_2 from the atmosphere and store it in solid form. It is estimated that the mulberry tree is capable of absorbing about 6.90 mt of CO_2/ha/year (equivalent to 2025 kg of carbon) and release 4.6 mt of oxygen/ha/year. In addition, mulberry leaves have high endurance and absorption capacity to air pollutants such as chlorine, hydrogen fluoride, and sulfur dioxide. Under 6 h of fumigation at 0.79×10^6 concentration of sulfur dioxide, 1 kg of dry mulberry leaves absorb 5.7726 g sulfur dioxide and 1 m^3 of mulberry forest absorbs 20 ml of sulfur dioxide gas each day (Lu and Jiang 2003; Qin et al. 2012). Thus, efforts have been made to develop new cultivars with wider adaptability to extend mulberry cultivation to areas where irrigation facilities are not available and prone to drought and salinity to reduce the adverse effect of global warming especially in the rural areas. Biotechnology tools have been employed to develop drought tolerant varieties. Similarly, recently the mulberry genome has been fully sequenced and annotated to provide much wanted information for the researchers to utilize in the crop improvement program. Using this information it is presumed that in the coming years new mulberry varieties with greater resistance to biotic and abiotic stress can be developed to increase the income of the farmers and to reduce the ill effect of industrialization in the world.

3.8.3 Recommendations for Future Research

Given the lack of inbred lines in mulberry to work out the genetics of most of the economically important traits, it is urgently required to develop homozygous lines through double haploidy by developing androgenic and gynogenic haploids. Similarly, to employ in the breeding programs, molecular markers tightly linked to economic traits need to be developed. QTLs for quantitative traits also should be identified for the improvement of those traits. Furthermore, the current impetus on the development of genomics resources and genetic engineering should be continued to facilitate development of new mulberry cultivars capable of producing qualitatively superior products.

Appendix 1

List of major Institutes engaged in research on mulberry (*Morus* spp.)

1. Central Sericultural Research &Training Institute, Srirampura, Manandavadi Road, Mysuru-570008, Karnataka, India. e-mail:csrtimys.csb@nic.in
2. Central Sericultural Research &Training Institute, Berhampore-721101, West Bengal, India. email:csrtiber.csb@nic.in
3. Central Sericultural Research &Training Institute, Pampore, Post bag No. 88, GPO, Srinagar-190001, Jammu and Kashmir. e-mail:csrtipam.csb@nic.in
4. Central Sericultural Germplasm Resources Centre, Hosur-635109, Tamil Nadu, India. e-mail:csgrchos.csb@nic.in
5. Karnataka State Sericulture Research & Development Institute, Thalaghattapura, Kanakapura Road, Bangalore-560 062, Karnataka, India. e-mail:csgrchos.csb@nic.in
6. Department of Sericulture Science, Mysuru University, Manasagangotri, Mysuru, India
7. Jiangsu University of Science and Technology, No.2 Mengxi Road, Zhenjiang, Jiangsu, 212003, P. R. China. e-mail: bgs@just.edu.cn
8. Sericulture Research Institute of China agricultural academy, Zhenjiang, 212018, Jiangsu, China
9. Sericultural research institute of Zhejiang, Hangzhou, 310021, China
10. Sericultural Research Institute of Sichuan, Nanchong, 637000, Sichuan, China
11. Sericultural Research Institute of Guangdong, Guangzhou,510640, Guangdong, China
12. Sericultural Research Institute of Shuanxi, Zhouzi county, 710400, Shuanxi, China
13. Department of Sericulture, Zhejiang University, Hangzhou, 310029, China
14. Department of Sericulture, Huanan Agricultural University, Guangzhou, 510642, Guangdong, China
15. College of Sericulture, South west Agricultural University, Chongqing, 630716, China
16. Institut National de la Recherche Agronomique, Unité Nationale Séricicole, 25 Quai J.J. Rousseau, 69350 La Mulatière, France
17. Sericultural Specialized Section of Padua, Experiment Institute for Agricultural Zoology of Florence, Ministry of Agricultural and Forestry Policies, Italy
18. National Institute of Agrobiological Sciences Ohwashi Tsukuba Ibaraki 305-8634, Japan
19. National Institute of Agricultural and Science Technology 61 Seodun-Dong Suwon, South Korea
20. Sericulture Experiment Station, 24 Mito Orozov Str., Vratza 3000, Bulgaria
21. Atatürk Central Horticultural Research Institute, 77102 Yalova Turkey
22. Erciyes University, Faculty of Agriculture, Department of Horticulture 38039 Kayseri, Turkey
23. Malatya Fruit Research Institute, 44100 Malatya, Turkey
24. Plant Breeding Research Center for Sustainable Agriculture, Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand

25. Bangladesh Sericulture Research &Training Institute Baliapukur P.O: Padma Residential Area, Rajshahi-6207 e-mail: info@bsrti.gov.bd
26. Sericulture Research Division, Pakistan Forest Institute, Peshawar 25120 Khyber Pakhtunkhwa—Pakistan. e-mail: dir.fedu.pfi@gmail.com

Appendix 2

A: List of mulberry genetic resources available in the ex situ germplasm of Japan, China, India and Korea

Species	Number of available accessions of mulberry			
	Japan	China	India	Korea
M. bombycis Koidz.	583	22	15	97
M. latifolia Poir.	349	750	19	128
M. alba L.	259	762	93	105
M. acidosa Griff.	44	–	–	1
M. wittorium Hand-Mazz.	–	8	–	–
M. indica L.	30	–	350	5
M. mizuho Hotta	–	17		–
M. rotundiloba Koidz.	24	4	2	–
M. kagayamae Koidz.	23	–	–	1
M. australis Poir.	–	37	2	–
M. notabilis C.K.Schn.	14	–	–	–
M. mongolica Schneider		55	–	–
M. boninensis Koidz.	11	–	–	–
M. nigriformis Koidz.	3	–	–	–
M. atropurpurea Roxb.	3	120	–	–
M. serrata Roxb.	3	–	18	–
M. laevigata wall.	3	19	32	1
M. nigra L.	2	1	2	3
M. formosensis Hotta.	2	–	–	–
M. rubra L.	1	–	1	–
M. mesozygia Stapf.	1	–	–	–
M. celtifolia Kunth.	1	–	–	–
M. cathayana Hemsl.	1	65	1	–
M. tiliaefolia Makino	1	–	1	14
M. microphylla Bickl.	1	–	–	–
M. macroura Miq.	1	–	–	–
M. multicaulis Perr.	–	–	15	–
Morus spp. (Unknown)	15	–	720	259

B. Total mulberry germplasm accessions available in different countries

Country	Mulberry accession
Japan	1375
China	2600
Brazil	–
South Korea	208
India	1271
Bulgaria	140
Italy	50
France	70
Indonesia	5
Taiwan	5
Argentina	2
Colombia	4
Mexico	5
Peru	2
USA	23

C. List of high yielding mulberry varieties developed in China

No.	Variety	Selection and breeding method	Year	Suitable zone
1	Xiansang 305	Mutation breeding	2001	The Huanghe River valley
2	Beisangyihao	Selected from local seedling mulberry	1995	The Changjiang River valley, The middle and lower reaches of the Huanghe River
3	Nongsang 8	Hybridization breeding	2000	The Changjiang River valley
4	Huangluxuan	Selection from local variety	1998	The Huanghe River valley
5	Jihu 4	Hybridization breeding	1989	Northeast zone
6	Dazhonghua	Polyploidy breeding	1996	The Changjiang River valley
7	Xinyiyuan	Mutation breeding	1995	The Changjiang River valley, The middle and lower reaches of the Huanghe River
8	Nongsang 14	Hybridization breeding	2000	The Changjiang River valley
9	Yu 237	Hybridization breeding	1989	The Changjiang River valley
10	Xuanqiu 1	Selected from local seedling mulberry	1989	Northeast zone
11	7307	Selected from local seedling mulberry	1989	The Changjiang River valley
12	Husang 32	Selection from local variety		The Changjiang River valley, The middle and lower reaches of the Huanghe River

No.	Variety	Selection and breeding method	Year	Suitable zone
13	Xiang 7920	Hybridization breeding	1995	The Changjiang River valley
14	Canzhuan 4	Selected from local seedling mulberry	2001	The Changjiang River valley
15	Huamingsang	Selected from local seedling mulberry	1994	Chizhou Xuanzhou Anqing in Anhui province and Linyi in Shandong province
16	7946	Hybridization breeding	1998	The Huanghe River valley
17	Yu 2	Hybridization breeding	1989	The Changjiang River valley
18	Shigu 11-6	Mutation breeding	1995	The Changjiang River valley, The middle and lower reaches of the Huanghe River
19	Xuan 792	Selection breeding	1989	The Huanghe River valley
20	Yu 711	Hybridization breeding	1995	The Changjiang River valley, The middle and lower reaches of the Huanghe River
21	Yu 151	Hybridization breeding	1989	The Changjiang River valley
22	Hongxin 5	Hybridization breeding	1995	The Changjiang River valley, The middle and lower reaches of the Huanghe River
23	Lunjiao 40	Selection from local variety	1989	The Zhujiang River valley
24	Wan 7707	Selected from local seedling mulberry	1994	chizhou、Xuanzhou、Anqing in Anhui province and Linyi in Shandong province
25	Huangsang 14	Selected from local seedling mulberry	1989	The Changjiang River valley
26	Lunjiao 40	Selection from local variety	1989	The Zhujiang River valley
27	Shi 11	Selection from local variety	1989	The Zhujiang River valley
28	Xinyizhilan	Introduced variety	1995	The Changjiang River valley; The Huanghe River valley
29	Jialing 16	Polyploidy breeding	1998	The Changjiang River valley
30	Tang10 × Lun 109	Hybrid mulberry seed	1989	The Zhujiang River valley
31	Nongsang 12	Hybridization breeding	2000	The Changjiang River valley

Source Vijayan et al. (2011c)

D. List of commercially exploited mulberry varieties in India

No.	Variety	Selection and breeding method	Year	Suitable zone
1	G4	Hybrid from *M. multicaulis* × S-13	2017	South India Irrigated
2	C2038	Hybrid from CF1 × C763	2017	Eastern and NE India Irrigated
3	Tr23	T25 × S-162	2017	Hills of Eastern India

No.	Variety	Selection and breeding method	Year	Suitable zone
4	Victory-1	Hybrid from S30 × C776	2010	South India Irrigated
5	Vishala	Clonal selection	2010	All India Irrigated condition
6	Anantha	Clonal selection from RFS-135	2010	South India Irrigated
7	DD (Viswa)	Clonal selection	2000	South India Irrigated
8	S-13	Selection from polycross (mixed pollen) progeny	2000	South India Rainfed
9	S-34	Selection from polycross (mixed pollen) progeny	2000	South India Rainfed
10	S-1	Introduction from (Mandalaya, Myanmar)	2000	Eastern and NE India Irrigated
11	S-7999	Selection from open pollinated hybrids	2000	Eastern and NE India Irrigated
12	S-1635	Triploid selection	2000	Eastern and NE India Irrigated
13	S-36	Developed through EMS treatment of Berhampore Local	2000	South India Irrigated
14	C776	Hybrid from Black cherry and *M. multicaulis*	2000	Saline soils
15	S-146	Selection from open pollinated hybrids	2000	N. India and Hills of J and K Irrigated
16	Tr-10	Triploid developed from T25 × S162	2000	Hills of Eastern India
17	BC $_2$59	Back crossing of hybrid of Matigare local × Kosen with Kosen twice	2000	Hills of Eastern India
18	Chak Majra	Selection from natural variability	2000	Subtemperate
19	China White	Clonal selection	2000	Temperate

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Chapter 4
Pear (*Pyrus* spp.) Breeding

Glacy Jaqueline da Silva, Fabíola Villa, Fernanda Grimaldi, Pricila Santos da Silva and Juliana Fátima Welter

Abstract Pear is an important temperate-climate fruit, cultivated since the beginnings of civilization. Due to the delicacy of its flavor, it is consumed by many different people, as fresh fruit or as a component of hot and cold dishes, as well as various drinks and as part of recipes handed down for generations. Due to its versatility and flavor, it has become an important commodity for several countries in Europe, and in China and the United States. Due to its broad dispersion around the world, major genetic breeding programs are necessary to obtain the best characteristics of market such as flavor, texture, color and odor, besides resistance to the most diverse types of pathogens found in the culture, like black spot, *Dysaphis pyri*, fire blight and scab; as well as adaptation to the various climatic and edaphic conditions in which the plant is cultivated. Another topic that needs attention within pear culture is the rootstock system, which influences cultivation around the world, contributing to the production of quality fruits. This chapter aims to provide an overview of the main topics related to pear genetic improvement programs around the world, as well as to update the literature on recent and innovative research studies mainly involving molecular markers and QTLs.

G. J. da Silva (✉)
Molecular Biology Department, Paranaense University, UNIPAR, Umuarama, PR, Brazil
e-mail: glacyjaqueline@prof.unipar.br

F. Villa
Horticulture Department, Western Paraná State University, UNIOESTE, Marechal C. Rondon, PR, Brazil
e-mail: fvilla2003@hotmail.com

F. Grimaldi · P. S. da Silva · J. F. Welter
Agricultural Sciences Center of Lages, University of Santa Catarina State, Florianópolis, SC, Brazil
e-mail: fernandagrimaldi@outlook.com

P. S. da Silva
e-mail: pricilassilva@hotmail.com

J. F. Welter
e-mail: julianawelter_@hotmail.com

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4.1 Introduction

The most important concerns when it comes to food security are the adaptations of different cultures to climate change, and increased production to meet the demands of human population growth. These adaptations require a great deal of effort from governmental organizations, research institutions, universities, researchers and curators of germplasm banks (Lobell et al. 2008).

The pear is a nutrient-rich fruit that grows mainly in temperate and subtropical climates, and is an important commodity for several countries such as China and the United States. Research to find cultivars adapted to the most diverse types of climate and tolerant to diseases of the culture, is paramount within programs of genetic improvement of pear trees (Sharma et al. 2010).

The high degree of heterozygosity of this fruit provides infinite possibilities within a breeding program; however, due to the extended juvenile period of the plant, obtaining new cultivars is time consuming and expensive (Reiland and Slavin 2015).

With the advent of biotechnology and the latest and most advanced techniques within molecular biology, it is possible to greatly reduce time and cost, more so after the complete pear sequencing, which enables researchers to develop extremely specific techniques that are delivering significant advances within a time previously unimaginable. The development of genetic maps from molecular markers, especially SSR and SNPs, combined with the discovery of QTLs associated with important agronomic characteristics, has allowed the solution of theretofore insoluble problems (Collard et al. 2005).

All such advances are impossible without the conservation of all species in germplasm banks, because it allows the search for new genes and characteristics that could be rescued from ancestral types and other cultivated species.

4.2 Origin, History and Production

The Rosaceae family consists of about 2500 species within 90 genera and includes several important food plants (Hummer and Janick 2009). It has three subfamilies: Dryadoideae, Rosoideae and Amygdaloideae (Potter et al. 2007). Economically the most important crops of the Rosaceae are pears (*Pyrus* spp.) and apples (*Malus domestica* Borkh.). Both belong to the subfamily Amygdaloideae and tribe Pyreae.

The pear tree belongs to the subfamily Pomoideae and genus *Pyrus*. It comprises around 20 species, native to Europe and Asia. It is a perennial, easy-growing tree from countries with a temperate climate, experiencing an accentuated winter. It is

widely cultivated in temperate regions of the world (Hummer and Janick 2009). The fruits vary in size, shape, texture and flavor.

Pears have a worldwide fruit production of approximately 25.2 million mt (FAOSTAT 2013). Asia is the main producer of this fruit, followed by Europe, South America, North America, Africa and Oceania. In Asia, China is considered the largest producer, accounting for 60% of world volume, followed by Japan (2.1%) and Korea (2.0%). In Europe, the main producers are Italy (4.9%), Spain (3.4%) and France (1.2%). In South America, Argentina (2.7%) and Chile (1.1%). In North America, the main producer is the USA (3.9%), predominantly from the states of Washington, California and Oregon. Finally, Africa is notable with South Africa (1.8%) and Oceania, with Australia (0.8%).

Four important *Pyrus* species with edible fruits are commercially grown: the Japanese pear (*P. pyrifolia* Nakai), European pear (*P. communis* L.) and Chinese pears (*P. bretschneideri* Rehd and *P. ussuriensis* Maxim.) (Bell et al. 1996). European pear cultivars are predominant in Europe, North America, South America, Africa and Australia; the Japanese pear is mostly cultivated in southern and central China, Japan and Southeast Asia. Other pears widely consumed in Asia include the ussur pear (*P. ussuriensis*) and hybrids of *P. pyrifolia* x *P. ussuriensis* (Jiang et al. 2016). The interest in Asian pears, especially Japanese cultivars, is increasing in Western Europe, North America, New Zealand and Australia. The European pears have little impact in Asia, except in northern Japan, where most Asian pears do not have enough winter resistance (Hancock 2008). Other pear trees species are used as rootstocks, such as *P. calleryana* Dcne and *P. betulaefolia* Bge (Feldberg et al. 2010), in industry, such as *P. nivalis* Jacp. (Paganová 2003) and ornamentals, such as *P. calleryana* (Culley and Hardiman 2009; Pasa et al. 2015).

In Brazil, European pear production is concentrated in Rio Grande do Sul and Santa Catarina states and Chinese pears (Yali cv.) are cultivated in Paraná, Santa Catarina and São Paulo states. Small areas of Japanese pear tree plantings are located in Santa Catarina (about 131 ha), Paraná, Rio Grande do Sul, São Paulo and Minas Gerais states. The cultivars Housui, Nijisseiki and the Japanese pear Kousui are planted in the south of the country. São Paulo state stands out for its plantations of Japanese pear trees of cvs. Atago and Okusankichi, which are of lower commercial quality (Faoro and Orth 2010).

Fruits are usually consumed fresh, cooked, dried or made into a fermented cider-like beverage called *perry* (Zhang et al. 2010). It can also be processed as canned halves, in fruit cubes for cocktails and as a puree for baby food (Yamamoto et al. 2006).

Pyrus is genetically diverse, with considerable variability in morphology and physiological adaptations (Jiang and Tang 2009). European and Asian pear breeders have used this variability to develop high-quality cultivars with fruits of large size, attractive appearance and adapted to local edaphoclimatic conditions. European pears are distinguished by their juicy, delicate flavor and aroma, while Oriental pears (Asiatic or Nashi) are known for their crispness and sweet taste (Reiland and Slavin 2015). US research has focused mainly on resistance to disease and cold (Saito 2016).

The first written record of pears is from Homer about 1000 BC, who referred to the fruit as one of the *gifts of the gods* (Bell 1991). According to Theophrastus (371–286 BC), pear culture was common in ancient Greece, where plants were propagated by grafting and cuttings. Cato (235–150 BC) described cultural methods for the pear tree which are very similar to the techniques practiced today. Pliny the Elder (23–79 BC) described at least 35 cultivars of pears grown in Rome, compared to only 3 cultivars of apples. At the end of the Song Dynasty (China, 1279), more than 100 pear tree cultivars were recorded (Hancock 2008).

In the Middle Ages, pear trees were widely grown in central and western Europe (Bell et al. 1996). Cordus (1515–1544) described cultivars of pear trees in Germany that had all the fruit characters of species currently cultivated, except for the pear with a buttery texture. France was the main pear-producing country in the sixteenth and seventeenth centuries and the most active in the development of new cultivars. In 1628, an amateur fruit collector named Le Lectier owned 254 pear cultivars in his garden, and in the early 1800s there were more than 900 pear cultivars growing in France, most of fruits with a crisp pulp (Hancock 2008).

In the eighteenth century, Belgium became the center of pear cultivation, mainly through the efforts of Nicolas Hardenpont and Jean Baptiste Van Mons (Bell et al. 1996). The Belgian breeders developed the first cultivars possessing a meaty and buttery mesocarp with some important cultivars such as Beurre Bosc, Beurre d'Anjou and Winter Nelis (Hancock 2008).

Pears are not considered native to England but their commercial cultivation probably emerged around 1200 BC. It is not known when the species was introduced to England, but is likely to have been before the Roman conquest (Bell et al. 1996). The British became experienced pear breeders in the 1800s, as evidenced by a catalog of the Royal Society of London (1826) listing 622 cultivars. The most widely cultivated pear in the world today, the Williams Bon Chretien (Barlett), was identified in 1796 (Faoro et al. 2017; Morgan 2015). Another pear cultivar called Conference (*Pyrus communis*), from the nineteenth century, is also heavily planted. Only two species were created in Europe, *P. communis* (common European pear) and *P. nivalis* (perry pear) (Volk et al. 2006).

Domestication of Chinese pear species began about 3300 years ago and commercial orchards have existed in China for more than 2000 years (Silva et al. 2014). However, modern pear breeding began in China only after 1956. Pears were cultivated as early as the eighth century in Japan (Saito 2016), but large orchards did not appear until 1868. Kikuchi started the first breeding program in Japan in 1915 and it remains active today (Bellini and Nin 2002).

4.3 Breeding for Enhanced Fruit Quality

High-quality fruit is the main goal of all breeding programs and covers a wide range of attributes including taste, texture, appearance, succulence, shelf life and incidence of physiological disorders. The most important attribute is food quality,

which depends largely on fruit taste and texture. Most programs emphasize fresh fruit quality; however, some are focused on fruit quality for processing into preserves and jellies. Considerable attention by breeders should be given to physiological disorders such as break-down, bitter pit and sunburn (Sha et al. 2011).

It is likely that high sugar content is the most important factor that determines a flavored pear. The acidity level is of secondary importance, because pears with high and low acidity levels are characterized as pleasant, even when containing large amounts of sugar (Franck et al. 2003). A high level of bitterness or astringency generally is not desirable in the Perry cultivar, used mainly in desserts, although these characteristics are important contributors to the bitter taste (Evans et al. 2008). Yamamoto et al. (2014) verified that the flavor heritability in *Pyrus communis* was considered relatively low, which was also confirmed by Wang et al. (2011). The flavor inheritance in *P. ussuriensis* hybrid populations is quantitative, with a lower presence of flavor and low dominance (Bell 2014).

The subjective perceptions of sweetness and acidity, as well as physical measurements of soluble solids and acidity, are inherited as independent quantitative characteristics. Estimates of heritability of soluble solids in *Pyrus pyrifolia* cultivars range from low to moderate (Yim and Nam 2016). Huang et al. (2014) stated that the succulence of the fruit is directly associated with the parent types, being regulated by a single or several genes.

It is important that pears can be stored in a cold chamber for a long period without the development of a physiological deterioration, such as internal collapse, so that they can be traded in the future when prices tend to be higher. Few genetic heritage studies have been conducted on this feature, although segregation patterns suggest that long-term maintenance quality is inherited quantitatively (Kaur and Dhillon 2015).

A large number of aromatic compounds are produced by pears, contributing significantly to fruit flavor. In the Bartlett cv., more than 77 volatile compounds have already been identified with different impacts on taste (Li et al. 2012). The esters of trans-2, cis-4-decadienoic acid are highly correlated with the intensity of Bartlett aroma (Zierer et al. 2016), although there are cultivars with high levels of those compounds that do not have this distinctive aroma (Qin et al. 2014). Seventeen volatile compounds, including toluene, ethyl butyrate, hexanal, (E) 2-hexanal, ethyl hexanoate and α-farnesene, are the most common constituents found in five Asian pear cvs. (Makkumrai et al. 2014).

The Ya Li cv. originating from *Pyrus* x *bretschneideri* and cv. Shinko, a putative interspecific hybrid, lacks the five volatile compounds present in three cultivars of *P. pyrifolia* (Chojuro, Hosui and Kosui) (Katayama et al. 2013). The genetics of these compounds are unknown, although Yim and Nam (2016) have found that some cultivars of *P. ussuriensis* have an aromatic quality that dominates the sweet and mild taste of cvs. *P.* x *bretschneideri* and *P. pyrifolia*.

The desired texture of pear flesh varies from region to region, in part due to differences between species. In Western Europe and North America, the preference is for soft and buttery texture, a typical feature of *Pyrus communis*, while in China and Japan dominant pears are crunchy and sandy, originated from *P. pyrifolia*, *P.*

× *bretschneideri* and *P. ussuriensis*. A small portion of the consumers in Asian and European countries still prefers more rigid fruits. Bell and Janick (1990) found heritability of 0.30 for texture in fruits of *P. communis*. A study conducted by Nishio et al. (2016a, b) found that pulp firmness may be highly heritable in populations of *P. pyrifolia*.

Several genetic studies have been conducted related to the epicarp and mesocarp of pear fruits. Kumar et al. (2017) claim that the pear background color affects an important gene, with dominance of yellow to green color. Also the dark × clear pulp characteristic is controlled by a recessive gene and the green and cream colors probably are regulated by other alleles in the same place.

The intense red exocarp color of Bartlett known as Cardinal Red and Max Red are regulated by a single dominant gene. It can be affirmed that the exocarp red color of cv. Sanquinole is dominant over the white color of cv. Conference (Hancock 2008). Not all the red exocarp mutations are sexually transmitted, as this coloring has influence only on the fruit epidermis, as observed in the Starkrimson cv. (Yang et al. 2015).

The exocarp's reddish color is acceptable if it is soft, even and with light tan for in natura consumption, but with these characteristics they are not acceptable for processing. In *Pyrus communis*, Bell (2014) affirmed that the control for this coloring was under quantitative control. The same author estimated that the heredity in *P. pyrifolia* was under the control of a single gene. However, Marino et al. (2013) proposed that the loci R and I could be involved. In this model, the RRxx genotypes are completely related to the reddish color of the epicarp and the Rrii genotypes are partially related. RrIx genotypes have a partial marker associated with high ethylene production, while a second marker has been associated with moderate ethylene production.

Low ethylene production does not have any of the above-mentioned markers. Wolko et al. (2010) and Yamamoto et al. (2014) cloned 13 cDNAs encoding cell wall hydrolases in European pear and followed their expression patterns during cold storage. Yamada et al. (2006) cloned two isoforms of invertase soluble acids of Japanese pear and monitored their development during maturation; one being particularly active in younger fruits. Tateishi et al. (2005) isolated a cDNA fragments from the soft-flesh pear enzyme, β-galactosidase. Several genes encoding the membrane-bound proteins were cloned, including the arabinogalactan proteins in *Pyrus communis* (Zhang et al. 2011a, b) and H (+)- pyrophosphatase in *P. pyrifolia* (Suzuki et al. 1999). Other additional genes that have been characterized in pear include alcohol dehydrogenase (Qin et al. 2017), polyphenol oxidase (Li et al. 2011) and α-L-arabinofuranosidase (Tateishi et al. 2005).

4.4 Trends in Tree Rootstocks

European pear is propagated through grafting with the use of rootstocks (Wertheim and Vercammen 2000). Many rootstock characteristics positively influence the tree-

top, increasing productivity, reducing plant size and juvenile period, and confer tolerance to climatic stresses and resistance to diseases and pests (Sharma et al. 2010). The most commonly used rootstocks in European pear-producing countries originates from the same species (*Pyrus communis*) and from quince (*Cydonia oblonga*) (Wertheim and Vercammen 2000).

In Europe, the clones most commonly used are *Cydonia oblonga* selections, such as quince A (EMA), quince C (EMC) and BA29, and in the United States with *Pyrus communis* clones such as Old Home × Farmingdale (OHxF), which improve the precocity, productivity and quality of some European pear cultivars (Ercisli et al. 2006).

Currently rootstock breeding programs aim to create clones that have fruiting precocity and less vigor, enabling cultivation at high-density, aiming to provide the farmer a short-term investment returns, management ease and labor savings (Werteim 2002). Examples are the series Old Home × Farmingdale (United States), Fox series (Italy), Pyriam series and Brossier (France), Pyrodwarf and Pyro 2-33 (Germany) and 708-36 (England) (Grimaldi 2014).

Pear cultivation in Brazil faces some problems that hinder its expansion, such as the low quality of cultivated plants due to the lack of cultivars adapted to the producing regions, resulting in low yields. Thus, there is a demand to develop treetop cultivars and high compatibility rootstocks and, at the same time, adapted to the soil and weather conditions of the main producing regions of Brazil (Pio et al. 2008).

Due to the great genetic variability existing in this crop, rootstocks tolerant to the specific abiotic stresses can be developed for the purpose of increasing and stabilizing productivity. In the same way, it is necessary to develop materials with resistance to pests and diseases, since the management of pathogens using chemicals is expensive and risky. Diseases like fire blight and scabies are difficult to control, even when application timing is strictly followed (Sharma et al. 2010).

Another breeding objective is to find new rootstocks that will result in plants with smaller crown diameter, vigor and high efficiency in the use of sunlight, accelerating production and increased yield. More compact plants allow planting at higher density, cultural practices become easier, and the use of mechanization in the orchards is facilitated, especially during the harvest season (Machado et al. 2012).

The use of quince rootstocks has provided a considerable reduction in plant vigor (Fig. 4.1) and precocity in the production of European pear; however, the problem faced by this combination lies in the morphological incompatibility between both (Pina and Errea 2009). As a solution, the use of Beurré Hardy and Old home has appeared as intergrafts. In addition, clones of *Pyrus communis* were developed by breeding as another alternative, since rootstocks of this species do not present incompatibility with European pear, such as the OHxF and the Fox series (Jackson 2003).

Nowadays, there are improvement programs for pear cultivation, such as in the United States, Australia, Italy, Germany, France and England, that have developed promising rootstock materials (Grimaldi 2014). In the major pear-producing regions of the world, quince rootstocks are used to reduce plant vigor, aiming to increase production efficiency and productivity, as well as to increase the precocity and quality of European pears. The most important existing quince rootstocks are East Malling

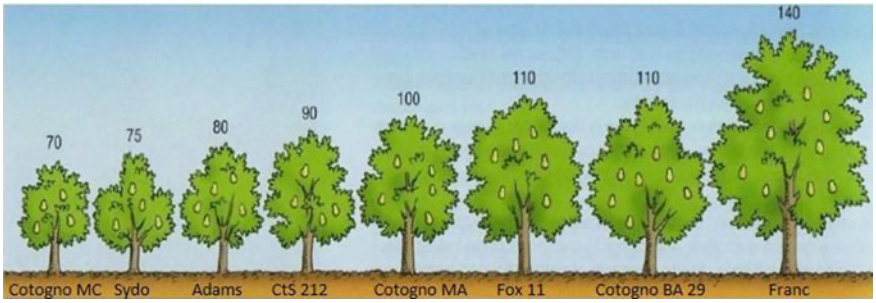

Fig. 4.1 Seven clonal rootstocks (six of quince, one of pear Fox 11 and one franc). The scheme represents the vigor induced by the different rootstocks with reference to quinine

A (EMA), East Malling C (EMC), Provence BA29 (BA29, Sydo, Adams, EMH, CTS 212) (Machado et al. 2012).

EMA rootstock (Fig. 4.2a) was selected in 1920 in England by the East Malling Experiment Station (Soares et al. 2001). EMA was developed from a D'Angers quince, with the main characteristic being the ease of propagation, it being the most commonly used rootstock in pear orchards in Southern Europe (Musacchi 2008). This rootstock presents high productive efficiency, being less precocious in relation to the production when compared to quince (Jackson 2003). EMA presents good development in deep and fertile soils, being able to be used in plant densities of 1500–2000 plants ha^{-1}. In addition, it adapts well to heavy, but not dry soils with lime content of more than 4–5% (Fachinello and Pasa 2010).

Adams rootstock (Fig. 4.2b) is a clonal selection of an Angers quince type, originating from Belgium in the 1960s. It has slightly higher vigor than the EMC (15%) but lower than the CTS 212 and EMA (Soares et al. 2001). Adams quince rootstock induces intermediate vigor between EMA and EMC in its effect on the canopy cultivar, inducing early production (Jackson 2003). The EMC rootstock (Fig. 4.2c) was selected and produced virus-free at the East Malling Experimental Station in the 1920s (Jackson 2003). This rootstock is the least vigorous; however, it has incompatibility with several cultivars, such as William's, Kaiser, the Conference and Abbè Fetél. As an ananizing clone, it shows good development in orchards with high plant densities ranging from 3000 to 7000 ha^{-1} (Fachinello and Pasa 2010). The reduced EMC vigor may be beneficial when grading pollinator varieties with excessive vigor, such as Comice and Carapinheira, because when they are grafted on the EMA or Provence BA29, they gain abundant vegetative development, becoming less productive, more subject to production alternation and more susceptible to certain diseases. In this way, the very vigorous varieties, when grafted on EMC, make it easier to control the vegetative plant activity (Soares et al. 2001).

The BA29 Provence quince (Bois Labbe Beaucouze-Angers) (Fig. 4.2d) was selected by the Angers Experiment Station (INRA) in France and registered in the national catalog of fruit species in 1967 (Barbera et al. 2007; Masseron and Trillot 1991). Among the most promising quince rootstocks, BA29 has been shown to be the

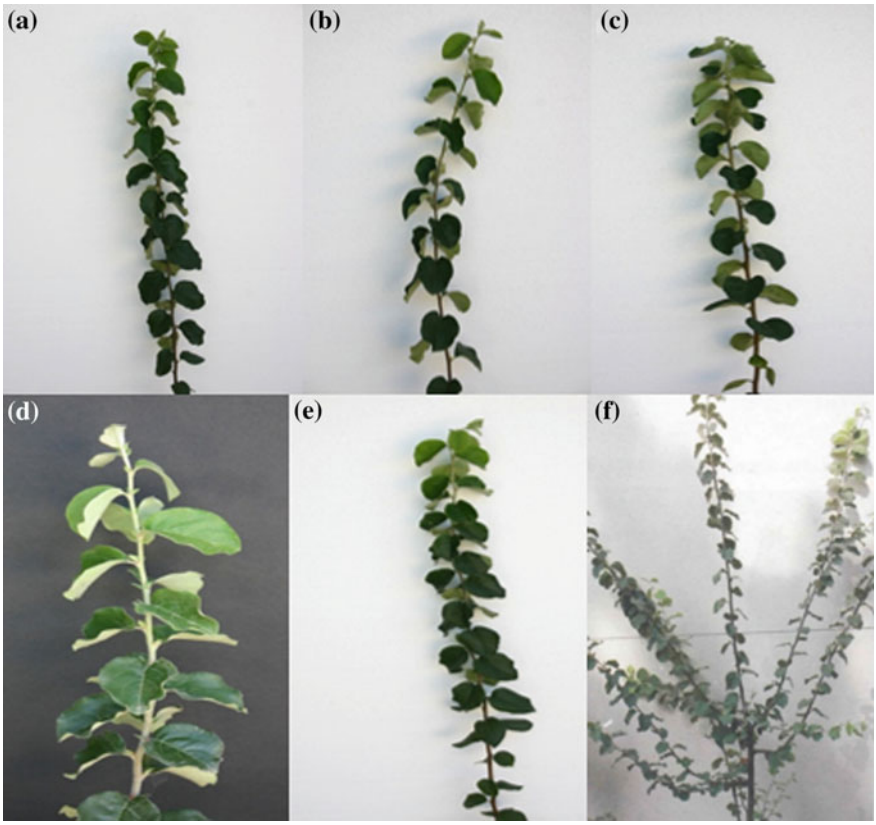

Fig. 4.2 Branch of the main quince rootstock studied. **a** Quince A. **b** Quince ADAMS. **c** Quince EMC. **d** Quince BA29. **e** Quince Sydo. **f** Quince CTS 212

most vigorous, giving rise to plants of greater arborescence compared to EMA and EMC, and its grafting with pears with weak vigor, such as Alexandrine Douillard, Coscia and to Crassane Pass (Soares et al. 2001).

In France, the main varieties grafted on BA29 were more vigorous (10–20%) and more productive than in the Sydo and EMA rootstocks, but in Italy these results were not the same (Barbera et al. 2007). At Rocha orchards, when grafted on BA29, planted in fertile soil, there was excessive vigor in the early years, with vegetative growth sometimes exaggerated. However, when Rocha grafted on BA29 enters fruiting, it is usually a slower process, when compared to the same cultivar grafted on EMA or EMC, showing an early fall of small fruits after the petals fall. This is a result of the strong competition between the vegetative releases and the fruits growth (Madeira 2012).

Sydo rootstock (Fig. 4.2e) belongs to the Angers quince group, and is the most popular clone in Europe (Barbera et al. 2007). Sydo's compatibility with Rocha is

lower than that of Provence BA29, but slightly higher than EMA. In the orchard, this rootstock has a lower vigor than the AMA and the BA29, but its productivity on average is slightly higher than BA29 and EMA. Its fruiting is faster than in Provence BA29 and EMA (Masseron and Trillot 1991).

Sydo also induces better caliber, with largest fruits, higher leaf and fruit Ca^{2+} concentrations (Soares et al. 2001; Quartieri et al. 2013). It is a very promising and more interesting rootstock than EMA and Provence BA29 in irrigated, semi-intensive or intensive cultivation (Sousa et al. 2001). Sydo grafted Rocha pear has a productivity index higher than BA29 and EMA rootstocks, yielding about 60% of pears with a diameter above 60 mm. Sydo rootstock can be used in fertile soils and with medium water resources. At medium plant densities, the productivity of this rootstock is shown to be slightly higher than the AMA (10%) and the BA29 (10–20%) (Soares et al. 2001). Sydo quince is more sensitive to lack of soil moisture and to active limestone (chlorosis in leaves) than the Provence BA29. The root system is very similar to EMA, reasonably branched, with a medium to good ground anchoring power, although less vigorous and less deep than the Provence BA29. However, it has a certain sensitivity to cold, drought, chlorosis and fire blight (Jackson 2003; Loreti 1994).

4.4.1 CTS Series

The CTS series of clones (Fig. 4.2f) was obtained from the Department of Cultivation and Defense of Ligneous Species, University of Pisa, Italy, from a selection of seedlings from a cross between EMA and Italian fruit quinces. This series consists of several clones differentiated by the great diversity of strength of the graft, ranging from low (CTS 212, 411, 412, 414) to high (CTS 407, 410) and productivity that is considered good (CTS 212, 411) to very high (CTS 407, 410). Among the aforementioned rootstocks, CTS 212 is the one that has shown greatest commercial diffusion and has intermediate strength between BA29 and Sydo (Loreti 1994).

4.4.2 Pyrus Genus Rootstock

Pyrus genus rootstocks are compatible with all European pear canopy cultivars and through plant breeding, had selected important features such as strength control, disease resistance, adaptability and production precocity.

Pyrus calleryana rootstock has good grafting compatibility, is resistant to wet ground, drought, nematodes and aphids, but is sensitive to cold and induces excessive vigor in grafted plants (Loreti 1994). It presents good adaptation to hot climates, sandy or clayey soils, has adequate anchorage, compatibility with existing canopy cultivars, and resistance to various diseases such as pear decline and fire blight. In

Brazil, it has been evaluated not only as rootstalk for pears, but also for apple and quince trees (Pio et al. 2008).

Pyrus betulaefolia rootstock is the most vigorous, tolerates decline of pear and fire blight, has resistance to extremely low temperatures and is therefore widely used in California. Although it adapts well to both dry and wet soils it does not tolerate waterlogged soils during the growth period. It is recommended as a rootstock for Asian pear trees (Faoro and Nakasu 2001; Leite and Denardi 1992; Webster 1998; Werteim 2002).

Pyrus betulaefolia plants are quite vigorous and have been used as rootstocks for cultivars of *P. communis*, which are less vigorous and produce red-colored fruits (Camellato 2003). Rootstocks of *P. calleryana* and *P. betulaefolia* are the most commonly used for pear tree cultivation in Brazil (Leite 2000). It is estimated that they used in 90–95% of the total cultivated area of pears (Rufato et al. 2004).

The Pyrodwarf clone was obtained from a cross between Old Home and Buona Luisa d'Avranches, made in Germany. This rootstock possesses high resistance to fire blight, has similar or inferior vigor to quince BA29 but has a delay in fruit production related to it (Colombo 2008; Mitcham and Elkins 2007). It shows good compatibility with cvs. William's and Abbé Fétel (Colombo 2008).

Clones of the Fox series were obtained by micropropagation from a free pollination selection of cv. Volpina at the University of Bologna, Italy. They were developed to reduce plant vigor, being considered a little less vigorous than the francs of *Pyrus communis*. The root system is slightly branched but has a good anchoring (Colombo 2008; Fideguelli and Loreti 2009). Clones Fox 11 (Fig. 4.3) and Fox 16, and Fox 9 are prominent among the rootstocks of the series, as they are undemanding in terms of soil and have good compatibility with the most popular pear tree cultivars (Machado et al. 2012).

Italian orchards with William's cultivar, grafted on Fox 11 and Fox 16 rootstocks, planted at a density of approximately 800–1000 ha^{-1}, plants expressed good fructification and precocious production. Some studies indicate that Fox 11 is slightly more vigorous than BA29 and allows a planting density of up to 2500 ha^{-1}, with good productive efficiency (Machado et al. 2012).

In the United States, rootstocks used in the orchards are of the OHxF series from *Pyrus communis*, developed to present different levels of vigor, resistance to cold, resistance to fire blight and pear decline, and are mainly highly compatibility with pear tree cultivars.

The rootstocks of the OHxF series that stand out are numbers 40, 69, 87, 97 and 333 (Fig. 4.4). These originate from *Pyrus communis*, from crosses conducted by the University of Oregon, aiming at resistance to fire blight by using highly-resistant parents. Not only are these rootstocks highly compatible with cultivars, they also have different levels of vigor, are precocious, tolerant to soils, chlorosis, resistant to cold (temperatures down to −28.9 °C), fire blight and pear decline.

Rootstocks of the 708 series were generated from a cross between Old Home and BP1. They are considered semi-dwarf to very dwarf, productive and early, with large fruits; propagated by semi-woody cuttings (708-2 difficult, 708-36 easier).

Fig. 4.3 Fox 11 rootstock sample

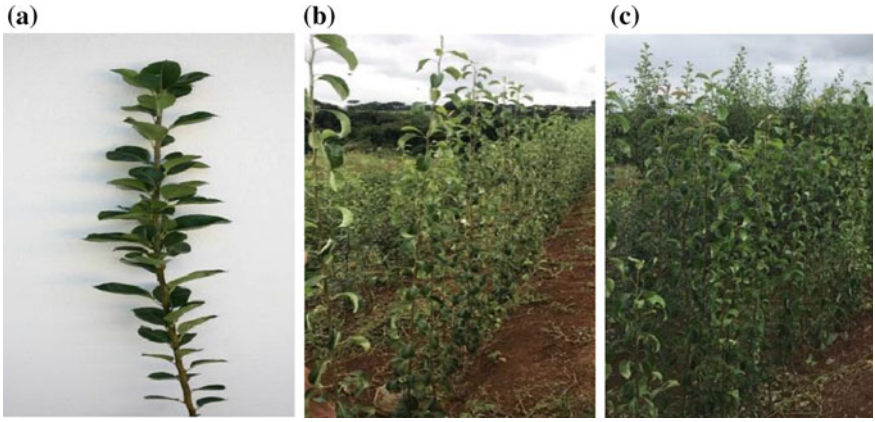

Fig. 4.4 Nursery rootstock of *Pyrus communis*, grafted with the canopy of Santa Maria 2017, Urubici-SC- Brazil. **a** OHxF 40, **b** OHxF 69, **c** OHxF 87

In Brazil, the important center for studies on fruit cultivation, the Center for Agroveterinary Science, State University of Santa Catarina (CAV/UDESC), began researching pear tree culture in 2006 in a commercial orchard in Vacaria, Rio Grande do Sul state. Since then, several studies have been carried out with pear culture, following different lines of research, with the objective of generating technical information for Brazilian producers, as well as to promote cultivation, since pears are among the main imported agricultural products. Among the Brazilian studies, the most important are the evaluation of growth patterns and production of European pear tree cultivars on quince rootstock, as well as the selection of *Pyrus communis* genotypes with rootstock potential and to develop specific micropropagation protocols.

Micropropagation consists of regeneration and multiplication of plants from cells and tissues, cultivated in nutrient medium. This method allows the mass production of plantlets, regardless of the time of year under excellent sanitary conditions. For pear culture, some limitations are experienced in micropropagation due to recalcitrant behavior during the multiplication phase, difficulty in the rooting induction and slow plant growth during acclimatization (Grimaldi et al. 2012). Despite these issues, the CAV/UDESC micropropagation lab has been able to successfully micropropagate pear rootstocks since 2013, with high multiplication rates (Fig. 4.5).

Currently, the best micropropagation protocol for pear rootstocks, including the *Pyrus communis* selections, the OHxF series and Pyrodwarf, is briefly described here. To establish the rootstocks in vitro young lateral buds are used, with basic asepsis using 70% alcohol for 1 min and 2.5% sodium hypochlorite for 15 min. The culture medium used in all phases is QL modified by Leblay (Leblay et al. 1991) supplemented with 30 g L^{-1} sucrose, 100 mg L^{-1} of inositol, pH of 5.8 and 6 g L^{-1} agar. During the establishment phase, no growth regulators are used in the medium and explants are kept in the dark for 7 days. For the in vitro multiplication phase, the medium is supplemented with 1 mg L^{-1} of 6-benzyl aminopurine (BAP); whereas, for the in vitro rooting phase, the medium is supplemented with 1.2 mg L^{-1} of indol butiric acid (IBA). During all the in vitro phases, the explants are under a photoperiod of 16 h and a temperature of 25 ± 2 °C. Acclimatization of the regenerated plantlets is done in a greenhouse with nebulization that delivers misted water for 2 s every 20 min, and is periodically augmentation with high levels of nitrogen.

4.5 Quantitative Trait Loci (QTLs) and Molecular Markers

A major problem facing pear breeders is to associate genotypic with phenotypic data, due to the high degree of self-incompatibility aligned with the juvenile developmental plant time. Thus, the use of marker-assisted selection (MAS) technology is a useful tool because of its sensitivity, making it possible to identify polymorphisms between genotypes, enabling accelerated breeding programs without the need to wait for the long period of maturity, thereby reducing costs and progeny size.

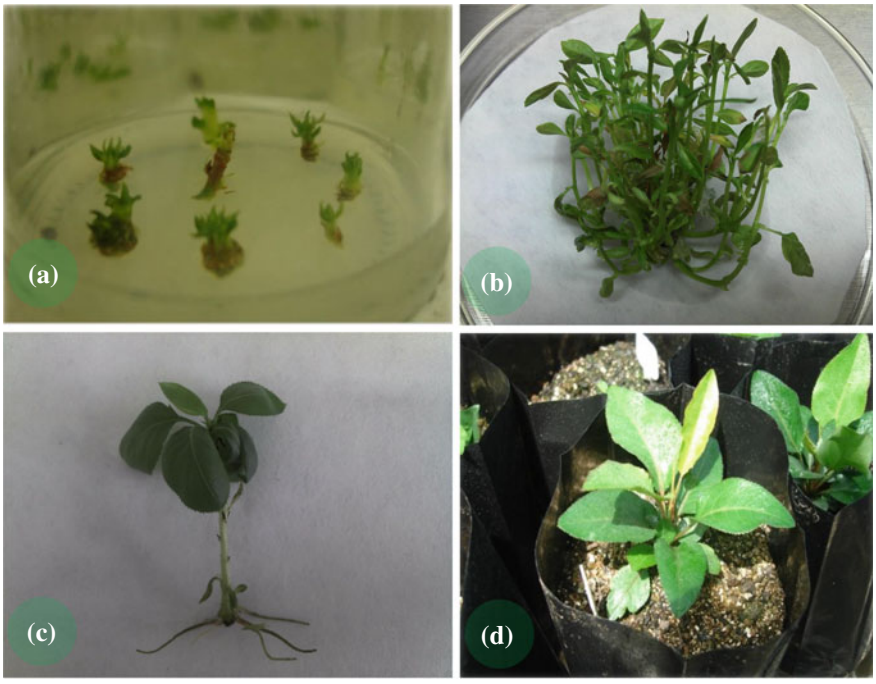

Fig. 4.5 Selections of *Pyrus communis* micropropagated by the CAV/UDESC laboratory. **a** Culture initiation from bud tissue. **b** Shoot multiplication. **c** Rooting. **d** Ex vitro plants successfully growing soil after and acclimatization

The molecular markers most useful for MAS are simple sequence repeats (SSRs), amplified fragment length polymorphism (AFLP), sequence related amplified polymorphism (SRAP), inter-simple sequence repeats (ISSR) and random amplified polymorphic DNA (RAPD) (Khorshidi et al. 2017; Kim et al. 2000; Monte-Corvo et al. 2001; Nishio et al. 2016a, b; Sawamura et al. 2004).

The construction of genetic linkage maps with molecular markers has become essential to support genetic breeding programs, in order to identify quantitative trait loci (QTLs) associated with important characteristics. QTLs are regions responsible for the expression of phenotypic characteristics with continuous distribution (Collard et al. 2005). In the pear, the most important and studied characteristics are diseases such as black spot, the aphid *Dysaphis pyri*, fire blight and scab, rate of fruit growth and leaf traits.

Yamamoto et al. (2002) identified 13 SSR markers from the Japanese pear (*Pyrus pyrifolia*); of these, 10 could be amplified in the European pear (*P. communis* L.). Similar work identified 19 SSR markers from European pear (Fernández-Fernández et al. 2006). A genetic linkage map with 894 SSRs was developed to facilitate genetic and breeding studies (Chen et al. 2015).

For black spot disease, two SSR markers were identified as tightly linked to the genes *Ani* and *Ana* in a Japanese cv. (Terakami et al. 2007, 2016). For psylla disease, 1 QTL was identified by SSR markers (Dondini et al. 2015).

Two populations were used to generate two genetic maps with SSRs, MFLPs, AFLPs, RGAs and AFLP-RGAs markers, with a total of 311 loci split in 37 linkage groups, and with this, it was possible to identify 4 QTLs for fire blight (*Erwinia amylovora*) disease (Fig. 4.6) (Dondini et al. 2004). More recently, 2 of these QTLs were repositioned and validated, besides the inclusion of new SSR markers on this linkage map (Le Roux et al. 2012). A similar study was conducted on apple and identified 2 more QTLs for fire blight in LGs 2 and 9 of the map, involved in epistatic interactions with apple (Calenge et al. 2005). In *Pyrus ussuriensis*, a QTL for fire blight was identified on the linkage group 11 (Bokszczanin et al. 2009). Evans et al. (2008) found that the *Dp-1* gene, the gene responsible for *Dysaphis pyri* resistance, is flanked by NH006b and NH014a markers in the linked group 17 and suggested a doubling in the linked group 9.

SSR molecular markers identified the gene (*Vnk*) involved in scab resistance in a Japanese pear cultivar at linkage group 1 (Terakami et al. 2006). Another gene (*Rvp1*) was mapped on linkage group 2 of the European pear genome, close to CH02b10 SSR marker (Bouvier et al. 2012). Linkage maps with SSR and AFLP markers were also constructed for scab pear in *Pyrus communis*, where it was possible to identify 2 QTLs in the groups 3 and 7 associated with scab resistance (Pierantoni et al. 2007). A similar study was conducted by Yamamoto et al. (2007) and it was possible to identify 124 SSR and 601 AFLP markers in 17 linkage groups in two F1 populations. Another strategy used for scab resistance were 5 DNA markers linked to scab resistance evaluated in a Japanese pear-breeding program and it was validated that individuals possessing these markers were classified as resistant (Gonai et al. 2009). Several other QTLs were discovered for scab resistance (Cho et al. 2009; Perchepied et al. 2015).

For fruit traits, 7 QTLs were detected using AFLP markers to construct a genetic linkage map (Zhang et al. 2011a, b). In another study, the same group identified 19 QTLs using SRAP, SSR and AFLP markers (Zhang et al. 2013). One functional marker (Md-Exp7SSR) was identified on the linkage group 1 of apple and pear genomes, where 1 QTL for fruit firmness was identified previously (Costa et al. 2008). For leaf traits, 11 QTLs were detected in 18 linkage groups with AFLP and SSR markers (Sun et al. 2009). Several other studies identified many QTLs for fruit traits with these molecular markers (Yamamoto et al. 2014).

By 2013, only a few hundred QTLs and markers had been identified in pear. However, after the new generation sequencing (NGS) and the Chinese pear sequencing (*Pyrus bretschneideri*) (Wu et al. 2013), it was possible to re-sequence several accessions of pear, as well as to verify variation of one nucleotide in all of the pear genome, aligning with the reference genome to verify in silico possible polymorphisms. After NGS and pear sequencing, it was possible to construct genetic maps with single nucleotide polymorphism (SNPs) markers. With SNPs, it is possible to identify all mutations of one nucleotide and increase dramatically the marker numbers and QTLs in a genetic map.

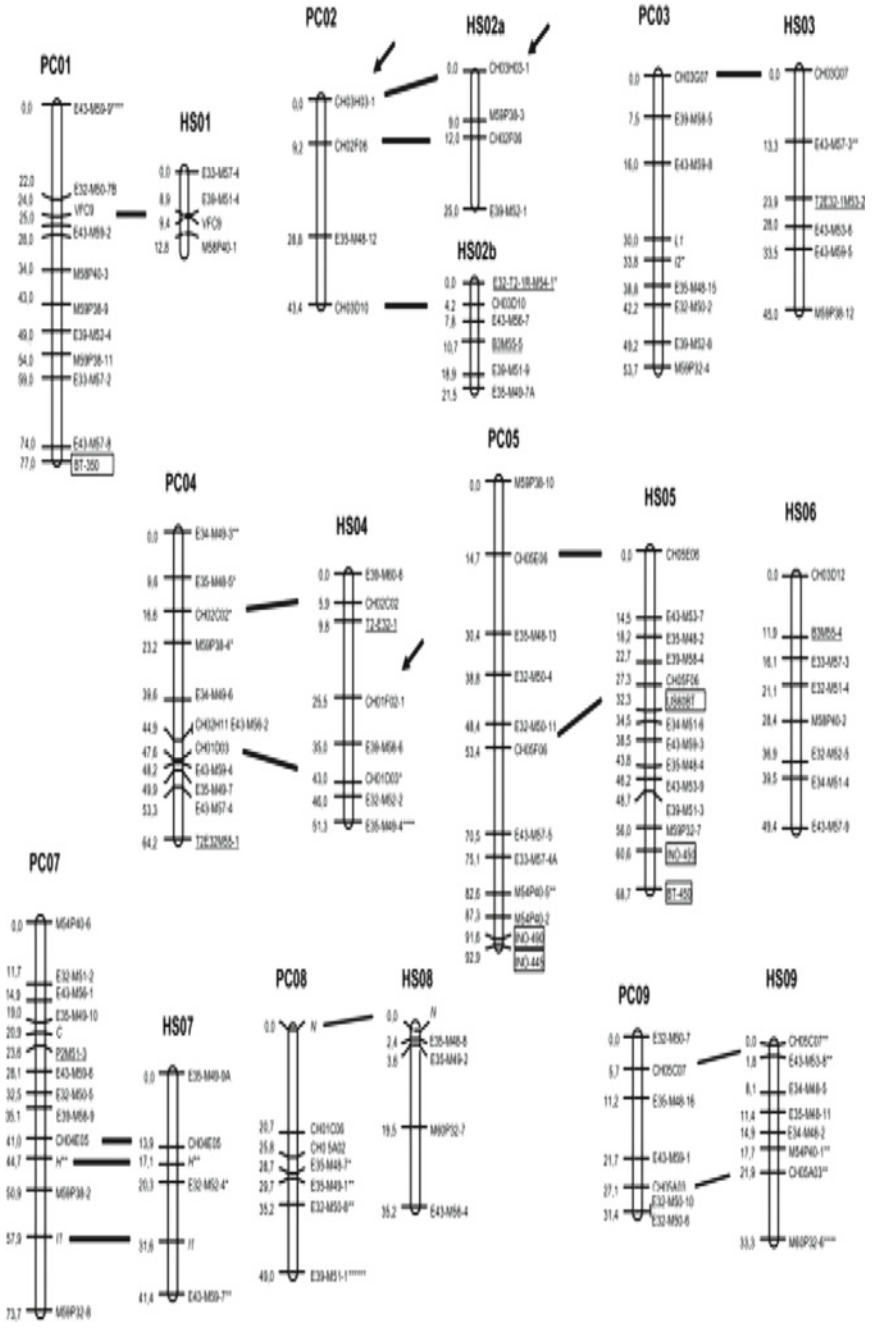

Fig. 4.6 Identifying QTLs for fire-blight resistance via a European pear (*Pyrus communis* L.) genetic linkage map. *Source* Dondini et al. (2004)

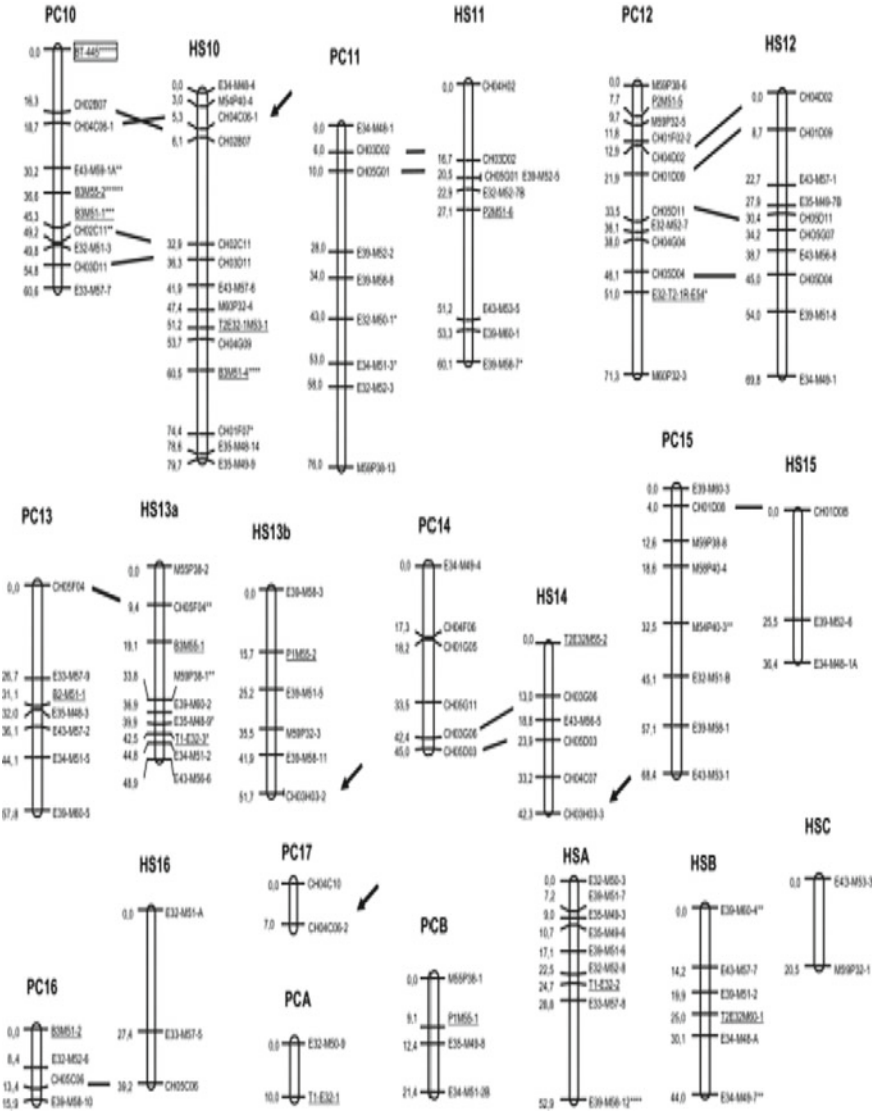

Fig. 4.6 (continued)

Only one high-density genetic map made of SNPs could generate 4797 SNPs that will be useful in a breeding program because it could identify important genes associated with economically-important characteristics (Wang et al. 2017). With a high density (SNP)-based genetic map, it was possible to identify the first QTL associated with vigor control and flowering traits in pear rootstocks, and with the same SNP array it was possible to identify a QTL for root development (Knäbel et al. 2015, 2017). An interspecific progeny derived from European and Asian pears was genotyped with SNPs markers for map construction and covered 17 linkage groups with 7 QTLs identified and effective against 3 scab isolates (Won et al. 2014).

For fruit traits, it was possible to develop a high-density linkage map of 3143 SNP and 98 SSRs markers used to detect 32 QTLs for 11 traits (Wu et al. 2014). For fire blight disease, a genetic linkage map constructed with SSR and SNPs markers was capable of detecting a major QTL on linkage group LG 2 of Moonglow, which was stable in two distinct environments and genetic background (Fig. 4.7) (Montanari et al. 2016). With all this information on the pear, we can infer that genetic linkage maps have been sufficiently saturated.

4.6 Transgenic Pears

In recent years, research centers have invested heavily in the development of transgenic pear clones. These transgenic plants have being studied, analyzed and developed. Several centers are trying to obtain cultivars resistant to fire blight disease, and studies with genetic transformation for this purpose have been developed. This is the case of a study with the cv. Pass Crassane that used the overexpression of the gene *Attacin E*, and thus it was possible to reduce the symptoms of fire blight disease in 6 genetic transformed lines (Reynoird et al. 1999).

Another study with *Pyrus communis* silenced the gene coding for ACC oxidase (ACO), and with this it was possible to decrease ethylene production by 85%, which could increase shelf life (Gao et al. 2007). Another work focused on the overexpression of transcription factors (TFs), aiming at knowledge of the metabolic route of anthocyanin biosynthesis (Yao et al. 2017). Matsuda et al. (2009) inserted the citrus *FLOWERING LOCUS T* (*CiFT*) gene in *P. communis* to induce early flowering. Another work aimed at the early flowering in *P. communis* was developed by Freiman et al. (2012), where the RNAi technique was used to silence the PcTFL1 gene, with promising results.

To date, there are no transgenic pear cultivars being marketed anywhere in the world. However, the Canadian company Okanagan Specialty Fruits, responsible for the development of Arctic® apple, is applying the same technology used in apple, to the pear and other fruits (Clive 2015).

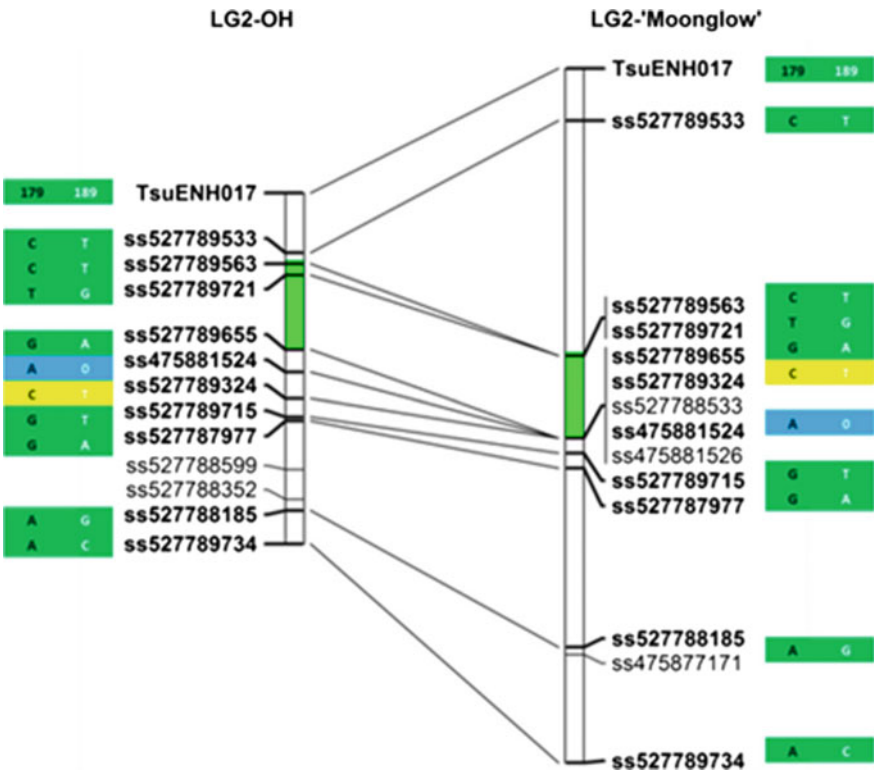

Fig. 4.7 Comparison of Old Home and Moonglow haplotypes within the region spanned by the quantitative trait locus (QTL) for fire blight resistance detected on LG2 of Moonglow. The position of the QTL peak is highlighted in light green on the LG bars. The common SNP markers between the two homologous regions are in bold and are connected with a line. The location of the SSR marker TsuENH017 on OH map is inferred from the Moonglow map. On the side of both regions, the haplotypes for the SNP markers in common and for TsuENH017 are reported: green color indicates collinearity between the two regions; inverted markers are highlighted with different colors. The resistant phase is in black, the susceptible phase in white. *Source* Montanari et al. (2016)

4.7 Impact of Climate Change

Climate change is no longer a simple assumption. In recent decades, the planet has been undergoing constant temperature rises caused by the accumulation of greenhouse gases in the atmosphere, led by carbon dioxide and methane. Computational simulation models predict an increase in the temperature of the European continent of between 0.1 and 0.4 °C per decade (Parry 2000). In recent years the earth has continued to experience record annual temperature increases; in the twenty-first century records have already been broken 15 times (Houston et al. 2017; NOAA 2017).

Global warming has a direct impact on agriculture, because there is instability throughout the world, with less severe winters, intermittent rains, out-of-season frosts

and unforeseen droughts. There are already several reports of fruit-production losses bordering 25% in isolated regions in the USA (Schrader et al. 2003; Schreiber 2016). These instabilities have a strong tendency to intensify, causing more widespread damage and can be said to be catastrophic if emergency measures are not taken in time (Hatfield et al. 2014).

There is major recent concern with perennial temperate fruit trees such as pear, especially in relation to their phenological phases, because the increase in temperature directly influences pollination, disease control, yield, and fruit quality (Grab and Craparo 2011; Vitasse et al. 2009). Studies have shown that many fruits, including pears, have blossomed ahead of time due to climatic and not necessarily genetic reasons (Grab and Craparo 2011; Parmesan 2007; Primack et al. 2009).

An important strategy being adopted by several fruit cultures is the use of freeze protectors, wind machines and methods to delaying bud break, among others. However, these methods most often require high-cost investments which limits their use.

Scientists and breeders are exerting continuing efforts to produce pears adapted to the new climatic conditions, such as low-chill varieties, cultivars adapted to warmer climates, disease resistant and with shorter flowering time (Dondini et al. 2004; Pierantoni et al. 2007). However, the greatest problem encountered in the pear tree is the very long juvenile period, which prolongs access to genetic information for many years (Iwata et al. 2016). However, as described in Sect. 4.5, the discovery of molecular strategies, along with the development of specific and timely molecular markers, have contributed greatly to decrease the time needed for cultivar development.

4.8 Germplasm Conservation

For the use of breeding strategies and biotechnology tools to be possible, it is extremely important to preserve and maintain the available genetic variability. Germplasm banks have the function of conserving the largest possible number of specimens of a species and to ensure that this variability is not lost in a diverse number of dangerous situations (Plucknett et al. 1983).

The U.S. Department of Agriculture has a germplasm bank of *Pyrus*, maintained as orchard trees, with a collection of about 2239 clones, with representatives of 36 *Pyrus* taxa from 56 different countries (USDA 2014). This bank is public and germplasm is available free of charge between research centers. In this way, it is possible to explore the genetic variability existing within the species in diverse research centers.

4.9 Conclusions and Prospects

The breeding of pear trees preferably aims at developing valuable commercial cultivars bearing fruits with desirable characteristics, such as succulence, good appearance, and long shelf life, among others. In order to expand the area of pear cultivation,

it is necessary to develop new plant materials by using the genetic pool to search for cultivars with desired traits such as tolerance to high or low temperature, humidity and drought, as well as to alkaline, saline or acidic soils.

Likewise, it is necessary to develop plant material, resistant to pests and diseases. Diseases like fire blight and scab are difficult to control, even when the application schedule is strictly followed. The search for resistant/tolerant plants is a major goal in pear-tree breeding worldwide.

Due to the extended plant juvenile period, obtaining new cultivars requires a long period of time, in addition to high cost, making it difficult to develop new cultivars. Through the use of molecular marker assisted selection (MAS), the discovery of new QTLs each day, the advent of transgenics, and from the complete sequencing of the pear genome, allied with the large gene pool and genetic variability in the culture, the future for breeding is promising. It is believed that programs can greatly accelerate the realization of new cultivars to meet field and market requirements by producing, in less time, specific fruit types to satisfy the preferences of all ethnic groups involved in pear consumption around the world.

Appendix 1

Research institutes and online resources

1. Institut de Recherche en Horticulture et Semences. 147 rue de l'Université 75338 Paris.
2. Better3fruit. Steenberg 36. B-3202 Rillaar, Belgium.
3. European Fruit Research Institutes Network, Research Center for Fruit npo (pcfruit npo), Fruittuinweg 1, 3800 Sint-Truiden, Belgium.
4. Prevar—Premium Apple and Pear products. Prevar Limited. 105 Eastbourne Street West. Hastings 4122—New Zealand.
5. Harrow—Vineland—Vineland Research and Innovation Centre. 4890 Victoria Avenue N, Box 4000, Vineland Station, ON L0R 2E0 | 905.562.0320. Ontario, Canada.
6. Plant and Food Research—Rangahau Ahumara Kai, 120 Mt Albert Road, Sandringham. Auckland 1025. New Zealand.
7. Inova Fruit. Stationsweg 28 5301 KH Zaltbommel. The Netherlands.
8. Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina—EPAGRI. Abílio Franco st, 1.500 C.P. 591 Zip: 89500-000 Caçador, SC, Brazil.
9. Empresa Brasileira de Pesquisa Agrícola—EMBRAPA Uva e Vinho. Livramento st, nº 515 Caixa Postal: 130 CEP: 95701-008—Bento Gonçalves, RS, Brazil.
10. USDA ARS Appalachian Fruit Research Laboratory, Kearneysville, West Virginia. 2217 Wiltshire Rd, Kearneysville, WV 25430, USA. e-mail: richard.bell@ars.usda.gov

11. Washington State University Tree Fruit Research and Extension Center, Wenatchee, Washington. 1100 N. Western Ave. Wenatchee, WA 98801-1230, USA. kate.evans@wsu.edu
12. CAHMRS Department of Horticulture, Genomics Lab, Washington State University, Pullman, Washington. Department of Plant Sciences, University of California, Davis. 387 N Quad, Davis, CA 95616, USA
13. Pear Horticulture Research Program. Todd Einhorn, Mid-Columbia Agricultural Res. & Extension Center, Oregon State University Corvallis, Oregon. 4017 Agriculture and Life Sciences Building, OR 97331, USA. e-mail: hortwebteam@oregonstate.edu
14. RosBreed. Mainlab Bioinformatics, Washington State University. Pullman, WA, USA
15. USDA ARS National Clonal Germplasm Repository, Corvallis, Oregon USDA-ARS, NAT. CLONAL Germplasm repository. 33447 Peoria Road Corvallis, OR 97333, USA
16. Washington Tree Fruit Research Commission. 1719 Springwater Ave., Wenatchee, WA 98801, USA
17. Pear Bureau Northwest 4382 SE International Way, Milwaukie, OR 97222, USA
18. California Pear Advisory Board 1521 I Street Sacramento, CA 95814, USA
19. Pear Pest Management Research Fund (PPMRF), 1521 I St. Sacramento, CA 95814, USA

Appendix 2

Genetic resource of the most common cultivars of pear in the world, maintained by the National Clonal Germplasm Repository, World Pear Collection, USDA Gene bank, Corvallis, Oregon, USA

Cultivar	Taxon	Origin	Traits
Abbe Fetel	*Pyrus communis*	France	Fire blight susceptible, low chill, quince compatible, diploid, heirloom, DNA standard, curator choice, parthenocarpic
Anjou	*Pyrus communis*	Belgium	Very late ripe, fire blight susceptible, quince compatible, diploid, heirloom
Anjou—Columbia Red	*Pyrus communis*	Oregon	Fire blight susceptible, red skin, diploid
Anjou—Dwarf	*Pyrus communis*	United States	Late ripe, fire blight susceptible
Anjou—Gebhard Red	*Pyrus communis*	Washington	Fire blight susceptible, red skin, diploid
Anjou—Naumes	*Pyrus communis*	United States	Late ripe, fire blight susceptible, quince compatible, diploid
Anjou—Russet	*Pyrus communis*	United States	Late ripe, russet fruit, diploid
Anjou—Striped	*Pyrus communis*	Oregon	Fire blight susceptible, striped fruit
Arbi	*Pyrus communis*	Tunisia	Low chill, diploid
Atago	*Pyrus pyrifolia*	Japan	Diploid
Ayers	*Pyrus hybrid*	Tennessee	Fire blight resistant, scab resistant, attractive, similar to Seckel, curator choice, diploid
Baldwin	*Pyrus sp.*	Alabama	Low chill 480 h, diploid
Bartlett	*Pyrus communis*	United Kingdom	Fire blight susceptible, virus indicator, diploid, heirloom, monolinia resistant, DNA standard
Beurré d'Amanlis	*Pyrus communis*	France	Quince compatible, polyploid, heirloom
Bon Chretien Bonnamour	*Pyrus communis*	France	Heirloom, diploid
Bon Chretien d'Hiver	*Pyrus communis*	Europe—Ancient	Very late ripe, ancient, heirloom, diploid
Bosc—Gebhard Russetless	*Pyrus communis*	Oregon	Fire blight susceptible
Bosc—Golden Russet	*Pyrus communis*	Oregon	Russet fruit, diploid, curator choice

Cultivar	Taxon	Origin	Traits
Bosc—OP-5	*Pyrus communis*	Belgium	Fire blight susceptible, virus indicator, scab resistant, russet skin, diploid, heirloom
Bou Tsu Li	*Pyrus pyrifolia*	China	Scab resistant, diploid
Carrick	*Pyrus communis*	Tennessee, Brazil	Fire blight resistant, low chill, promising in Brazil, quince compatible, diploid
Chojuro	*Pyrus pyrifolia*	Japan	Black spot resistant, diploid, heirloom
Clapp Favorite	*Pyrus communis*	Massachusetts, Canada	Fire blight susceptible, monolinia resistant, diploid, heirloom
Conference	*Pyrus communis*	United Kingdom	Quince compatible, diploid, apparently self-fertile (parthenocarpic), heirloom, DNA standard
Dangshansu Li	*Pyrus ussuriensis*	China	Heirloom, DNA standard, genome sequenced
De Cloche	*Pyrus communis*	France	Perry, polyploid
PDoyenne du Comice	*Pyrus communis*	France	Late ripe, fire blight susceptible, frost resistant, quince compatible, diploid, heirloom, curator choice, DNA standard
Farmingdale	*Pyrus communis*	Illinois, Asia	Late ripe, fire blight resistant, diploid
Flemish Beauty	*Pyrus communis*	Belgium	Fire blight susceptible, cold hardy, mildew resistant, quince compatible, diploid, heirloom
Flordahome	*Pyrus hybrid*	Florida	Fire blight resistant, low chill 260 h
Forelle	*Pyrus communis*	Germany, Brazil	Late ripe, fire blight susceptible, diploid, heirloom
Fragrante	*Pyrus communis*	Italy	Very late ripe
French Resistant	*Pyrus communis ssp. pyraster*	United States	Rootstock
Garber	*Pyrus hybrid*	Pennsylvania	Very late ripe, fire blight resistant, low chill, heirloom
Giant Seckel	*Pyrus communis*	Oregon	Fire blight resistant, cytoplasmic male sterility, diploid
Hand Winter	*Pyrus communis*	Armenia	Late ripe
Harrow Delight (HW 603)	*Pyrus communis*	Ontario, Canada	Fire blight resistant, quince compatible, cold hardy (fruited in Anchorage)

Cultivar	Taxon	Origin	Traits
Harrow Sweet (HW 609)	*Pyrus communis*	Ontario, Canada	Fire blight resistant, diploid
Harvest Queen (HW 602)	*Pyrus communis*	Ontario, Canada	Quince compatible
Highland	*Pyrus communis*	New York	Fire blight susceptible, quince compatible, diploid
Hood	*Pyrus hybrid*	Florida	Fire blight resistant, low chill 260 h, diploid, apparently self-fertile, scab resistant (Australia)
Horner	*Pyrus communis*	Oregon	Rootstock
Hortensia	*Pyrus communis*	Germany	Scab resistant, late autumn ripe
Hosui	*Pyrus pyrifolia*	Japan	Fire blight susceptible, black spot resistant, scab resistant, diploid, curator choice, DNA standard
Khan Tangoo I	*Pyrus pyrifolia hybrid*	Pakistan	Diploid
Kieffer	*Pyrus hybrid*	Pennsylvania	Late ripe, fire blight resistant, low chill 680 h, quince compatible, diploid, heirloom
Kikusui	*Pyrus pyrifolia*	Japan	Fire blight susceptible, black spot resistant, mildew resistant, scab resistant, diploid
Kosui	*Pyrus pyrifolia*	Japan	Black spot resistant, scab resistant, diploid, curator choice
Le Conte	*Pyrus hybrid*	Georgia	Fire blight susceptible, low chill 680 h, heirloom
Le Lectier	*Pyrus communis*	France	Monolinia resistant, late ripe, heirloom
Leopardo Morettini	*Pyrus communis*	Italy	russet fruit, curator choice
Louise Bonne d'Avranches (L.B. de Jersey)	*Pyrus communis*	France	Quince compatible, monolinia resistant, diploid, Heirloom
Lukavanski	*Pyrus communis*	Czech Republic	
Madame Verte	*Pyrus communis*	Belgium	Very late ripe, heirloom
Magness	*Pyrus communis*	Maryland	Fire blight resistant, scab resistant, quince compatible, cytoplasmic male sterility, diploid
Maxine	*Pyrus communis*	Ohio	Fire blight resistant, mildew resistant, quince compatible, diploid, heirloom

Cultivar	Taxon	Origin	Traits
MoongLow	*Pyrus communis*	Maryland	Fire blight resistant, diploid
Nelis	*Pyrus hybrid*	United States	Rootstock
Niitaka (=Singo)	*Pyrus pyrifolia*	Japan	Fire blight susceptible, black spot resistant, scab resistant, diploid
Nijisseiki	*Pyrus pyrifolia*	Japan	Fire blight susceptible, diploid, heirloom
Nijisseiki × D'Anjou	*Pyrus hybrid*	United States	Late ripe
Nijisseiki × Old Home	*Pyrus hybrid*	United States	Rootstock
Nijisseiki × Old Home (Decline Susceptible)	*Pyrus hybrid*	United States	Rootstock, scab resistant, pear decline susceptible
OHxF 1 to 97	*Pyrus communis*	Oregon	Rootstock, fire blight susceptible
Okusankichi	*Pyrus pyrifolia*	Japan	Very late ripe, fire blight susceptible, heirloom
Old Home	*Pyrus communis*	Illinois	Fire blight resistant, rootstock, quince compatible, cold hardy, diploid
Old Home × P. betulifolia-1	*Pyrus hybrid*	United States	Fire blight resistant, scab resistant
Orient	*Pyrus hybrid*	California	Fire blight resistant, low chill 680 h, polyploid
Packham Late	*Pyrus communis*	Australia	Very late ripe
Packham's Triumph	*Pyrus communis*	Australia	Late ripe, fire blight susceptible, diploid, heirloom
Parker	*Pyrus communis hybrid*	Minnesota	Fire blight susceptible, cold hardy
Parrot	*Pyrus communis*	United Kingdom	Apparently self-fertile, heirloom
Passans du Portugal	*Pyrus communis*	Portugal	Heirloom
Passe Crassane	*Pyrus communis*	France	Very late ripe, fire blight susceptible, virus indicator, compact habit, frost resistant, quince compatible, monolinia resistant, heirloom, diploid, DNA standard
Passe Crassane Rouge	*Pyrus communis*	France	Very late ripe, fire blight susceptible, quince compatible, compact habit
Plant de Blanc	*Pyrus communis*	France	Perry, diploid
Santa Maria Morettini	*Pyrus communis*	Italy	Diploid

Cultivar	Taxon	Origin	Traits
Seckel	*Pyrus communis*	Pennsylvania	Fire blight resistant, diploid, apparently self-fertile, heirloom, curator choice
Seigyoku	*Pyrus pyrifolia*	Japan	Fire blight susceptible, scab resistant, large fruit, diploid
Seuri Li	*Pyrus pyrifolia*	China	Fire blight resistant, mildew resistant, diploid
Shin Li	*Pyrus pyrifolia*	California	Scab resistant, diploid
Shinseiki	*Pyrus pyrifolia*	Japan	Black spot resistant, scab resistant, cold hardy, diploid
Shinsui	*Pyrus pyrifolia*	Japan	Curator choice, early ripe, diploid
Sidi Bou Ali	*Pyrus communis*	Tunisia	Low chill, diploid
Sierra	*Pyrus communis*	Canada	Fire blight susceptible, quince compatible, diploid
Smith	*Pyrus hybrid*	United States	Diploid
Sucre Verte	*Pyrus communis*	France	Fire blight resistant, ancient, heirloom, diploid
Summercrisp	*Pyrus hybrid*	United States	Cold hardy (fruited in Anchorage), diploid
Tse Li	*Pyrus ussuriensis*	China	Late ripe, ancient, heirloom, low chill 500 h, fire blight resistant, Psylla resistant, diploid
Tzu Ma Li	*Pyrus ussuriensis*	China	Diploid
Uta	*Pyrus communis*	Germany	Scab resistant, fire blight resistant, very late ripe, graft incompatible with quince
Verte Longue d'Automne	*Pyrus communis*	France	Heirloom, diploid
Warren	*Pyrus communis*	United States	Fire blight resistant, low chill, quince compatible, diploid
Williams Precoce Morettini	*Pyrus communis*	Italy	Diploid
Winter Bartlett	*Pyrus communis*	Oregon	Heirloom, diploid
Winter Nelis	*Pyrus communis*	Belgium, Asia	Fire blight susceptible, late ripe, diploid, heirloom
Ya Li	*Pyrus _bretschneideri*	China	Late ripe, fire blight resistant, low chill 500 h, Psylla resistant, early bloom, mildew resistant, diploid, scab resistant (Australia)

Source USDA (2017)

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Chapter 5
Plum (*Prunus* spp.) Breeding

Tomo Milošević and Nebojša Milošević

Abstract Despite over 6000 plum cultivars from 19 to 40 species that have originated in Europe, Asia and America, used for fresh market, processing, canning, drying and deep freezing, there is a continuing need to develop new cultivars through different breeding tools as the requirements of industry and market change. Most plums grown commercially are either hexaploid, *Prunus domestica* L. (European or domestic) or diploid, *P. salicina* L. (Asian or Japanese). The main goal of breeders of European plums are cold hardiness, modest tree size, self-fertility, regular productivity, excellent fruit internal and external quality, storage ability, resistance to *Plum pox virus* (PPV) and, recently, tolerance to climatic change and poor soils. Problems that compromise the production of Japanese plums are susceptibility to spring frosts, insufficient winter hardiness and limited soil adaptations. Basically, selection criteria are compatible in both Japanese and European plum breeding. Hybridization is still very popular in plum breeding programs. However, plum breeding using hybridization techniques is a very slow and expensive, so breeding work was stopped or been suspended for some time in many countries over the last few decades. Recently, research began on biotechnology with the aim of speeding up the breeding process by applying biotechnological methods and cytoembryological analysis. New cultivars in the near future will be developed using both conventional and genetic transformation techniques, especially in the private sector. This review is a synopsis of new European and Japanese cultivars and interspecific *Prunus* hybrids recently released around the world. Also, new breeding technologies are discussed.

T. Milošević (✉)
Department of Fruit Growing and Viticulture, Faculty of Agronomy, University of Kragujevac, Cara Dušana 34, 32000 Čačak, Republic of Serbia
e-mail: tomomilosevic@kg.ac.rs

N. Milošević
Department of Pomology and Fruit Breeding, Fruit Research Institute, Kralja Petra I/9, 32000 Čačak, Republic of Serbia
e-mail: nmilosevic@institut-cacak.org; mnebojsa@ftn.kg.ac.rs

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5.1 Introduction

5.1.1 Botanical Classification and Distribution

Plums are a temperate zone fruit tree and/or shrub belonging to the genus *Prunus* L., subfamily *Amygdaloideae* (syn.: *Prunoideae*) and family *Rosaceae* Jussieu (Potter et al. 2007). The basic chromosome number of plum is 8 ($x = 8$).

Prunus L. includes about 400–430 species, classified into subgenera (Das et al. 2011). The most widely accepted infrageneric classification of *Prunus* by Rehder (1940) consists of 5 subgenera: *Amygdalus* (peaches and almonds), *Cerasus* (sweet and tart cherries), *Prunus* (plums), *Laurocerasus* (evergreen laurel-cherries) and *Padus* (deciduous bird-cherries). Later, Ingram (1948) added to them the subgenus *Lithocerasus* (flowering, sand cherries). The members of these subgenera are mainly distributed in north temperate areas of the boreal hemisphere as a component of forest and desert communities (Ucchesu et al. 2017; Yilmaz et al. 2009) and widely cultivated for fruit and ornament. Some important species of this genus are found in the Indo-Malaysian Mountains, the Andes and other tropical mountains (Depypere et al. 2007; de Boucaud et al. 2002; Kalkman 1965). Fruits from this genus are commonly called *stone fruits*.

Recently, data from different published studies broadened this outlook and revealed that the *Prunus* genus contains ornamental plants such as cherry plum (*P. cerasifera* Ehrh.), a wide range of ornamental cherries, fully fertile hybrids i.e. sour (tart) cherry (*P. cerasus* L.), invasive plants (black cherry *P. serotina* Ehrh.), stone fruits [plums (*P. domestica* L., *P. insititia* L., *P. cerasifera* Ehrh., *P. spinosa* L.)], apricot (*P. armeniaca* L.), sour cherry (*P. cerasus* L.), sweet cherry (*P. avium* L.), peach (*P. persica* L. Batsch.) and almond (*P. dulcis* (Mill.) D. A. Webb. (Cici and Van Acker 2010; Ucchesu et al. 2017). The subgeneric classification and relationships among groups are still not fully understood (Bortiri et al. 2001). Despite new knowledge over the last 30–35 years related to this complex taxonomic problem (Bortiri et al. 2006; Potter et al. 2007), the most widely accepted classification of *Prunus* is that of Rehder (1940) complemented by Ingram (1948).

Prunus spp. have been grown throughout the world for centuries. Among the commercial species, the plum is the most commonly cultivated. The genus originates from five centers in general; Western Asia for *Prunus insititia* L. (Damson plum), Europe for *P. domestica* L. (European plum), Western and Central Asia for *P. cerasifera* Ehrh. (cherry plum), North America for *P. americana* Marsh. (American plum) and China for *P. salicina* Lindl. (Japanese plum) (Watkins 1976).

Plums represent the fruit species with the most controversial genetic origin. Therefore, numerous genetic breeders have carried out concentrated studies of the problem (Zhivondov 2012).

European or domestic plum (*Prunus domestica* L.) is one of the most economically important fruit trees in temperate regions and represents a major crop in Europe and southwest Asia (Ramming and Cociu 1991; Watkins 1995; Zohary et al. 2012). *Prunus domestica* L. is assumed to be a relatively younger species and its wild state is unknown (Hartmann and Neumüller 2009). Earlier, Luther Burbank indicated the Caucasus Mountains near the Caspian Sea as the origin place (gene center) for *P. domestica* and also of its ancestors (Das et al. 2011). Some authors report Asia Minor as the place where natural hybrids between *P. ceracifera* Ehrh. and *P. spinosa* L. first originated and disseminated as seeds of such hybrids from Iran and Asia Minor and may have been the progenitors of *P. domestica* L. in Europe (Crane and Lawrence 1934, 1952). These authors suggest that the hexaploid *P. domestica* L. ($2n = 6x = 48$) is an amphydiploid or allopolyploid of diploid cherry plum (*P. cerasifera* Ehrh., $2n = 2x = 16$) and tetraploid sloe also called blackthorn (*P. spinosa* L., $2n = 4x = 32$). On the basis of extensive research, Rybin (1936) discovered spontaneous interspecific hybrids in the Caucasus region. Among others, one of the natural hybrids had $2n = 6x = 48$ chromosomes and similar morphological traits to European (domestic, common) plum (Mišić 2006; Hartmann and Neumüller 2009). After intraspecific research into the genus *Prunus* in the Caucasus region of Georgia, Bajashvili (1991) presumed that the domestic plum (*P. domestica*) may have originated not only from hybridization between myrobalan and sloe, but in other ways too-directly from hexaploid *P. divaricata* or from *P. spinosa* (alloploidization). That author once again reported that the Caucasus is considered to be one of the origin places of hexaploid plums. However, although the origin of *P. domestica* dates back millennia, surprisingly and inexplicably, no wild types of *P. domestica* have been found in this area by Rybin (1936), Bajashvili (1991) or other researchers.

Moreover, the domestic (European) plum is a member of *Prunophora* Focke subgenus which is subdivided into two sections according to geographical prevalence: *Prunocerasus* and *Eurocerasus* (Hegi 1906). Section *Prunocerasus* contains North American species such as *Prunus americana* March., *P. angustifolia* Marsh., *P. hortulana* Bailey, *P. munsoniana* Wight & Hedrick and *P. maritima* Marsh. Section *Euprunus* contains plums present in Europe and Asia, such as, *P. domestica* L., *P. cerasifera* Ehrh., *P. spinosa* L. and *P. salicina* Lindl.

Thus far, research related to the origin of the domestic plum has not provided clear answers, but it is widely accepted that myrobalan and sloe played a major role in the genesis of these plum species. In the near future, multidisciplinary research is likely to provide more precise answers.

Japanese plum (*Prunus salicina* Lindl., formerly *P. triflora* Roxb.) is diploid ($2n = 2x = 16$) and is very widespread, but in warmer climates. The wild form of *P. salicina* is uncertain as well. Wild strands of *P. salicina* are still reported in Hubei and Yunnan areas in China (Okie and Weinberger 1996). According to Yoshida (1987), it may originate from Yangtze River basin in China. In a very early period, this species was introduced to Japan either via the Korean Peninsula or by a Chinese

monk who brought plum trees as a gift to the Emperor (Matsumado 1977). In the latter half of nineteenth century, Japanese plum was introduced into the USA and Australia, and recently to other areas with suitable climatic conditions. For example, Okie and Ramming (1999) reported that improved cultivars of this species, Kelsey and Abundance, were introduced into the USA from Japan over 100 years ago. In addition, stones of Japanese plum have been found dating back to about 200 BC, whereas its cultivation was mentioned around 500 AD (Yoshida 1987), indicating that the plum as a fruit species in Japan and Korea is very old, but it is not possible to tell if they were ever part of the native range for plums (Okie and Ramming 1999).

5.1.2 Plum Importance

Plums are grown under different climatic, soil and orographic conditions, i.e. from the cold Siberian zone to the warm subtropical regions of the Mediterranean. In the last 30 years, world plum production and harvested area have risen dramatically. In 1987, world plum production was 6,065,184 mt from 1,288,280 ha of harvested area, whereas in 2014 statistics were 11,282.527 mt from 2,521,100 ha of harvested area. Both plum production and harvested area for this period have doubled. Among the continents, the largest producer is Asia (7,609,652 mt), followed by Europe (2,473,880), South America (508,380), Africa (365,489), North America (235,498) and the geographic region of Oceania (17,703 mt) (FAOSTAT 2017).

The ten leading countries in plum production, in descending order, are China, Romania, Serbia, Iran, Chile, Turkey, Spain, USA, India and Italy. Production of plums in 2014 in China was 6,256,906 mt (99% Japanese plums), whereas in the other nine countries total production was 2,692,057 mt (FAOSTAT 2017), meaning that China produced 2.3 times more plums than the next nine producers combined. Based on previous data, it can be said that the plum has a great economic, social, partially political importance for the people living in these countries. In some regions of the world, especially in certain countries such as Serbia, Bosnia and Herzegovina, plum is a *cult* plant, because *the economic power of the household is determined by the number of plum trees in the orchard and the amount of alcoholic beverages, commonly named rakija or šljivovica, in the cellar* (Milošević 2002; Milosevic et al. 2010).

Prunus species such as *P. salicina, P. domestica*, *P. cerasifera* and *P. institia* are widely grown. The European (domestic) plum (*P. domestica*) and the Japanese plum (*P. salicina*) are most important in terms of commercial production (Ozbek 1978). Japanese plum (*P. salicina*) and its hybrids with other diploid plum species provide over 70% of the world's global plum production. Only in Europe, whose share in world production is about 25%, domestic (European) plum dominates. Other plum species and their cultivars are cultivated on a much smaller scale.

Plum fruits are used for fresh consumption (table fruit) as well as for drying. The main processed foods made from plums include compotes, mousse, pulp, candied fruit, frozen fruit, jelly products, jams, *pekmez* (obtained by cooking without sugar),

marmalade, various culinary specialties such as pies and cakes, *slatko* (a traditional Serbian product intended for guests, prepared by cooking of skinless fruit halves with a sugar supplement) and plum alcoholic beverages. Nearly 60 human food products can be obtained from plum fruits (Milošević 2002). From the standpoint of possible processing into various products, plum fruits are considered to be the most valued in comparison with other fruit species.

Plums are considered one of the most delicious temperate fruits and are highly appreciated by consumers for their taste and aroma. Plum fruit is a rich source of minerals and vitamins, and contributes greatly to human nutrition because of its richness in fiber and antioxidants (Kim et al. 2003). Plums have a high content of organic acids, such as neochlorogenic and chlorogenic acid, which contribute to their antioxidant properties (Heo et al. 2007). Consuming plums is positively associated with nutrient intake, improved anthropometric measurements and reduced risk of hypertension (Beals et al. 2005). Plums have saccharides which positively affect human health and as a source of energy for metabolism (Stacewicz-Sapuntzakis et al. 2001). The saccharides present in plums include free mono- and disaccharides (glucose, fructose and sucrose) particularly, and sugar alcohols, sorbitol for the most part, and also important amounts of soluble solids (Milošević 2013; Milošević and Milošević 2012a; Rato et al. 2008). These and other organic and inorganic substances determine the nutritive value, flavor and taste of plums (Crisosto et al. 2007; Ertekin et al. 2006). In addition to the economical and fruit characteristics (yield, fruit weight, shape and skin color, weight and separability of stone, skin firmness and resistance to diseases), the chemical parameters of the fruit (dry matter, saccharides and polyols) also play an important role in the commercial use of plums (Bohačenko et al. 2010).

5.1.3 Domestication, Selection and Early Improvements

Plums were domesticated on the continents of Asia, Europe and North America (Okie and Weinberger 1996). In prehistoric times, people used the plums for food. On the basis of archaeological evidence, excavation sites of lake dwellers in Switzerland contained stones of *Prunus spinosa* and *P. insititia*. Fruit plums were used by ancient peoples in the areas of the Caucasus Mountains and Caspian Sea. From these areas, the plum was spread to ancient Syria, Mesopotamia, Egypt and Crete, about 6000 BC (Faust and Surányi 1999).

Much later, thanks to wars and conquests, migration and trade movements, the plum became known to the Greeks and Romans. The first written record of plums comes from the Greek lyric poet Archilochus (680–645 BC) and iambic poet Hipponax (541–487 BC), whereas the ancient Greek philosopher Theophrastus (370–287 BC) described 3 plum cultivars. The Romans brought domestic plum from Damascus (Syria) after they conquered in 65 BC. Pliny the Elder (AD 23–79), a Roman naturalist and natural philosopher, in his work *Naturalis Historia*, speaks of the origin of plums, and described 14 cultivars. Moreover, Palladii (fourth century AD), in the work *De Re Rustica*, described a large number of plum cultivars. From Rome, plums spread

throughout the European continent (Neumüller 2011). The Roman emperors Diocletian (AD 244–312) and Probus (AD 232–282) dispersed plums to the western part of Balkan Peninsula, especially to some areas of the former Yugoslavia (Milošević 2002; Mišić 2006). At that time, a number of plum orchards were established on the banks of the Drava and Sava rivers between the second and third centuries (Faust and Surányi 1999; Ramming and Cociu 1991). Since then, Bosnia has been the leading plum producer in Europe, especially for the cv. De Bosnia (syn.: cv. Požegača), which is today the most important plum cultivar in Europe (Hartmann and Neumüller 2009). According to the same authors, cv. Požegača was introduced into Germany in the seventeenth century and called *Zwetsche* or *German Prune*.

The ancient Slav tribes in the area between the Carpathians and the Baltic Sea consumed plums in their diet. Later, the southern Slavs found plums when settling the Balkan Peninsula (between third and seventh centuries) and continued with its humble growing (Milošević 2002). In Serbia, plums were somewhat more cultivated on small farms in the thirteenth and fourteenth centuries. In the seventeenth century in Serbia and Bosnia, plum production reached a more significant level. The plum, probably cv. Požegača, was dried and traded. The first large quantities of dry plums were exported in 1856 from Brčko (Bosnia) to Vienna and Trieste. Furthermore, in 1899, 40,530 mt of dry plums were exported from Serbia. The beginning of the twentieth century is considered to be the *golden age* of plum production in these countries. Namely, in 1904 and 1908, about 108,000 and 85,000 mt, respectively, of dry plum of Požegača and Bilska Rana cvs. were exported from Serbia and Bosnia to Europe and North America (Milošević 2002).

Plums are mentioned in France (742–814), Sweden (tenth century), Moravia (thirteenth century), Norway (fourteenth century), Finland (fifteenth–sixteenth centuries), Czech Republic and Slovakia (1579), and Russia (1654) (Mišić 2006).

European settlers carried European and Damson plum to North America, although the native peoples consumed the indigenous plum fruits much before the colonists arrived (Milošević 2002).

Janick (2005) stated that the garden plum and the Japanese plum emerged as important fruit crops about 300 BC. Today, plum fruit growing and associated food industries are based on European and Japanese plums, whereas other plum species and their cultivars are represented in minor amounts.

The main objective of this chapter is to review available information about the origin of plums, domestications, breeding tools, newly-released cultivars and intraspecific hybrids and new methodologies which can be used in improvement program with special emphasis on European and Japanese plums, and new interspecific hybrids within the *Prunus* genus.

5.2 Cultivation and Traditional Breeding

5.2.1 Current Cultivation Practices

Current intensive conventional planting of plums is characterized by several components: small-scale cultivation with more trees per unit area, early precocity and regular productivity, decrease in labor costs per unit of fruit production, especially pruning and harvest, decrease in usage of pesticides for plant protection and weed control, excellent external and internal fruit quality, and the yield of young plantations must be high and economically justified (directly proportional to number of trees per unit area) (Milosevic et al. 2008), and finally must be sustainable (Jemrić et al. 2017).

A key factor in producing a successful fruit crop is the choice of both good cultivars and rootstocks, i.e. a harmonious combination of rootstock-cultivar. Both cultivar and rootstock vary country-by-country. However, the choice of the appropriate rootstock is much more difficult as compared to the choice of the cultivar, because there is no universal plum rootstock suited to different ecological conditions, growing systems and intensity of growing technology (Milosevic et al. 2008). Different cultivars produce fruits at different times of the season. The fruit of early ripening European plums tends not to keep well whereas the fruits of later ripening cultivars are suitable for multiple use including fresh, processed, drying, canning, cooking and deep freezing.

In modern commercial plum orchards, cultural practices have been developed (training, pruning, irrigation, soil maintenance, fertilization, pest and disease protection and weed control). These are to assure regular and optimal yield, good quality and health-safe fruits and environmental protection (Jemrić et al. 2017). The concept of integrated fruit production (IFP), developed in Europe, is recommended for plum growers (Damos et al. 2015; Djouvinov and Vitanova 2002; Jörg 1993). For example, several cultivars of European plum such as Hanita, Katinka, Elena, Jojo, Top 2000, Topfirst, Toptaste, Tophit and Čačanska Najbolja are suitable for IFP (Mezey 2009).

Also, in recent decades, there has been increased interest of growers and consumers in organic plum production (Jemrić et al. 2017), although pest management for organic production is very complex and difficult (Jaastad et al. 2007). According to our experience, for this purpose, some local (autochthonous, folk, primitive) cultivars of European and/or Damson plums have better potential compared to commercial cultivars due to their higher resistance to pest and disease attacks (Milošević and Milošević 2012b; Milosevic et al. 2010). Generally, commercial plum growers want information about refining production and marketing practices to produce healthy foods and enhance profitability. It seems that an integrated cropping system that improves efficiency, uses fewer inputs and protects natural resources is a good approach to efficient plum growing.

5.2.2 Current Agricultural Problems and Challenges

In the coming decades, the world's human population is expected to double and increase future food demand (Jemrić et al. 2017). Horticultural has an important role to play in meeting future food demands.

As for the future breeding of European plums, Neumüller (2011) has defined several key challenges. In the short-term these are: (a) the hexaploid genome hampers the study of the inheritance of single features; it will be necessary to reduce ploidy degree by interspecific hybridization with related diploid species in order to transfer the feature of interest into a diploid hybrid plant, (b) breeding for resistance to negative abiotic and biotic factors, especially major diseases, such as *Plum pox virus* (PPV) or Sharka disease, *Monilinia* spp. and *Xylella fastidiosa* Wells et al. (authors' remark) are obligatory as fundamental efforts, (c) endocarp splitting as well as caverns in the fruit flash cause increasing economic losses, (d) crossing within the group of European plum may result in a larger diversity of plum fruits in order to improve fruit size and firmness; for example, crossing between cv. Mirabelle and large-sized prunes or cv. Reine Claudes, (e) economically-important features present in European plum obtained through interspecific hybridization could be transferred to other stone fruits—Japanese plum, peach, apricot and its rootstocks, also obtained by interspecific hybridization.

5.2.3 Improvement Strategies

Fruit production and its food industries face important challenges to remain competitive; among them are: (a) climate change (irrigation, diseases, water and temperature stress, greater losses and increased costs), (b) labor (cost, availability, efficiency), which should promote mechanization and c) consumer expectations (evolving towards quality, food safety and traceability) (Retamales 2011). Regarding fruit crops, some data are very interesting. Nearly 50% of the total global volume of fruit is produced by 5 countries: China, USA, Brazil, Italy and Spain (FAOSTAT 2017). Although world population has increased about 70% in the last 30 years, global fruit consumption per capita is only 20% higher (Retamales 2011). Among temperate fruit crops, there was a marked expansion in the area harvested, with a large increase for plums (6.4 times) in the period 1961–2014 (FAOSTAT 2017).

5.2.4 Traditional Breeding Methodologies and Limitations

Over millennia, people cultivated various fruit species, including plums and other *Prunus* spp. Although these fruit species have been cultivated for some 2000–4000 years, it was only during the last 100 years that formal breeding pro-

grams began to develop new cultivars (Okie and Hancock 2008). New plum cultivars originated for a long time from chance seedlings. Scientific plum breeding was started by Thomas Rivers (1798–1877), Luther Burbank (1849–1926) and Ivan Mitschurin (1855–1935).

Plums and other fruit selection and improvement have been relatively slow over the centuries. Available literature contains various data on the number of known plum cultivars in the nineteenth century. Mišić (2006) states that there were about 400 plum varieties in the nineteenth century, while much earlier Hedrik (1911) pointed out that during that period more than 1,000 cultivars of European plum were known. There is disagreement regarding the number of plum cultivars today; Mišić (2006) stated that about 2,500 plums are known whereas Blazek (2007) reported that there are over 6,000 cultivars. However, the number of commercially-grown plums is quite limited. Basso and Faccioli (1978), Surányi and Erdös (1998) and Hartmann (2003) provide detailed descriptions of commercially-important plum cultivars.

Currently, new-cultivar development is the result of a cyclical process, each cycle consisting of several overlapping phases (Poehlman and Sleper 1995). The phases include: (a) assembling and generating new diversity, (b) selection and testing to identify superior recombinants and (c) release, distribution and commercialization of new cultivars.

Among the stone fruits, plum breeding is one of the most dynamic; new cultivars released each year have originated from *P. domestica* and partially from other plum species (*P. insititia*) (Blažek et al. 2004). Plum-breeding programs are focusing on improving fruit external and internal quality, prolonging the harvest season, good storage performances and developing resistance and/or tolerance to biotic and abiotic stress, especially to PPV (Blazek 2007; Glišić et al. 2017; Jacob 1998, 2002, 2007; Milošević and Milošević 2011a, b, 2012c; Milošević et al. 2016a, b; Neumüller et al. 2010, 2012; Zhivondov et al. 2012). Current European plum-breeding programs, however, seem to favor fresh fruit suitable for the dessert markets (Neumüller 2011).

Modern breeding programs for Japanese plum are currently focused on satisfying both consumer and grower preferences (Carrasco et al. 2013). Consumer preferences relate mainly to fruit quality, and breeding programs are directed towards creating fruits that have acceptable taste and desirable texture. On the other hand, growers require highly-productive cultivars, resistance to pest and disease, different harvest dates to prolong the period of fruit production and high storability (Byrne 2005). Generally, the procedure of creating, then gaining recognition of a new the cultivar and its potential introduction into the market, is very long, difficult and uncertain. Practically every producing country has had at one time a plum-breeding program. Basic research in new-cultivar development is typically funded by governmental research programs. Recently, private breeding programs have become very important. Contributions to fruit crops improvement are coming from hobbyists, selectors, nurserymen and growers as well as professional breeders working in the private sectors. For example, earlier in this century, about 68% of plant breeding jobs in the USA were in the private sector (Bliss 2006).

Basically, there are several methods of plant breeding: (1) conventional methods (selection, hybridization, polyploidy, induced mutation), (2) biotechnological methods (in vitro cultivation of plant cells and regeneration of plants from cultured cells, in vitro selection and somaclonal variation, somatic hybrid plants) and (3) genetic engineering (restriction fragment length polymorphism (RFLP), gene transfer, transgene expression, selection and plant regeneration). Conventional breeding methods for plums are still largely used in the majority of programs.

The conventional method is dominant, and within this method, the most important is hybridization (intra- and interspecific) and open-pollination (Carrasco et al. 2013). Intraspecific hybridization is the way in which the largest number of European plum cultivars has been created, especially in European countries (Blazek 2007; Minev and Balev 2002).

Interspecific hybridization within *Prunus* is common for the improvement of rootstock cultivars. In the 1950s, at the East Malling Research Station, Kent UK, hybrid No. 21 was created by crossing *P. avium* and *P. pseudocerasus* which was later disseminated as cherry rootstock cv. Colt (Webster 1989). The plum rootstock cv. Ishtara is a complex interspecific hybrid established from *P. domestica*, *P. cerasifera* and *P. armeniaca* (Sansavini et al. 1989). Jaspi cv. plum rootstock was obtained from the parental combination *P. salicina* Metley × *P. spinosa*.

A breeding program to create PPV-resistant rootstocks was established at the Technische Universität München. Genetic crosses between European plum (*P. domestica*) genotypes with hypersensitivity resistance to PPV were used as one parent and either *P. spinosa* or *P. cerasifera* as the other parent, resulting in either cv. Dospina (*P. domestica* × *P. spinosa*) or cv. Docera (*P. domestica* × *P. cerasifera*) rootstock series, respectively (Neumüller et al. 2013). Earlier, as a result of interspecific hybridization Marianna cv. rootstock was established from the parental combination of *P. cerasifera* × *P. munsoniana.*

Very interesting are the new fruit forms obtained by crossing different taxa of *Prunus*, especially the European plum (*P. domestica*) and common apricot (*P. armeniaca*), and vice versa (Fig. 5.1). The plum-apricot F_1 hybrid called a Plumcot® is part plum and part apricot. It was named by Luther Burbank, combining *plum* and *apricot*. Later, Burbank crossed the Japanese plum (*P. salicina*) and common apricot. Today, the name *plumcot*® designates all of the plum-apricot hybrids (Zhivondov 2012). In obtaining hybrids of F_1 generation, in each of them the plum and apricot parent has 50% participation. Generally, most of the existing Plumcots® are hybrids of *P. salicina* or *P. cerasifera* with apricots (*P. armeniaca* or *P. mume*) (Okie 1995).

The second crossing of Plumcots® with either plum or apricot, results in the second hybrid generation (F_2), and these hybrids have different commercial names. If a Plumcot® is crossed once more with the plum parent, the latter has a 75% share in the new hybrid and the apricot parent 25%. The name of these hybrids is *Pluot*®, without regard for the direction of crossing [(*Prunus domestica* × *P. aremeniaca*) × *P. domestica* or (*P. salicina* × *P. armeniaca*) × *P. salicina*]. At the end of twentieth century, the first pluots were established by breeder Floyd Zaiger. Currently, Pluot® is a registered trademark of Zaiger Genetics Inc., Modesto, California.

Plumcot® cv. Spring Satin
(Source:
https://www.starkbros.com/products/fruit-trees/plum-trees/spring-satin-plumcot)

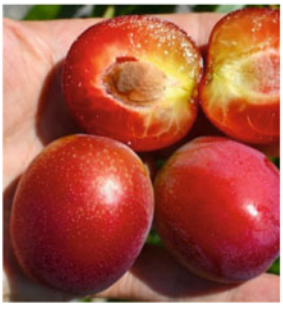
Pluot® cv. Dinosaur Egg
(Source:
http://www.davewilson.com-product-information)

Aprium® cv. Aprisali®
(Source:
http://www.artevos.de/en/list-of-varieties/sorte/aprisali.html)

Pluerry™ cv. Sweet Treat™
(Source:
http://www.davewilson.com/product-information/product)

Peacotum cv. Bella Gold
(Source:
https://cuesa.org/article/getting-bottom-peacotum)

Nectaplum cv. Spice Zee®
(Source:
https://www.groworganic.com/nectarine-nectaplum-a-spice-zee.html)



Cherrycot cv. Aprikyra®
(Source:
http://www.shop.zahradnictvolimbach.sk/de/APRIKYRA

Honeysun
(apricot × peach hybrid)
(Source:
https://www.haeberli-beeren.ch/de/produkt/pfirsich)

Susincocco
cv. Biricoccolo Vesuviano
(Source:
http://www.biogiardino.it/2017/01/ecco-il-biricoccolo-nato)

Fig. 5.1 Interspecific genotypes of *Prunus*

More than 20 registered plum cultivars are grown in the USA for fresh consumption and/or processing into jellies, sauces, syrup, juices, and also dried fruit (Crisosto et al. 2007). Aprium® is a relatively recent hybrid fruit tree developed in the late 1980s by Floyd Zaiger, different from Plumcots® and Pluot® (Frecon and Ward 2012).

Aprium® was obtained by a second crossing (F_2) of Plumcot® with apricot. Genetic participation share of the apricot and plum parent is 75 and 25%, respectively [(*Prunus aremeniaca* × *P. domestica*) × *P. aremeniaca* or *P. armeniaca* × (*P. salicina* × *P. armeniaca*)]. The first cultivar of this product is Honey Rich. Later, other cultivars such as Cot-N-Chandy, Flavor Delight and Flavor Candy were created. Fruits are consumed fresh or dried and processed into various products.

Biricoccolo cv. derives from a natural crossing between *Prunus cerasifera* and *P. armeniaca*; it is also called cv. Susincocco. From a botanical point of view it can be classified as *Prunus* × *dasycarpa* Ehrh. (purple apricot and/or black apricot). It is an ancient fruit, known since 1700, but not widespread. Initially it was cultivated in Emilia, Lombardy, Veneto and then later in Campania, in the Vesuviana area in Italy (Ruggiero 2015). Albicocco del Papa is the most common name in the sale of nursery trees.

Nectaplum® is a tradename for cultivars that are a hybrid of nectarines and plums developed by Floyd Zaiger. Both nectarine and plum traits are easily detectable. An application to trademark the name *Nectacotum* in the USA for cultivars derived from nectarine-type peaches was made in 2004 but later abandoned.

Peacotum® is a peach-apricot-plum hybrid developed by Zaiger Genetics. This product is a trademark of Dave Wilson Nursery Inc. A triple-header of a hybrid fruit–touted as the first *three-in-one ever* has made cameo appearances at a few markets in the USA. Bella Gold is the interesting cultivar of this hybrid.

Pluerry is a complex *Prunus* hybrid, primarily of Japanese plum and sweet cherry (*P. salicina* × *P. avium*) with dominant parentage of plum and having fruit resembling a plum. It was developed by Zaiger Genetics. Cherrycot is a hybrid between *P. pumila* var. *besseyi* and *P. armeniaca* with the Aprikyra® cv. which is a trademark of Artevos GmbH (Germany). Honey Sun® is another Zaiger Genetics hybrid obtained recently by crossing of apricot and peach.

Much more information about new fruit forms, i.e. hybrids between different *Prunus* taxa is provided by Zhivondov (2012).

There is no question that classical breeding techniques are currently the only ones used to produce new prune cultivars because most of them were developed either by randomly identifying selected seedlings or by using classic breeding techniques in traditional breeding programs (Lavi et al. 2006). However, conventional methods have a great number of limitations (Botu and Botu 2007). The major limitations include: a large volume of work, engaging a large number of workers, especially for cross-breeding, extended spaces (land area), long selection period and high costs.

As an imperative, there is a need for the faster introduction of new breeding methods and techniques which will contribute to eliminating the greatest number of restrictions in the creation of new plum cultivars (Lavi et al. 2006; van Nocker and Gardiner 2014) and as a support of conventional breeding methods. Current methods

and techniques can be complementary to any of the conventional breeding (Botu and Botu 2007).

5.2.5 *Plum Breeding Activities*

Over centuries, the first plum cultivars derived from chance seedlings. The first breeders who used scientific methods in this job were Thomas Rivers, Ivan V. Mitschurin and Luther Burbank. Burbank's important introductions of European plum were the cvs. Improved French prune (1898), Sugar prune (1899) and Standard prune (1911) (Karp 2015). Today, the main breeding objectives in Europe (80% of all activities by *P. domestica* and 20% by *P. salicina*) are fruit quality, ripening time, resistance to pest and disease, especially to PPV, storage ability and climatic robustness (Bellini et al. 2010; Butac et al. 2010, 2013).

Specific objectives of plum breeding varied among countries. They includes late blooming, self-fertility, short growing period, different ripening time, spur fructification, regular and good productivity and frost resistance.

In Europe, there are over 20 breeding program in nearly 20 countries (Table 5.1).

In the countries where production has declined, or funding is no longer available, plum-breeding programs have been reduced or stopped, whereas some programs have become more private with less public funding (Hartmann 2010; Ikase 2015). On the basis of conventional breeding methods such as controlled hybridization, open pollination, selection from wild population of *Prunus* spp., mutagenesis using X-ray and improvement of the old local cultivars, 170 new plum cultivars have been registered (Butac et al. 2013).

Burmistrov (1992) reported that there were 10 breeding programs in the former USSR. In recent years, work on the creation of new cultivars in Russia intensified.

Table 5.1 excludes new plum cultivars developed in some Baltic and Nordic countries and Ukraine; plum-breeding programs in these countries are discussed in the next sections. Recently, several plant-breeding programs are incorporating, into their conventional plant breeding and plant physiology strategies, innovative biotechnological methods such as functional genomics, mutations and molecular markers in order to increase their efficiency in the development of new cultivars (Carrasco et al. 2013).

5.2.5.1 European (Domestic) Plum

Czar is the first plum cultivar from *P. domestica* obtained by controlled hybridization, developed by Thomas Rivers in England using cvs. Prince Engelbert and Rivers Early Pacific, as parental components in 1843; it was introduced in 1873. Later, in 1894, cv. President, previously found as a chance seedling, was also obtained by Rivers. Although this is a cultivar, today it has commercial value in parts of some countries such as the UK, France, Serbia and Ukraine. Also, cv. President is used as

Table 5.1 Countries, plum breeding centers, breeders and objectives in Europe

Country	Breeding center	Breeder(s)	Objective of breeding
Serbia	Fruit Research Institute, Čačak	N. Milošević I.S. Glišić	Fruit size and quality, yield, early and late ripening time, resistance to *Plum pox virus* (PPV)
	Faculty of Agronomy, Čačak	T. Milošević	Fruit external and internal quality, productivity, resistance to PPV
Romania	Research Institute for Fruit Growing, Pitesti Fruit Growing Research Station, Valcea Research Station for Fruit Growing, Bistrita Research Station for Fruit Growing, Voinesti Research Station for Fruit Growing, Targu Jiu	M. Butac M. Botu I. Zagrai	Improvement of old cvs. Tuleu Gras, Grase Romanesti, Vinete Romanesti, fruit quality, PPV resistance, ripening season extension, self-fertility
Germany	University Hohenheim, Stuttgart Hochschule Geisenheim University, Geisenheim	W. Hartmann M. Neumüller H. Jacob	Fruit quality, PPV resistance by hypersensitivity
France	INRA, Bordeaux	R. Bernhard R. Renaud	Fruit quality, PPV resistance by transgenic plants
Italy	University of Bologna, Florence, Forli and Rome Private programs	S. Sansavini E. Bellini V. Nancetti A. Liverani	Japanese and European plum breeding: quality, fresh consumption, resistance to PPV
England	East Malling Research Station, Kent	K Tobutt R. Jones T. Laxton	Late blooming, frost resistance, PPV resistance
Bulgaria	Fruit Growing Institutes in Dryanovo, Troyan, Kyustendil and Plovdiv	V. Bozhkova A. Zhivondov	Late blooming, extended ripening period, fruit quality, resistance to PPV

(continued)

Table 5.1 (continued)

Country	Breeding center	Breeder(s)	Objective of breeding
Latvia	Horticultural Plant Breeding Experimental Station, Dobele	L. Ikase E. Kaufmane I. Grâvîte	Winter hardiness, vigor, precocity, fruit quality, self-fertility, early ripening
Belarus	Institute for Fruit Growing, Minsk	V.A. Matveev M. Vasiljeva Z. Kozlouskaya	Fruit quality, frost resistance
Moldova	Research Institute for Horticulture and Alimentary Technologies, Chisynau	M. Pintea A. Juraveli	Resistance to frost, fruit quality
Czech Republic	Research and Breeding Institute of Pomology, Holovousy	J. Blazek	Fruit quality, PPV tolerance
Sweden	University of Agricultural Sciences, Balsgård	I. Hjalmarsson V. Trajkovski	Short period of vegetation, fruit quality
Norway	Ullensvang Research Centre Division Njos, Lofthus	S.H. Hjeltnes	Fruit quality, storage
Hungary	Research and Extension Centre for Fruit Growing, Ujfeherto	T. Lakatos	Fruit quality, processing
Poland	Research Institute of Horticulture, Skierniewice	T. Jakubowski E. Żurawicz J. Dominikowski E. Rozpara Z. Grzyb J. Szymański	Fruit quality, productivity, resistance to PPV
Russia	North-Caucasus Zonal Horticulture and Viticulture Research Institute, Krasnodar Krymsk Experiment Breeding Station, Krymsk Far East Experiment Station, Pavlovsk, St. Petersburg Maicop Experiment Station, Maicop, Krasnodar Region Volgograd Experiment Station, Krasnoslobodsk	R. S. Zaremuk E. M. Alekhina G. Eremin	Cold hardiness, fruit quality, productivity

Source Milošević and Milošević (2011a, b), Butac et al. (2013), Milošević et al. (2016b)

a parent component in some breeding efforts in Eastern Europe. By the early 1900s, plum breeding was being carried out at research stations at Long Ashton (later East Malling) and John Innes (Roach 1985).

Important steps related to plum breeding in government institutions were made at the beginning of the twentieth century, first in England—East Malling Research Station, Kent (1913) and Belarus—Institute for Fruit Growing, Minsk (1925) (Butac et al. 2013). After World War II, plum breeding in different countries showed an upward trend, beginning in Moldova—Research Institute for Horticulture and Alimentary Technologies (Butac et al. 2013; Chisynau 1946); in Serbia (1947–1949) (Milošević et al. 2016a; Ogašanovic et al. 1994, 2006; Paunovic 1988; Paunovic et al. 1968); Romania (Cociu et al. 1978; Butac et al. 2013) and Bulgaria (1950) (Zhivondov et al. 2012).

A major program of plum breeding in Serbia started in the Fruit Research Institute in Čačak led by S. Paunović, later by D. Ogašanović and today by N. Milošević and I. S. Glišić. A total of 14 plum cultivars have been named and released by the Institute. Cultivars Čačanska Lepotica and Čačanska Najbolja are used as parent components in different breeding programs in some European countries, especially Germany, Czech Republic, Bulgaria and Serbia (Milošević et al. 2016a).

Cultivar Čačanska Rodna is also very important. It can be categorized as a prune. It has excellent fruit quality and produces heavy crops of fruits, but is very sensitive to PPV. In the last decade of the twentieth century, breeding activities at Čačak decreased due to war events in the former Yugoslavia, economic sanctions, NATO bombing of Serbia and retirement of breeders, but have recently been reactivated.

Several promising new cultivars with high commercial potential were named and released after 2000. These are cvs. Mildora, Timočanka, Boranka, Krina, Pozna Plava, Zlatka and Nada, developed by Ogašanović and collaborators (Milošević et al. 2016b) (Fig. 5.2).

From 1991 to 2007, at the Faculty of Agronomy in Čačak, new plums were created, led by T. Milošević (PPV resistance, larger fruit, external and internal fruit quality, high and regular yield, long shelf life). Among 3013 hybrids obtained by crossing cvs. Čačanska Lepotica, Čačanska Najbolja and Stanley as a parent components, 7 promising hybrids (P_1–P_7) were created with good pomological properties and commercial potential (Milošević and Milošević 2011a, b, 2012c). However, due to technical and financial reasons, the program ceased.

In Romania, plum-breeding programs were started by N. Constantinescu, continued by V. Cociu, and are led today by I. and M. Botu, E. Turcu, I. Zagrai, I. Ivan, N. Minoiu and M. Butac (Butac et al. 2013). Plum programs exist at Pitest, Bistrita, Valcea, Targu Jiu and Voinesti; Pitest and Bistrita are the most active (Butac et al. 2010). The total number of new cultivars named and released in this country is a remarkable 40 (M. Butac pers comm). Between 2000 and 2012, 17 new cultivars were released in this country. Among others, very interesting are Alutus, Topval and Romanţa, named and released in 2010, 2011 and 2012, respectively.

The Bulgarian plum-breeding program is strongly influenced by Atanasoff (1935), who emphasized the importance of breeding against *Plum pox virus* (PPV). Plum breeding activities are underway in Dryanovo, Troyan, Kystendil and Plovdiv. As a

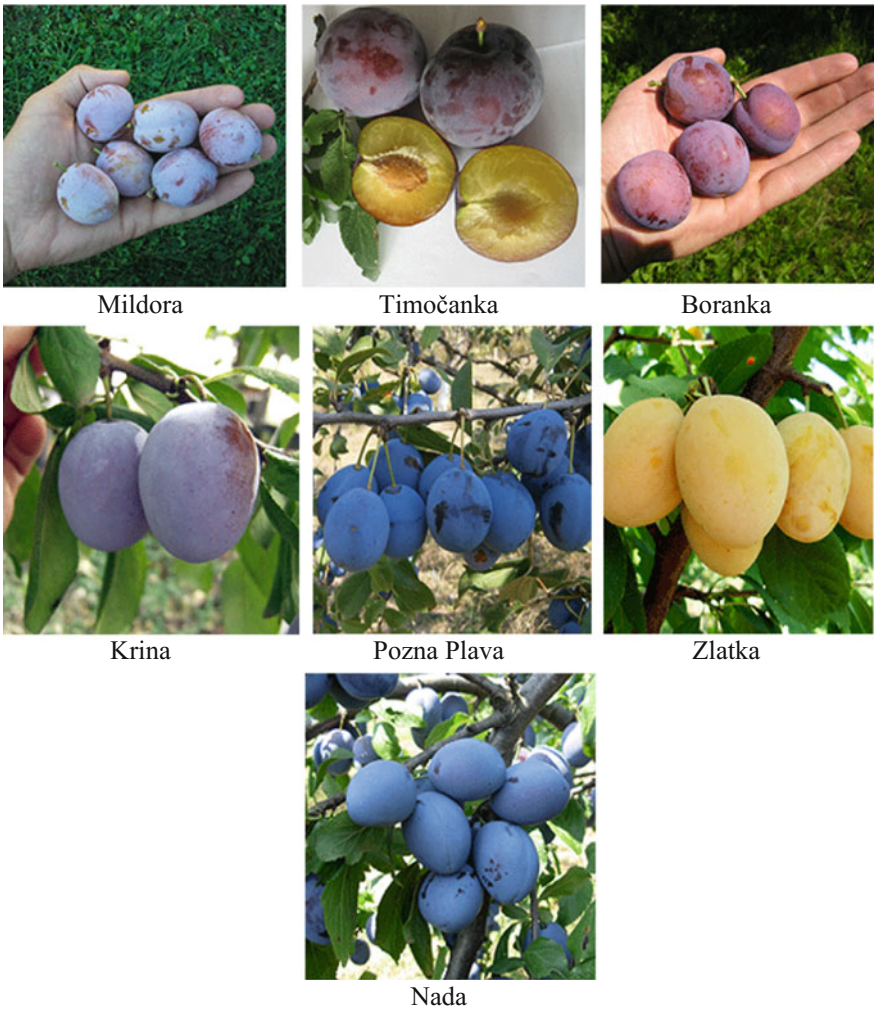


Fig. 5.2 Seven new plum cultivars named and released 2004–2012 by the Fruit Research Institute Čačak, Serbia (*Photo* N. Milošević and I.P. Glišić)

result of these programs, 11 new plum cultivars have been recognized to date (Butac et al. 2013); however, Zhivondov et al. (2012) stated that 29 new plum cultivars were created in Bulgaria during the 60-year period of the breeding programs. Recently, Bozhkova (2013) described 20 new hybrids tolerant to PPV, created in Plovdiv. They derive from open-pollination of cvs. Stanley, Ruth Gerstetter and Althan's Gage in most cases or by crossing cvs. Althan's Gage × Grand Duke and Althan's Gage × Syneva. Bulgarian breeding activities have had their ups and downs. In the late 1980s and early 1990s, in order to obtain new fruit forms and to transfer

PPV resistance, *Prunus domestica* and *P. armeniaca* were crossed; however, the program subsequently declined (Zhivondov 2012). As previously reported, more interspecific hybrids were obtained and some are fertile and produced fruits with specific traits. Among all released cultivars belonging to *P. domestica*, the most important are Plovdivska renkloda, Syneva and Ulpiya (Zhivondov et al. 2012).

The first stage of plum breeding in Poland began at the Research Institute of Pomology and Floriculture at Skierniewice in the early 1960s, and a second stage was initiated in 1991 (Jakubowsky 1998). As a result, 7 new cultivars were released and introduced to commercial production (Z. Grzyb and M. Sitarek pers comm). Kalipso, Emper and Polinka cvs. were created by hybridization, and were recognized as new cultivars in 2009 and 2012. Dąbrowice Prune cv. is a seedling of the common plum from open-pollination, while Nectavit, Tolar and Promis are clones of the common plum, released in 1991 and 1998.

Institutional plum breeding in the former Czechoslovakia started in 1960 at the Institute for Fruit and Ornamental Plants in Bojnice, Slovakia (Okie and Ramming 1999). Several promising prune hybrids were created and tested (BO II/65, BO III/77 and BO IV/39). However, published information of what became of these selections is not available.

Since 1988 in the Czech Republic, breeding for PPV resistance has been done at the Research and Breeding Institute of Pomology at Holovousy (Drobny1990). As a result, 13 new European plum cultivars were named and released: Amátka, Dwarf, Kamir, Hololepa, Simona, Samera, Lipniská, Malenovická, Paní Háje, Stáňa, Těchobuzická, Valentýnka and Vítek. The first 6 cultivars were created by J. Blažek at the above Institute using cvs. Čačanska Lepotica and Čačanska Najbolja as pollen parents or from open-pollination of cv. Čačanska Lepotica from the Fruit Research Institute, Čačak (Blažek and Šecová 2013; J. Blažek and B. Krška pers comm).

The plum-breeding program in France (INRA, Bordeaux) is linked to researchers R. Bernhard and R. Renaud, to obtain a series of cultivars suitable for drying (prunes) and dessert plums (plums) adapted to French environmental conditions (Renaud and Roy 1990). The total number of released new cultivars is 6 (Butac et al. 2013). Cultivars Primacotes, Lorida, Spurdente and Tardicotes are the most famous prunes, whereas Fermareine and Ferbleue® are the first of a series of dessert plums that will be released (Renaud 1994; Renaud and Lafargue 1998). However, plum-breeding programs have stopped.

The main goal of plum breeding at the Horticultural Research International in East Malling, Kent, UK in the twentieth century included late bloom to improve productivity, large fruit size (>40 mm) and dessert quality (Jones and Wilson 1987). Three new plum cultivars were released (Butac et al. 2013). Avalon and Excalibur cvs., released in 1988, have large, high-quality fruit suitable for the dessert market. However, this program was suspended when R. P. Jones retired, but selection testing is continuing (Okie and Ramming 1999).

In Moldova, the Research Institute for Horticulture and Alimentary Technologies, Chisynau, in the last 20 years, released 10 new plum cultivars. Among them, the most interesting are cvs. Naiboleco and Udlinennaia şi Cabardinca.

In Switzerland, a new breeding program began in 1984 under M. Kellerhals at the Swiss Federal Research Station in Wadenswil. The main objectives were creation of early- and late-ripening plums for fresh use, high quality, long shelf life and resistance to pest and disease, especially to *Monilinia* spp. (Kellerhals and Rusterholz 1994). However, plum breeding programs in both Moldova and Switzerland were suspended (Hartmann and Neumüller 2009).

In Italy, a plum-breeding program was started in 1970 by E. Belini at Florence to develop new early-ripening dessert plums with large, high quality fruit, vigorous productive trees and resistance to biotic and abiotic stresses (Bellini et al. 1990). Their first release was cv. Firence 90. Smaller breeding programs exist at Bologna, Forli and Rome. Private programs are still active. As a result of realization of determined goals, 20 cultivars were released in Italy: 14 originating from *P. domestica* and 6 from *P. salicina* (Butac et al. 2013). The more attractive European new plum cultivars are Liablu, Sugar Top, Prugna 29 and Maria Novella.

In Norway, plum breeding has continued intermittently since 1934; modern breeding began at the Agricultural University in Ås in 1979 and moved in 1990 to the Research Station at Njøs in order to develop cultivars with high quality, large attractive fruit with good shelf life, and high annual cropping (Okie and Ramming 1999). Only one plum cultivar, named Edda, was created in 1983 (M. Meland pers comm), whereas Butac et al. (2013) stated that two new Norwegian plum cultivars were released. The breeding program in Norway was interrupted in 1997 because PPV destroyed most of the material, but was renewed in 2001; it has not yet released registered cultivars.

Sweden has been very active in the creation of new cultivars of European plums. The main goals of the breeding programs, created by I. Hjalmarsson and V. Trajkovski at Balsgård, are: compact tree habit, high cold hardiness, short vegetative cycle and larger fruits with good quality (Okie and Ramming 1999). Sweden has released 12 new plum cultivars including Herman, Meritare, Jubileum, Anita, and dwarf plums Violetta and Madame. Swedish cvs. Opal and Violetta have shown good tree traits, fruit quality and yield under Serbian pedoclimatic conditions (Milošević et al. 2012).

In other Nordic (Finland, Denmark) and Baltic countries (Lithuania, Latvia and Estonia) plum-breeding programs exist independently or in mutual cooperation with the public or private sector. Earlier, the programs served Denmark and Finland by producing seedlings for evaluation in their climates (Hjeltnes 1994), whereas in Lithuania breeding at present is done in cooperation with Graminor AS, Norway (Ikase 2015).

Institutional fruit breeding in Estonia, financed by the government, started in 1945 at the Horticultural Institution in Polli, recently renamed the Polli Research Institute for Horticulture and Apiculture. Up to 2000, 15 new plum cultivars had been released (Kask et al. 2010). Some of the cvs. Ave, Julius, Kadri, Kressu, Liisu, Polli Munaploom, Villu, Reeta, Kaidi and Suhkruploom demonstrated high cold hardiness and good quality.

In Latvia, plum breeding started in 1981 by A. Spolîtis at the Institute of Biology and by P. Upîtis in Dobele (Ikase 1992). In 1996–2001 a common breeding program with Sweden (Swedish Pomological Science Center) was carried out. The main

goals were created by E. Kaufmane, I. Grâvîte and L. Ikase. Since the 2000s, 8 new plum cultivars have been released by hybridization at the Institute of Horticulture in Dobele, independently or in cooperation with V. Trajkovski from Balsgård (I. Grâvîte pers comm). The new cultivars are Agra Dzeltena, Minjona, Zemgale, Adele Lāse, Minjona, Ance, Sonora, Adelyn, Laine, Lotte and Zane (Ikase 2015; Kaufmane et al. 2012). The last two cultivars not yet registered.

In Lithuania, new cultivar development has focused on improving cold hardiness, resistance to diseases and pests, and yield, as well as on shipping and shelf life qualities, plant development and biotechnological applications (Sasnauskas et al. 2017). The breeding programs of apple and stone fruits started in 1952 at the Institute of Horticulture, Lithuanian Research Centre of Agriculture and Forestry in Babtai. As a result of breeding efforts, five plums were named and released: cvs. Gynė, Jūrė, Rausvė, Kauno Vengrinė and Aleksona (Lukoševičius 2000).

During the last few decades a number of new plum cultivars were bred in Germany (Hartmann 1998, 2006). Plum breeding was revived in 1980s at the University of Hohenheim by W. Hartmann, later led by M. Neumüller. The main goals are: the extension of ripening time, high fruit quality and PPV resistance (Hartmann 2007). From 1990 to 2006, 13 new cultivars were released. Stanley cv. has been a good parent to transmit tolerance to PPV.

As a result of extensive efforts, a large number of hybrid seedlings were created, some recognized as a new cultivars such as Katinka, Hanita, Presenta, Haganta®, Haroma®, Joganta®, Colora®, Elena®, Hanka®, Juna and Tipala. Also the first absolute PPV-resistant cv. Jojo was released and further used as donor to an extensive resistance breeding program (Hartmann and Neumüller 2009). For example, cv. Jofela, also resistant to PPV, was created by crossing cvs. Jojo and Felsyna. About 2000 seedlings derived from Jojo and its sisters are awaiting selection. Jojo, Hanita and Katinka cvs. showed very good productivity and fruit quality under Serbian conditions when grown under high-density planting systems (Milošević 2013).

Since about 1985, a successful breeding program, established by H. B. Jacob, has existed in Geisenheim (Jacob 1998, 2002). The most important goals in this program were improving cultivars for seasonal markets, PPV resistance, sufficient hardiness to pest and disease and climatic robustness. Between 1993 and 2005, 14 new cultivars were named and released. The most important are Topfirst, Topfive, Topking, Toptaste, Topper, Top, Top 2000, Topgigant Plus, Topstar Plus, Tophit Plus and Topend Plus. Results of their evaluation were published by Jacob (2007). However, when H. B. Jacob retired in 2005, the program was suspended and no crosses have been made since in Geisenheim.

Although the breeding program at Geisenheim ceased, activities continued at the Technical University of Munich—Freisyng Weihenstephan and Bavarian Centre of Pomology and Fruit Breeding (Hartmann and Neumüller 2009). The main goals of this effort are to create new cultivars with large, tasty fruits with good shelf life. An overview on the plum breeding activities was made by Neumüller et al. (2016).

Plum breeding in Hungary was initiated in 1960 by F. Nyujtó, continued by E. Tóth at the early 1960s, and later by I. Csöbönyei, Z. Erdős, D. Surányi and T. Lacatos, at research stations in Újfehértó and Cegléd (Butac et al. 2013). Clonal selection of

cv. Besztercei i.e. Požegača in order to develop clones with larger fruits and eventual tolerance to PPV and improvement of foreign cultivars were the main goal of these activities. As a result, 8 new European plum cultivars have been registered and more clones of Besztercei were obtained and named as a Tiszavidék cv. (Tv. 5, 9, 39, 51, 53, 58, 59, 61, 62) (Surányi and Erdös 1992). The Cegléd Station developed, among others, clones cv. Besztercei Bb. 398, Besztercei Bt. 2 and Besztercei 105-58, whereas Érd Station released cvs. Besztercei Bt. 2, Besztercei Nm.122 and Besztercei Nm.150 (Markovitz-Zsohár et al. 2017). However, a big breakthrough has not yet happened. Currently, all activities regarding stone fruit breeding is carried out by the NARIC Fruitculture Research Institute, founded 1 January 2014, which is the legal successor of research stations located in Budapest, Érd, Cegléd and Fertőd. The main breeding aims are production safety, increasing tolerance/resistance to diseases and pests and fruits having outstanding taste, aroma and market value.

In Belarus, at the Institute for Fruit Growing in Minsk, fruit breeding programs began for plums in 1928 (Matveev 2010). The Belarus climate is temperate and continental, characterized by a warm and damp winter, comparatively cool and rainy summer, wet autumn and sunny but variable weather in spring. Winter-hardy forms of European plums with good productivity, fruit quality and multiple resistance to various abiotic and biotic stresses, are the main goal of the breeding program (Kozlouskaya 1994). As a result of breeding efforts, some 15–20 European plum cultivars and more hybrids were developed by E. P. Syubarova, V. A. Matveev and Z. A Kozlouskaya. The newly-released cvs. at the Institute for Fruit Growing in Minsk are Venera, Charadejka, Dalikatnaya, Ranaya Lošickaya, Vengerka Belorusskaya and Volat (Matveev and Malyukevitch 1987; Matveev and Vasileva 2013, 2015). Recently, the Grodno Zonal Research Institute of Agriculture, Schuchin, released a new cv. named Nagrada Nemanskaya.

In Ukraine, fruit cultivation research is done at two smaller institutes (Institute of Irrigated Horticulture, Melitopol and Mlyevsky Institute of Pomology, Mlyevsk) and one of the largest (Institute of Horticulture of the National Academy of Agrarian Sciences of Ukraine, Kiev) with two branches—in L'viv and Donets'k (i.e. Artemivsk city). Also, this work is done in research stations such as: Prydnistrovska in Chernivtsi, Podil'ska in Vinnytsia, Krasny Kut in Kharkiv, Chernihiv in Chernihiv city, Crimean in Symferopol and Sevastapol, a station of the Vavilov Institute of Plant Industry in St. Petersburg, now belonging to Ukraine (Gladon et al. 1994). Generally, the main goal of European and cherry plum is breeding of cultivars by using sources and donors of useful traits (e.g. disease resistance, fruit shelf-life and high content of biologically active constituents) with the purpose of obtaining cultivars for modern environmentally-friendly, resource-saving technologies (Tolstolik et al. 2012). As a result of breeding activities, several plum cultivars were released. In the 1980s, the Donetsk branch of the Institute of Horticulture in Kiev, developed cvs. Renklod Karbysheva (1984), Ughorka Donets'ka Rannia (1984), Uhorka Donets'ka (1985) and Renklod Rannii (1988). Also, in this period, the Mlyevsky Institute of Pomology, Mlyevsk, released two cultivars: Voloshka (1980) and Oda (1986); after 2000, cvs. Sentiabrs'ka (2003), Nen'ka (2007), Oryhinal'na (2008), Kantata (2008), Pam'iat Materi (2008), Trudivnytsia Mliieva (2009) and Okrasa Sadu (2017).

At the end of the twentieth century, there were 10 plum breeding programs in Russia (Burmistrov 1992). A brief list of the released cultivars until 2000s was provided by Okie and Ramming (1999). Today, there are 12 breeding programs for the European plum, 7 for the Japanese plum and 3 for cultivars originating from *P. cerasifera* and other plum species. The breeding goals are extensive due to the fact that Russia as a large country having different climatic conditions—from very cold to warm with 12 climatic zones. Dominant aims are cold hardiness, modest tree size, productivity, self-fertility, larger fruit size (>30 g), higher sugar content (>13%), purple color, earliness, resistance to PPV and other pests and diseases.

From 2000 to 2017, a large number of new plum cultivars were released by the Russian state institutes. Regarding European plum, at the All-Russian Research Institute of Fruit Crop Breeding in Orel, 3 new cultivars was released, Aleksiy, Bolhovčanka and Kantemyrovka; at the North-Caucasian Zonal Research Institute of Horticulture and Viticulture, Krasnodar (former Crimean Experimental Breeding Station of the All-Russian Research Institute of Plant Growing) 13 cvs. were released: Ballada, Osenniy Suvenir, Prikubanskaya, Debjut, Golubaja Mechta, Milena, Podruga, Gertsog, Krasotka, Charodeyka, Osennyaya, Krasnodarskaya and Beglyanka (Eremin and Zaremuk 2014; Zaremuk and Alekhina 2013; Zaremuk et al. 2017). At Nizhny-Volzhsky Research Institute of Agriculture, Moscow, cvs. Vengerka Korneevskaya, Nižegorodskaya and Renklod Scherbinskiy were bred. The Samara Zonal Experimental Station of Horticulture, Samara released 4 cvs. 2000–2017: Vecherniy Zvon, Viola, Galateya and Pamyat Finayeva. Results of the plum breeding at Tatar Scientific Research Institute of Agriculture in Kazan for the above period were 5 new plum cvs.: Volžanka, Kazanskaya, Pamyat Hasanova, Rakitovaya and Teknovskaya Golubka. Breeders from the All-Russian Horticultural Institute of Breeding, Agrotechnology and Nursery in Moscow named and released 6 cultivars: Zanyatnaya, Syniy Dar, Suhanovskaya, Utro, Yahontovaya and Kantemirovka (Simonov and Kulemekov 2017); the Kuibyshev Experimental Station for Horticulture, Samara, released cv. Indira. Modern plum breeding exists at the Dagestan Breeding Experimental Station of Fruit and Berry Crops, Buynaksk; 2 cvs. were developed: Predgornaya and Sverhrannyaya. The breeding program at the Maritime Fruit and Berry Experimental Station of the Maritime Research Institute of Agriculture in Vladivostok, released cv. Antonina. New plum cultivars with high cold hardiness were released at the All-Russian Research Institute of Genetics and Breeding of Fruit Plants in Michurinsk: Startovaya, Zarecnaya Rannaya, Renklod Kursakova and Nochka. Other smaller programs at the Nikitsky Botanical Garden—National Science Center, Yalta and the Scientific Research Institute of Horticulture of Siberia, Barnaul are aimed at developing plums suitable for growing under these climatic zones. The cultivars released were Viktorina and Baykalskaya, respectively.

Regarding breeding programs of European (domestic) plum in other continents and countries, some data are available.

In Canada, plum breeding is a very old practice, beginning in 1913 at the Horticultural Research Institute of Ontario in Vineland, Ontario. A modern breeding program was initiated by G. Tehrani. Breeders targeting a better frost tolerance, outstanding fruitfulness and blue skin color. The 7 fresh market cvs. Valor, Vanier, Verity, Vision,

Veeblue, Voyageur and Victory are the results of this breeding work (Tehrani 1990; Tehrani and Lay 1992). However, the breeding program has been suspended.

In the USA, there are several breeding programs of European plum. At the New York Agricultural Experiment Station, Geneva, breeding work began at the end of the nineteenth century. Breeding work continues dealing with creating cultivars suitable for the fresh market and for multiple purposes. Several cultivars have been released, such as Hall, American Mirabelle, Stanley and Albion created in the 1920s, and Iroquois, Mohawk, Oneida and Seneca in the 1960s and 1970s (Okie and Ramming 1999). Among them, cv. Stanley (1926) is the most widely-grown cultivar worldwide. For example, in Serbia its fruits are used for fresh consumption, processing, deep freezing, drying and production of a traditional alcoholic beverage called *rakija* or *šljivovica* (Milošević 2002; Milosevic et al. 2010). Also, cvs. Castleton, Longjohn and Polly, all dessert plums, were released by this New York Station.

Another important breeding center in the USA is the Missouri State Fruit Experiment Station. It released three European plum cvs. in 1947: Bluebell, Bluefre and Radiance.

The main goal of breeding work under the leadership of T. DeJong at the University of California at Davis is to develop prunes which ripen before and/or after cv. Improved French (also called Prune D'Agen, Petite Prune or Prune D'Ente) in order to meet standard production practices for dried fruit. The program was initiated in 1985 and cvs. Sutter and Tulare Giant were released in 2000 and Muir Beauty in 2005 (De Jong and Castro 2015; De Jong et al. 2002). Emperor cv. was earlier released for dessert use (Okie and Ramming 1999).

Since 2005, in cooperation with R. Scorza, USDA Kearneysville WV, and W. Hartman and M. Neummler to obtain sources of PPV resistance that can be incorporated into the breeding material, the program of refinement of cv. Improved French has continued, using previously created seedlings and selections. Several promising genotypes were selected (e.g. cvs. G16 N-19, G47S-61, H13S-58, H10 N-38 and F11S-38). They are in the process of testing and evaluation (De Jong and Castro 2015).

Other programs have existed, the most important being the USDA breeding at Prosser, Washington and later Beltsville, Maryland, Md. resulting in the recent release of cv. Bluebyrd plum by H. W. Fogle (Scorza and Fogle 1999).

Currently research is underway at the USDA Kearneysville, WV, centered on developing plums highly resistant to PPV using gene manipulation methods (Scorza et al. 2013). The first transgenic cultivar is Honey Sweet (Fig. 5.3), into which a gene from the virus coat protein was inserted.

Additionally, some minor breeding efforts in the USA existed earlier: Oregon Agricultural Experiment Station with the Gardner cv. released in 1923, and the USDA in Cheyenne WY, with Hildreth cv. released in1982 (Okie and Ramming 1999).

Fig. 5.3 Honey Sweet, the first transgenic plum cultivar (*Photo* T. Milošević)

5.2.5.2 Japanese Plum

About 90% of the plums produced in Europe are European plums, whereas in Asia 82% are Japanese plums (Surányi and Erdös 1998). In North America, 53% of the plums produced are European, 38% Japanese and nearly 9% *P. americana*, *P. simonii*, *P. nigra* and *P. munsoniana* (Surányi and Erdös 1998). Ramming and Cociu (1991) estimated the production of Japanese plums for different countries; for example, 40% for Italy, 55% for Spain, 85% for Australia and 99% for Pakistan. In China, 99% are Japanese plums. Therefore, it can be said that the world production of Japanese plums is from cultivars with high genetic potential of productivity and fruit quality (Fig. 5.4). Some of them are very old, of unknown origin. For example, cv. Zhui Li dates back more than 2000 years or cv. Tam Hoa widely grown in Vietnam around Bắc Hà town, in Lào Cai province is also very old.

Cultivars of the Japanese plum species were greatly improved in Japan and then introduced into the USA in the latter half of the nineteenth century, where subsequent breeding produced many more cultivars, generally with larger fruit. As previously noted, Luther Burbank was the first breeder to create new Japanese plums based on scientific methods. The 113 named cultivars of plums introduced by Burbank were by far the most numerous and arguably the most important of his horticultural accomplishments (Karp 2015). His crowning achievement was cv. Santa Rosa (1906), which in 1945 accounted for 36% of the California plum harvest. Many of Burbank's other cultivars of primarily *P. salicina* ancestry were extensively cultivated in California in the early and middle 1900s, including Beauty (introduced 1911), Burbank (1888), Duarte (1911), Eldorado (1904), Formosa (1907) and Wickson (1895).

The first breeding efforts of *P. salicina* cultivars have been concentrated in California and the southern USA. Interestingly, in California about 200 Japanese plum cultivars are in commercial production (Okie and Ramming 1999). However, the top

Blackamber TC Sun

Fig. 5.4 Fruits of some cultivars of Japanese plum (*Prunus salicina*) grown under temperate climate conditions in Prislonica village near Čačak, Serbia (*Photo* T. Milošević)

3 cultivars, Friar, Angeleno and Blackamber, have black skin and currently account for ≈40% of California's total production.

In the USA, active plum-breeding programs are located in California, Georgia, Texas and Florida. In collaboration with the USDA, the University of Davis started a breeding program in 1932 and released some commercially-important prunes for drying. Active plum breeding in California is by the USDA-ARS in Fresno. It was established there by J. S. Weinberger in the 1950s and is currently directed by D. W. Ramming (Hartmann and Neumüller 2009). About 67,000 hybrids have been grown and tested. They released cvs. Frontier, Friar, Blackamber, Calita, Queen Rosa, Fortune, Black Splendor, Owen T. and John W. (Topp et al. 2012).

In the USA, several private companies exist which are highly respected for their large fruit breeding programs: Zaiger Genetics (Modesto), Bradford Farms (Le Grand) and Sun World International (Bakersfield). Cultivars created by Zaiger Genetics include Hiromi Red, Joanna Red, Golden Globe® (syn.: Zairobe), Earliqueen and Crimson Glo. Zaiger Genetics has released some 50 cultivars between 1997 and 2008 (Topp et al. 2012).

Bradford Farms has a remarkable breeding program producing 10,000–15,000 seedlings per year, and they applied for 35 plant patents from 2000 to 2007. Sun World International is also intensively creating new Japanese plums. Their more important cultivars include: Black Diamond®, Red Giant™, Applum™, Black Giant®, Red Diamond®, PL1347RB and PL454YY selections, respectively.

In California, some private breeders and growers have selected new cultivars such as the late-ripening Angeleno® (syn.: Suplumsix), Green Sun, October Sun, Tracy Sun and TC Sun, selected by J. Garabedian. Since 2003, 18 new Japanese plum cultivars and several from interspecific crossings have been released (Ramming 2006).

The most important breeding program outside of California is located in Byron, Georgia. Between 1964 and 1995, about 40,000 seedlings were grown and 8 new

cultivars released (Okie and Ramming 1999). A comparison of the plum cultivars bred at Byron with cv. Santa Rosa was made by Okie (2006). The breeding program continues to cross Japanese plum cultivars with native plum species.

At the University of Florida, a low-chill breeding program has been developed (Sherman et al. 1992); cvs. Gulfbeauty, Gulfblazze, Gulfruby, Gulfgold and Gulfrose were released by this institution (Sherman and Rouse 2001). A sixth cultivar, Hypoluxo is an open-pollinated seedling of cv. Gulfruby which was named and patented by S. Farnsworth of Miami (Rouse and Sherman 1997).

A short overview of the new Japanese cultivars released by other public institutions and the private sector in the USA was provided earlier by Okie (1999), Okie and Ramming (1999), Hartmann and Neumüller (2009) and Topp et al. (2012).

In the Southern Hemisphere, there are some breeding programs such as in Brazil, which has five Japanese plum breeding programs in traditional plum-growing areas. Some cultivars with low chill requirements were released in 1970s and 1980s, such as Carmesim, Rosa Paulista, Grancuore, Gema de Ouro, Golden Talisma, Rosa Mineira, Januaria, Kelsey-31 and Centenaria (Okie and Ramming 1999).

In South Africa, plum breeding has been carried out at the Infruitec Center for Fruit Technology, 1972–1993, initiated by C. Smith and M. Oosthuizen. The aim was to achieve large-fruited plums with resistance to diseases and good shipping quality. Several new cultivars were released from 1970s to 1990s, especially by the company ARC Infruitec-Nietvoorbij in Stellenbosch (Western Cape). Some of the cvs. are Songold (syn.: Sun Breeze), Harry Pickstone, Reubennel, Redgold, Laetitia, Celebration, Sapphire, Golden Kiss, Souvenir and Celebration, Ruby Red and Pioneer.

In Australia, plum breeding was established by B. Topp in 1967 at the Horticultural Research Station in Queensland. Two new cultivars were named and released in 1988: Queensland Bellerosa and Queensland Earlisweet. In 1998, the cultivar Teak Gold was released by R. A. Yates (Okie 1999). Cultivar Queen Garnet developed as a high anthocyanin and high antioxidant plum under the program at the University of Queensland in 2001 was first grown commercially in 2007 (Netzel et al. 2010).

New Zealand has a small plum and Plumcot® breeding program established to develop cultivars with larger fruits and improved flavor, better storage, different maturity time, smaller tree habit and disease-resistance, suitable for local usage and export. Earlier, at HortResearch, now known as Plant & Food Research, M. Malone created seedlings from open-pollinated populations of cv. Flavorella, other Plumcots® and local plums (Okie and Ramming 1999). Recently, as a result of breeding program of Summerfruit New Zealand in Alexandra, in partnership with the Plant and Food Research and private company Nevis Fruit, Bannockburn, new cvs. Humdinger and Malone, are being evaluated for commercial cultivation.

In Japan, plum-breeding activities are mostly in the hands of private breeders and growers (80%) and to a lesser degree in the public sector (20%) such as National Institute of Fruit Tree Science (NIFTS) in Tsukuba, Ibaraki Prefecture initiated by M. Yamaguchy (H. Itamura pers comm). At NIFTS, two cvs. (Honey Rose and Honey Hart) were named and released in 1994 and 1995, respectively (Yamaguchy et al. 1998, 1999). Honey Rose was developed from the open-pollinated seedlings of cv.

White Plum to improve fruit quality of early season maturing cultivars, whereas cv. Honey Hart was developed from seedlings of cvs. Sordum × Nishida in order to improve fruit quality of mid-season maturing cultivars. In Fukushima prefecture, breeder O. Roshio has released many cultivars including Oishiwasesumomo, Oishinakate and Gekkou (H. Itamura pers comm).

In China, the main goals in cultivar improvement are late ripening time, large fruit size, good cropping, eating quality, postharvest behavior and resistance to spot and other diseases. At Liaoning, under J-Y Zhang, a large plum germplasm collection has been established. Several selections were developed in Changchun, Jilin province, by P. Li such as cvs. Changli #15, Changli #84 and Changli #109 (Li 1993). In Heilongjiang province a new cold-hardy plum cv. Sui Li 3 was released (Wu 1993). Breeding to improve *Prunus humilis* Bunge for fruit size and quality was made in Taigu, Shanxi (Du et al. 1993). Breeding work in China has increased in recent years. A yellow-fruited cv. Crown Chinese plum was released recently as a mutation of the very old cv. Qing Nai (Li et al. 2015).

In South Korea, a small breeding program began in the 1980s at the National Horticultural Research Institute in Suwon. Breeding is for improved fruit quality; cvs. Purple Queen and Honey Red have been released (Jun et al. 2008). Summer Fantasia is a new cultivar derived in 1999 at the National Institute of Horticultural and Herbal Science of the Rural Development Administration in South Korea. It was first selected and propagated in 2008 for testing and evaluation (Jun et al. 2015).

In 1994, several breeding programs for Japanese plum were started in Thailand. The main goals were developing of low-chill cultivars with large and high-quality fruits, suitable for long storage. The effort was a partnership between the Royal Project Foundation and Kasetsart University, under the direction of U. Boonprakob (Okie and Ramming 1999). A similar program began at the Taiwan Agricultural Research Institute in Wu-Feng by Wen Jen-Chie, which is currently managed by Shyi-Kuan Ou.

In the Russian Federation, especially in Siberia and Krasnodar, the main goal of Japanese plum breeding programs is cold hardiness (Eryomine 1990; Burmistrov 1992).

A short list of cultivars released to 2000 was made by Okie and Ramming (1999). After 2000, 30 new Japanese plums were released and registered at eight government stations with similar breeding programs, for cold hardiness, productivity, fruit quality, self-fertile and resistance to pest and disease. The largest number of cultivars since 2000 so far has been created at the Scientific Research Institute of Horticulture of Siberia, Barnaul (7), followed by All-Russian Research Institute of Fruit Crop Breeding, Orel (6), All-Russian Research Institute of Plant Growing, Vladivostok (5), Maritime Fruit and Berry Experimental Station, Vladivostok (4), Sverdlovsk Horticultural Breeding Station, Ekaterinburg (3), South Ural Research Institute of Fruit and Vegetable Production and Potato Growing, Shershin (3), Buryat Scientific Research Institute of Agriculture, Ulan Ude (2) and Maikop Experimental Station, Podgorny (1) (Table 5.2).

Table 5.2 New Japanese plum cultivars released and registered in the Russian Federation from 2000 to 2017

All-Russian Research Institute of Fruit Crop Breeding, Orel	Buryat Scientific Research Institute of Agriculture, Ulan Ude	Eastern Experimental Station of the All-Russian Research Institute of Plant Growing, Vladivostok	The Scientific Research Institute of Horticulture of Siberia, Barnaul
Alenushka (2001)	Amtatai (2006)	Podarok Primoryu (2006)	Goryanka (2004)
Orlovskiy Suvenir (2001)	Baykalskaya (2009)	Virovskaya (2009)	Timoshka (2004)
Krasa Orlowchiny (2006)		Nelya (2017)	Uzyuk (2004)
Krasivaya Vecha (2010)		Smuglyanka (2017)	Chemalskaya Synyaya (2004)
Orlovskaya Mechta (2009)		Habrovskaya Desertnaya (2017)	Kseniya (2005)
Nezhenka (2010)			Frosya (2016)
			Daka (2017)
Sverdlovsk Horticultural Breeding Station, Ekaterinburg	Maikop Experimental Station of the All-Russian Institute of Plant Industry, Podgorny village	South Ural Research Institute of Fruit and Vegetable Production and Potato Growing, Shershin	Maritime Fruit and Berry Experimental Station, Vladivostok
Pionerka (2000)	Irkutiyanka (2008)	Krasnoselskaya (2002)	Podorok Primoryu (2006)
Sodruzhestvo (2003)		Kuyashskaya (2002)	Liudmila (2007)
Zavet (2004)		Uralskaya Zolotistya (2004)	Sharovay (2011)
			Nadezhda Primorya (2011)

Source State registration of selection achievements, appropriate for use. Ministry of Agriculture of the Russian Federation, Volume 1, Plant Cultivars, Moscow (2017)

In Europe, Japanese plum breeding is relatively new but will be important in the future because of the increasing demand for large-fruited plums. Several breeding program exist in different countries.

Breeding programs of Japanese plum were initiated in Italy (Rome, Forli, Bologna and Florence). The Horticulture Department of the University of Florence began Japanese plum breeding in 1989, initiated by E. Bellini and V. Nencetti, to develop new cultivars suitable for the climatic conditions of central-north Italy (Bellini and Nencetti 1998).

These breeders have released cvs. DoFi-Guidy and DoFi-Sandra (Bellini and Nencetti 2002). At Bologna (CMVF-DCA Bologna) several cultivars were released recently such as Dark Sunlight® and Dark Sunshine®. At Azienda Agricola Martelli

company released Early Fortune® and Dark Sunlight®, Golden Plumza* at Vivai F.lli Zanzi and Anne Gold and Carmen Blue at Vivai Calderoni, both protected by the EU.

In France, small breeding programs for Japanese plum exist in the private sector. For example, at Earl Les Hebrard, the cutivar Ruby Crunch® was named and released.

In Spain, a coordinated Japanese plum breeding program between CEBAS-SCIC and IMIDA in Murcia began in 2011. The main goal is to create early ripening, self-compatibility and resistance to PPV, with high fruit quality and productivity (Ruiz et al. 2016). From 4000 seedlings, 43 promising hybrids were selected which are currently under trial.

Ben Dor Fruits is a very old private company (1880) located in Yesud A'maala, Israel. They develop new fruit cultivars, including Japanese plum, with a special emphasis on skin color, fruit shape, flavor and aroma (Fig. 5.5). As an approved plant breeder, the company recently released about 30 new Japanese plum cultivars grouped into several types: plumegrante (black skin with red flesh), watermelon plums (red to burgundy flesh), lamoon plums (darker yellow skin with red touching) (González et al. 2016), black plums (black color skin with light color flesh), pink plums (pinkish purple skin), shark teeth plums (shark teeth shape fruits with different colors or multiplied color) and red plums (speckled skin combine with light red flesh color).

All these cultivars and selections were tested at Univeg. RuBisCO (Pty) Ltd. in South Africa. The more important members of all types with some features are presented in Table 5.3. This overview did not include the large number of selections in the evaluation process.

5.2.5.3 Breeding of Other Plum Species and Interspecific Hybrids

In some countries, special breeding programs include interspecific hybridization as a tool to create new plum cultivars. In most cases, the major goal is to breed for high tolerance or resistance to PPV and high fruit quality (Zhivondov and Djouvinov 2002), and more recently resistance to climatic change and new pests and diseases. In these programs, the main challenges are climatic adaptation, yield potential, ripening time, fruit characters and breeding for resistance in the created cultivars (Neumüller 2011).

Earlier, at the Vavilov Experimental Breeding Station in Crimea, genotypes of *Prunus cerasifera* with very early (June) and very late (September) ripening, large fruit (>30 g), winter hardy, drought-tolerant, with good taste and canned fruits qualities, high content of antocian, ascorbic acid and other valuable substances are of great interest (Eremin 1978). Crossing *P. cerasifera* cvs. with *P. salicina* cvs., and other diploid cultivars, made it possible to have large-fruited cultivars with good taste, early ripening, high winter-hardiness and disease resistance. Promising hybrids were obtained when cherry plum cvs. Purpurovaja, Chernilnaja, Ashtarakskaja N_2, Pionerka, Riony and Kok-Sultan were used as parent stock. At the All-Russian Scientific Research Institute of Horticulture in Michurinsk, crossing of cv. Green Gage and *P.*

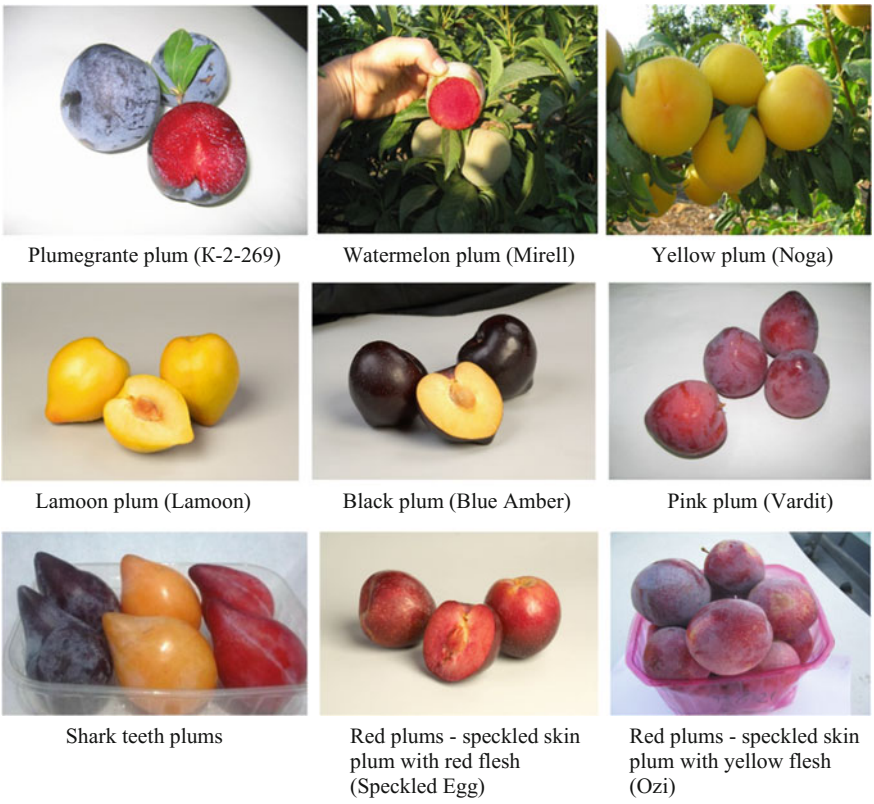


Fig. 5.5 Some Japanese plums named and released at Ben Dor Fruits Company, Yesud A'maala, Israel (*Source* http://www.bendorfruits.com/plums.html)

domestica subsp. *insititia*, resulted in cv. Renklod Kolhozniy. Sodurzhestvo cv. is the product of crossing two Ussuri plum selections, cvs. Usuriyskaya Immunaya × Usuriyskaya 14-26 at Sverdlovsk Horticultural Breeding Station, Ekaterinburg.

After 2000, a few new cultivars from *Prunus cerasifera* were introduced. Cultivars from North-Caucasian Zonal Research Institute of Horticulture and Viticulture, Krasnodar, include cvs. Globus (2002), Dinnaya (2002), Evgeniya (2002), Kolonovidnaya (columnar type) (2002), Podarok Sad-Gigantu (2002) and Kometa Pozdnaya (2006). The Moscow Agricultural Academy, led by K. A. Timiryazev, released cvs. Kleopatra (2004), Nesmeyna (2005) and Zlato Skifov (2005), whereas the Nikitsky Botanical Garden—National Science Center, Yalta, also released 3 cultivars, Andromeda (2014), Desertnaya Rannaya (2014) and Rumyanaya Zorka (2014). All are productive, large-fruited, winter-hardy cultivars, resistant to a complex of major diseases.

In Ukraine, at the Donetsk Experimental Gardening Station in Artyomovsk, several new cherry plum cultivars were released: Donchanka Rannia (1997), General

Table 5.3 New Japanese plum cultivars released recently at Ben Dor Fruits Company in Yesud A'maala, Israel

Type of cultivar	Cultivar	Soluble solids content (°Brix)	Fruit maturity (date)
Plumegrante type	Sweet Aroma	23	01 June
	Early Aroma	17	05 June
	Brave Heart	17	10 June
	Selek	15	15 June
	Madlen	23	20 June
	Plumegrante	23	15 July
	Black Egg	21	15 July
	Mark	23	10 August
	Marcco	22	15 September
Watermelon type	Damask Heart	18	10 June
	Avatich	16	10 June
	Mirell	22	25 June
	Crocodile Dandy	20	01 July
	Silver Red	20	15 August
	Green Red	25	20 August
	Red Sunset	25	20 September
Lamoon type	Kesem	16	10 July
	Lamoon	18	15 July
	New Lamoon	24	01 August
	Earlamoon	20	01 August
	Late Lamoon	20	20 August
	Latemoon	20	20 September
Yellow type	Vahav	13	05 July
	Candy	29	05 July
	Big Sun	16	05 July
	Zehava	18	15 July
	Sun Queen	22	05 August
	Zehava Giant	16	10 August
	Jobbi	22	10 August
	Golden Queen	24	15 August
	Yellow Giant	20	25 August
	White Sun	18	25 August
	Noga	24-26	15 September
	Nov	24	20 September
	Yellow Beauty	20	25 September

(continued)

Table 5.3 (continued)

Type of cultivar	Cultivar	Soluble solids content (°Brix)	Fruit maturity (date)
Black type	Ziv	16	10 June
	Bluebee	17	05 July
	Ami	17	10 July
	Blue Amber	20	20 July
	New Queen	23	20 July
	Black Queen	23	25 July
	Blue Giant	14	30 July
	Yasmin	16	05 August
	September	20	10 August
	Black Stone	18	20 August
	Black Giant	18	20 August
Pink type	Pink Diamond	18	25 July
	Pink Varda	20	20 August
	Varda	20	30 August
	White Angeleno	18	05 September
	Vardit	23	25 September
	Vardy	22	10 November
Shark teeth type	Black Shark Teeth	13	10 July
	Early Galit	18	25 July
	Zigallow	18	25 July
	Red Flesh	19	04 August
	Yellow Galit	18	15 August
	Dolphin	18	15 August
Red plum types			
(a)*	Namrur	18	15 June
	Winner	20	15 June
	Merry Diamond	20	20 June
	Bandora	16	20 June
	Speckled Egg	24	15 July
	Inbar	20	25 July
	Maria	22	25 August
	Malka Red Heart	16	25 August
(b)**	Ozi	18	01 July
	Muskat	20	01 August
	Shchada	16	10 August

Source http://www.bendorfruits.com; * Speckled skin plums with red flesh, ** Speckled skin plum with yellow flesh

(2007) and Tetiana (2007) and an apricot-plum hybrid named Cernyj Princ. The Central Botanical Garden, Kiev and the Donetsk branch of the Institute of Horticulture, developed cvs. Kyivs'ka Hibrydna (2001) and Slivovidnay (not yet registered), respectively.

Bulgarian breeding efforts restarted in 1997 and several cultivars were included in the hybridization experiments such as Stanley, Green Gage, Pacific, President, Scoldus and some local cultivars, all European plums (*P. domestica*). Other species, such as *P. armeniaca, P. dasycarpa, P. cerasifera* and *P. ussuriensis* were used as parents in crossing with *P. domestica* and/or *P. salicina*. More promising cultivars and new fruit forms were obtained (Zhivondov 2012).

Also in Bulgaria, resistance to PPV was obtained under natural infection in 166 plum hybrids from the crosses: *P. spinosa* × *P. domestica* Green Gage, *P. spinosa* × *P. domestica* Zh'lta Ablanshka and *P. spinosa* × *P. cerasifera* (Minev and Dragoiski 1995). In a similar program, as a result of crossings between some plum species (*P. cerasifera, P. spinosa* and *P. domestica*), 17 promising hybrids were selected. Due to their fruit-bearing capacity and specific traits, as well as disease resistance, they are interesting as original hybrids for further breeding work (Minev and Balev 2002).

Earlier, at the Institute of Horticulture in Dobele (Latvia) a new program named 3 hybrids of *P. ussuriensis* Kov. & Kost. × *P. cerasifera* Ehrh. (Ikase 1992). As a result of this effort, cv. Inese was released. A cultivar of cherry-plum hybrid Zolushka was developed at the Fruit Growing Institute in Minsk, Belarus. In addition, the first winter-hardy cultivars, Mara (*P. cerasifera* × *P. salicina* var. *ussuriensis*), Naidyona and Vetraz were developed at this institute (Kozlouskaya 1994).

There are numerous breeding programs around the world that deal with interspecific hybridization of certain species *Prunus*, especially using *P. salicina* and *P. cerasifera* as seed parents, and *P. armeniaca*, *P. persica* and *P. avium* as pollen parents. Private programs dominate, especially in the USA, Spain, Japan, China and Australia. One of the interesting public programs of interspecific breeding is in South Korea, created by J.H Jun (Jun and Chung 2007; Jun et al. 2009).

At the National Institute of Horticultural and Herbal Science in South Korea, since 1999, interspecific crosses between Japanese plums and apricots have been made to breed new Plumcot® cultivars (Jun et al. 2009). As a result of controlled interspecific cross between *P. salicina* Lindl. Soldam cv. (seed parent) and *P. armeniaca* L. Harcot cv. (pollen parent), several cultivars were made in the first decade of the twenty-first century. They are Beniasama, Red Pricot, New Castle Gold and Plum Apricot (Jun and Chung 2007).

Several interspecific hybrids were developed recently by a number of private companies and breeders such as Zaiger Genetics. A peach-plum hybrid (*P. persica* hybrid) having fruit resembling peach with one cultivar named Tri-Lite. Cherry Plum is a hybrid between *P. cerasifera* and *P. salicina* have been developed by Zaiger Genetics, with the cultivar name Delight. The most important cultivars of the Pluerry™ [complex *Prunus* hybrid, primarily of Japanese plum and sweet cherry (*P. salicina* × *P. avium*) with dominant parentage of plum and having fruit resembling plum] are Candy Heart, Sweet Treat and Sugar Twist, all developed by Zaiger Genetics.

Aprikyra® is cherrycot cultivar developed by crossing *P. pumila* var. *besseyi* and *P. armeniaca*, whereas Aprisali® is an interspecific hybrid obtained by crossing *P. armeniaca* and *P. salicina* (Aprium®); both of them are trademark of Artevos GmbH group (Germany).

Cultivars Biricoccolo or Susincocco (*Prunus* × *dasycarpa*) are Biricoccolo Bolognese and Biricoccolo Vesuviano (Ruggiero 2015).

In Italy, the N. Sgaravatti & C. Sementi Company released several new Japanese plum and interspecific cultivars. Most interesting is the Pluerry cv. Sorriso di Primavera.

An interesting program of interspecific breeding efforts was realized by B. Q. Genetics and Kingsburg Orchards, San Joaquin Valley, USA. Several Aprium® cvs. developed recently are Black Velvet™, Blue Velvet, Crimson Velvet, Gold Velvet, Red Velvet and Ruby Velvet. Generally, for Plumcots® worldwide, low productivity has been a problem. A detailed overview of some new Plumcot® cultivars was made by Okie (1999) and Zhivondov (2012).

5.3 Germplasm Biodiversity and Conservation

The diversity of plum germplasm is attributable to natural and artificial selection for adaptation, and other characteristics over a long period of time and under a wide array of ecological conditions. The basic problem with the utilization of plum germplasm, for either cultivation or breeding purposes, is the lack of reliable pomology data or reference repositories to enable positive identification of cultivars (Milošević and Milošević 2012b).

A total of 2254 accessions of plums and prunes are included in the European *Prunus* (EP) database. Outside Europe, 128 accessions of *P. domestica* are held in the US Germplasm repository. However, more than 2000 other cultivars of the crop, mostly present in East European countries, have not yet been submitted in the European database (mostly collected in Ukraine and Russia). More than 1500 accessions, of *P. salicina* are preserved in Russia, China and Japan (Blazek 2007). A rich source of germplasma of European plums (about 100 accessions) in Iran has been described by Khadivi-Khub and Barazandeh (2015). Autochthonous (local, folk, traditional, primitive) plums are very important genetic resources in improving new plum production due to the fact that they breed in relative isolation and are selected according to their resistance or tolerance to biotic and abiotic factors (Bálint et al. 2016). They could be used as a highly effective source of germplasm through the development of new resistant cultivars and rootstocks for stone fruits (Milošević and Milošević 2012b).

Autochthonous cultivars are numerous and well-adapted to local agro-ecological conditions (see Appendix 2: Plum Genetic Resources). Despite its importance, little is known about plum phenotypic and genetic diversity. Using genotyping-by-sequencing (GBS), Zhebentyayeva et al. (2015) characterized 90 genotypes representing different types of European plum and its possible progenitors, diploid species

Prunus spinosa L. and *P. cerasifera* Ehrh. Halapija-Kazija et al. (2014) assessed 62 plum accessions (42 traditional Croatian accessions, 6 well-known traditional accessions collected from Serbia and Bosnia and Herzegovina, and 14 international reference cultivars) using microsatellite markers and distinctness, uniformity, and stability (DUS) plum descriptors. In Serbia, phenotypic variation of plums was traditionally assessed using morpho-logical characterization (Milošević and Milošević 2012b; Milošević et al. 2010; Paunović and Paunović 1994). Local autochthonous genotypes were also evaluated in other European countries: Bulgaria (Ivanova et al. 2002), Romania (Botu et al. 2002; Butac et al. 2017), Bosnia and Herzegovina (Drkenda and Kurtović 2012; Vukojevic et al. 2012), Croatia (Jelačić et al. 2008; Halapija-Kazija et al. 2014) and Slovenia (Usenik et al. 2007). However, only a very small percentage of global genetic resources has been utilized in plum breeding so far or has been properly screened for this purpose. Their successful application may be possible (Blazek 2007). In addition, American plum species, ancient Oriental cultivars and autochthonous European cultivars represent important germplasm resources that require preservation for use in future breeding.

5.3.1 Different Approaches for Germplasm Conservation

Conservation and utilization of genetic resources depends largely on the availability of information regarding phenotypic and genotypic characteristics of the species of interest (Rao and Hodgkin 2002). Different approaches to germplasm conservation can have considerable impact on the amount of genetic diversity captured. Gene banks of fruit species are mostly maintained in situ, in the field. Field collections are convenient for breeding and evaluation of genotypes, but demand high requirements in terms of land, labor and costs. These collections are under threat by environmental factors and high pest pressure. Plum genetic resources are kept in collections that include wild species, local populations, cultivars and rootstocks of *P. domestica*, *P. salicina, P. cerasifera* and *P. spinosa*. These collections are located mainly in research and development institutes or fruit-growing stations. Collections of plum germplasm exist in Romania (Botu et al. 2017), Latvia (Kaufmane et al. 2002), Sweden and Norway (Sehic et al. 2015), Serbia (Milošević and Milošević 2012b), as well as in France, Germany, the USA and China (Okie and Hancock 2008).

Establishment of a modern germplasm collection of fruit plants necessarily involves the use of in vitro techniques as an important supplement to conventional field preservation of germplasm (Reed et al. 2008). Maintenance of a germplasm collection by in vitro techniques has the advantages of requiring less space and limited labor costs (Towill 1988).

A few in vitro techniques exist for the genetic conservation of fruit species. Among them, slow growth in vitro has been carried out in a wide range of species including *P. domestica* and *P. cerasifera* and the duration of a subculture cycle can be extended from a few weeks to 6–12 months or more (Gianni and Sottile 2015).

For long-term germplasm conservation, cryopreservation (storage at −196 °C) has been mostly used. Cryopreservation is the only alternative, safe and cost-effective method for long-term storage of plant genetic resources, particularly for stone fruits (*Prunus* spp.) (Engelmann 2004). De Carlo et al. (2000) and Vujović et al. (2015, 2016) indicated that cryopreservation could be a very useful tool for preservation of indigenous plum genotypes and different protocols exist.

5.4 Molecular Breeding

5.4.1 Molecular Marker-Assisted Breeding

Marker-assisted selection (MAS) is a promising strategy for improving classical breeding methods which allows the pre-selection of the most important traits years before they can be evaluated in the field (Knapp 1998). This saves significant time and space in the development of new cultivars and allows selection to be focused on genotypes carrying suitable alleles that will be passed on to descendants. Also, DNA-based markers, and particularly microsatellites (or SSRs), are very useful tools for distinguishing plum cultivars since they directly reflect the genotype.

Over the last 50 years, genetic improvement methods in *Prunus* spp. have changed very little (Dirlewanger et al. 2004) due the polygenic nature of most useful fruit quality traits, with genes distributed throughout the entire genome, making it very difficult to develop linked markers. *P. domestica* is a less-investigated species, mostly due to its hexaploidy and molecular markers which could be used for selection are not available.

Random amplified polymorphic DNA (RAPD) markers can be used to distinguish different cultivars, which focus on the utilization of DNA fingerprints in identifying plant cultivars and also to develop a new strategy to properly utilize DNA marker s in the separation of plum cultivars (Yu et al. 2013); RFLP markers are used to estimate polymorphism and genetic diversity of plum cultivars (Ben Mustapha et al. 2015), whereas SSR markers are available for molecular genotyping (Decroocq et al. 2004; Xuan et al. 2011). Genomic linkage maps for plum have not yet been developed.

In the case of Japanese plum, these tools can be used for genetic characterization of cultivars (Ahmad et al. 2004; Carrasco et al. 2012; Klabunde et al. 2014), but very little in genotyping and identification of fruit-quality traits (Salazar et al. 2017). Therefore, more investigations focused on this species are necessary in order to incorporate MAS into the breeding programs for *P. salicina* fruit quality.

5.4.2 Bioinformatics in Breeding

The most important objectives of plant bioinformatics are encouragement for submission of all sequence data into the public domain, through repositories, to provide rational annotation of genes, proteins and phenotypes, and elaboration of relationships both within the plants data and between plants and other organisms (Vassilev et al. 2006). This new tool has found application in generating of large amounts of genomic, genetic and breeding data in some species in Rosaceae family (apple, strawberry, *Prunus mume*) but not in plum (Jung and Main 2014).

5.5 Tissue Culture and Genetic Engineering

5.5.1 Micropropagation

In tree fruits, tissue culture has large application in the propagation of rootstocks, own-rooted scion cultivars, elite genotypes and trees free from viruses (Fig. 5.6).

The application of in vitro methods in the production of own-rooted planting material of plum has significantly increased the production capacities and contributed to the improvement of its quality and health status (Popov and Kornova 2009).

Micropropagation methods have been developed for some species of *Prunus*, including plums, using stem node, shoot tip, axillary bud, leaf, cotyledon and seed explants (Wolella 2017). However, there is no universal medium for in vitro culture because plant species and cultivars are genetically specific with regard to different components of the medium.

Currently, this method was mostly used for plum rootstocks propagation (Paunović et al. 2011). Propagation of rootstocks by micropropagation in vitro has been widely used in commercial nurseries, because it allows the production of a large number of plants from one mother tree.

Some protocols were created for propagation of cultivars of *P. domestica* (Ruzic and Vujovic 2007; Wolella 2017) and *P. salicina* (Kassaye and Bekele 2015; Zou 2010). In the production of plum cultivars by micropropagation, the greatest handicaps are rejuvenation, later precocity, somewhat higher tree vigor, while there were no differences in yield and fruit quality (Popov and Kornova 2009). It appears that Japanese plum is more suitable for micropropagation as compared to European plum (de Magalhães Bandeira et al. 2012).

5.5.2 Genetic Transformation

Genetic transformation and regeneration in plums has only been applied to PPV-resistance detection. The lack of naturally-resistant germplasm makes genetic engi-

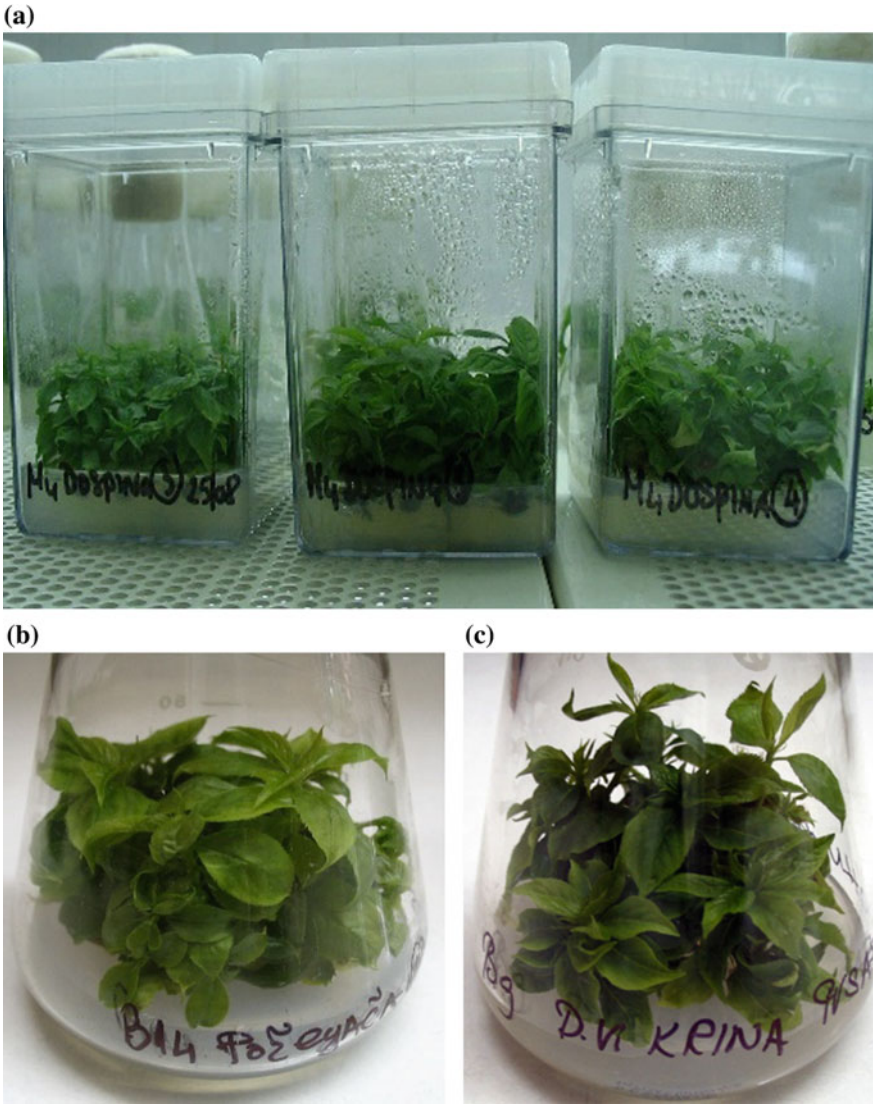


Fig. 5.6 Micropropagation in vitro of **A** Dospina rootstock, **B** the European plums Požegača cv. and **C** Krina cv. at Fruit Research Institute, Čačak, Serbia (*Photo* T. Vujović)

neering an important alternative approach to develop PPV resistance in European plum. As an enhancement to classical breeding, genetic engineering was used to produce transgenic clones that contain the PPV coat protein (CP) gene, applying the principle of pathogen-derived resistance. The result of this new approach was the development of transgenic clone C5 (experimental name) or Honey Sweet (commercial name) (Fig. 5.3) (Ravelonandro et al. 2013). This transgenic plum clone was

identified as highly resistant to PPV through aphid- and graft-inoculation and did not express PPV-CP and produced barely-detectable levels of CP mRNA. Clones that did express the CP gene proved to be susceptible, which suggested a resistance mechanism that was not protein-mediated (Polák et al. 2017; Ravelonandro et al. 2013). This type of resistance is not an advantage of the known quantitative resistance in existing cultivars as the genetically modified plants can get infected with the virus, e.g. when the PPV sensitive rootstock is infected and can serve as host of PPV (Neumüller 2011).

At this level of knowledge about gene transfer, it could be said that genetically-modified plum cultivars are not necessary for plum production and they can find their purpose in further experiments, while classical breeding techniques could be still used for improvement of plum genotypes.

5.6 Conclusions and Prospects

Traditional plum breeding is a long, expensive, difficult and uncertain process with fundamental limitations such as long juvenile period, large tree size and many inapplicable technologies (backcrossing and hybrid seeds). However, in this way, a large number of cultivars of European, Japanese and a few cultivars of plums originating from other plum species have been created in research centers and in the private sector. A large number of intraspecies hybrids between taxa of the genus *Prunus* have also been created. There is a logical question: is this large number of cultivars needed? Obviously not, because some of them are very old, they have numerous defects in production conditions or are unacceptable to consumers. Nevertheless, the process will continue in the future and its dynamics will depend primarily on the financial power of public research centers, private companies and independent breeders and growers. Despite optimism about continued yield, fruit quality, resistance to pest and disease, primarily to PPV, self-fertility tolerance to climate change, and improvement from conventional breeding, new biotechnological techniques will be needed to maximize the probability of success. Modern biological techniques are not yet widely used in fruit-plant breeding. One reason is that the genetics of fruit trees is still not well understood. We hope that in the near future new plum cultivars, with better properties than the existing ones, will be created by new techniques and methods.

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Appendix 1

Research institutes concerned with plum

Institute	Specialization and research activities	Contact information including website
Fruit Research Institute, Čačak	Selection and introduction of fruit trees, fruit growing technology	http://www.institut-cacak.org
Research Institute for Fruit Growing, Pitesti	Genetics and breeding, propagation, tissue culture	http://www.icdp.ro
Hochschule Geisenheim University, Geisenheim	Creation of new plum cultivars and rootstocks	http://www.hs-geisenheim.de
University Hohenheim, Stuttgart	Creation of new European cultivars resistant to Sharka virus	http://www.hs-geisenheim.de
University of Bologna	Breeding of new plum cultivars, especially Japanese	http://www.unibo.it
Fruit Growing Institute in Plovdiv	Breeding of new European plums and intraspecific cultivars	http://www.fruitgrowinginstitute.com
Horticultural Plant Breeding Experimental Station, Dobele	Fruit breeding, pome, stone and small fruits	http://www.hridir.org/countries/latvia
Institute for Fruit Growing, Minsk	Breeding and introduction of fruit, small fruit, nut bearing and vine crops and their rootstocks	http://www.belsad.by
Research and Breeding Institute of Pomology, Holovousy	Genetic resources, plum breeding, creation of new rootstock	http://www.vsuo.cz
University of Agricultural Sciences, Balsgård	Plant breeding, including plum	https://www.slu.se
NARIC Fruitculture Research Institute	Fruit growing technology, fruit breeding, creation of new rootstock	http://fruitresearch.naik.hu
Research Institute of Horticulture, Skierniewice	Research program covers all areas related to fruit, vegetable, ornamental plant and bee sciences	http://www.inhort.pl
North-Caucasus Zonal Horticulture and Viticulture Research Institute, Krasnodar	Resource-saving technologies in horticulture and viticulture, fruit breeding	www.kubansad.kubannet.ru
East Malling Research Station (NIAB EMR), Kent	Conducting of high-quality strategic and applied research in horticultural and environmental sciences	http://www.emr.ac.uk

Institute	Specialization and research activities	Contact information including website
Horticultural Research Institute of Ontario, Vineland	Fruit breeding	http://www.hriresearch.org
Bavarian Centre of Pomology and Fruit Breeding (Bayerische Obstzentrum), Hallbergmoos	Fruit and Plum breeding resistant to PPV, fruit growing technology	http://www.obstzentrum.de
INRA, Bordeaux	Creation of new rootstock, fruit cultivars, growing technology	http://www.bordeaux-aquitaine.inra.fr
Lithuanian Research Centre of Agriculture and Forestry in Babtai	Genetic control of orchard plant traits and creation of new breeding methods, modification of plant genetic structure using biotechnological methods, breeding of fruit and decorative plants	https://www.lammc.lt
USDA-Kearneysville, WV Centerson	Development of biological systems for controlling fruit decay, genetic improvement of fruit crops through functional genomics and breeding, improving stress and disease resistance in tree fruit srops	https://www.ars.usda.gov
Zaiger Genetics	Fruit and rootstock breeding	http://www.davewilson.com
Bradford Farms	Breeding of stone fruits	http://www.bradfordgenetics.com
Sun World International	Fruit and grape breeding	http://www.sun-world.com

Appendix 2

Genetic resources of plum

Cultivar	Important traits	Cultivation location
Grase de Becs	High productivity, fruit quality, tolerance to PPV	Romania
Grase de Pesteana	High productivity, tolerance to PPV	Romania
Negru Pletos	High productivity, tolerance to PPV	Romania

Cultivar	Important traits	Cultivation location
Galbene Mari Aurii	High productivity, fruit quality	Romania
Mirabele mari locale	Fruit quality	Romania
Uriase de Sibiu	Fruit quality, tolerance to PPV	Romania
Timpurii de Turlesti	Fruit quality	Romania
Stanigele	Tolerance to PPV	Romania
Bjelica sitna	Fruit quality	Bosnia and Herzegovina
Prskulja	Fruit quality	Bosnia and Herzegovina
Bilska rana	Drying, fresh consumption, processing	Bosnia and Herzegovina
Alu Bokhara Amritsari	Large fruit size, high sugars content	Punjab, India
Sanacore	Fruit quality, nutritional value, shelf life	Sicily, Italy
Ariddu di Core	Fruit quality, nutritional value, shelf life	Sicily, Italy
Alu bukhara	Productivity, fruit quality, different skin colour	District Poonch, Pakistan
Aluchi	Productivity, fruit quality	District Poonch, Pakistan
Goulina	Fruit quality	Greece
Asvestochoriou	Fruit quality	Greece
Krikon	Productivity, fruit quality, cold hardiness	Norway, Sweeden
Shengjine Tapizes	Fruit quality, tolerance to abiotic and biotic factors	Albania
Sauvage	Fruit quality, drough tolerance	Tunis
Zenou	Fruit quality, productivity	Tunis
Jelya	Fruit quality, drough tolerance	Tunis
Chaaraouiya	Fruit quality, drough tolerance	Tunis
Auerbacher	Fruit quality, productivity, self-fertility	Germany
Bühler Frühzwetsche	High productivity, self-fertility	Germany
Ersinger	Early ripening season, fruit quality, productivity	Germany
Ortenauer	Late ripening season, fruit quality, productivity	Germany

Cultivar	Important traits	Cultivation location
Wangenheims	Fruit quality, productivity, winter hardiness	Germany
Green Gage	Fruit quality, processing	France, England
German prune	Fruit quality, processing, drying, sensitive to PPV	Europe
Italian prune	Fruit quality, processing, drying, sensitive to PPV	USA, Europe
Prune D'Agen	Fruit quality, drying	France, California
President	Fruit quality, late ripening	Europe, USA
Fellenberg	Fruit quality, drying, fresh sensitive to PPV	USA
Mirabelle de Metz	Fruit quality, fresh consuption	Europe, USA
Chrudimska	Tolerance to PPV, fruit quality, productivity	Czech Republic
Durancia	Fruit quality, productivity, late ripening season	Czech Republic
Požegača	Drying, processing, fresh consumption, sensitive to PPV	Serbia
Crvena ranka	Alcholic distillation, rootstock, fresh consumption	Serbia
Belošljiva	Alcholic distillation, rootstock, sensitive to PPV	Serbia
Petrovača	Early ripening, rootstock, fresh consumption	Serbia
Moravka	Fruit quality, alcoholic distilation	Serbia
Trnovača	Processing, alcoholic distillation, rootstock	Serbia
Metlaš	Fruit quality, alcoholic distillation, rootstock	Serbia
Obični piskavac	Fruit quality, alcoholic distillation	Serbia
Mudovalj	Fruit size, processing	Montenegro
Domača češplja	Fruit quality, productivity, sensitive to PPV	Slovenia
Miholjčanka	Fruit quality, alcoholic distillation	Croatia
Ružica	Fruit quality, alcoholic distillation	Croatia

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Chapter 6
Genetic Improvement of Strawberry (*Fragaria* × *ananassa* Duchesne)

Ather-uz-Zaman, Jameel M. Al-Khayri and Rafiul Islam

Abstract The modern cultivated strawberry (*Fragaria* × *ananassa* Duchesne) is one of the world's most appetizing and delicious fruits. The genotypic diversity, highly heterozygous nature and broad range of environmental adaptation have made this fruit unique. It is a good source of fructose, glucose, vitamin-A, vitamin-C, and minerals like potassium, calcium and phosphorus. The anticancer effects of individual phytochemical constituents of strawberries, as well as whole strawberry extracts, have been demonstrated. Research on the antioxidant content of strawberries is providing us with more and more evidence about decreased lipid peroxidation in blood vessel linings following consumption, and less malondialdehyde formation as well. Breeding and induction of somaclonal variation are useful to create new genetic variability for the improvement of strawberry genotypes. The application of classical breeding and biotechnological approaches are potential tools to improve yield and taste of strawberry. Different diseases like crown rot and *Verticillium* significantly impede yield. Somaclonal variation was discussed as a breeding tool for future improvement of strawberry varieties. Although the chapter is predominantly written and arranged emphasizing the specific research on biotechnology and genetic improvement of strawberry; nevertheless information on origin, taxonomy, breeding, mutagenesis and parthenocarpy are also presented.

Keywords Anthocyanin · Biotechnology · Breeding · In vitro · Germplasm Medicinal · Molecular markers · Mutagenesis

Ather-uz-Zaman (✉)
Department of Agro-Biotechnology, GETCO Agro Vision, Dhaka, Bangladesh
e-mail: dr.zaman.ather@gmail.com

J. M. Al-Khayri
Department of Agricultural Biotechnology, College of Agriculture and Food Sciences, King Faisal University, P.O. Box 420, Al-Hassa 31982, Saudi Arabia
e-mail: jkhayri@kfu.edu.sa; jmkhayri@yahoo.com

R. Islam
Department of Botany, Rajshahi University, Rajshahi, Bangladesh
e-mail: rislamakm@yahoo.com

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6.1 Introduction

The modern cultivated strawberry (*Fragaria* × *ananassa* Duchesne) is one of the most tasty, energizing and attractive fruits of the world. Strawberry is the fruit with the highest production among berries with a global production of over 8 million mt (FAOSTAT 2017). Worldwide it is the most extensively distributed fruit crop due to its genotypic diversity, highly heterozygous nature and broad range of environmental adaptations. It is among the few fruit crops that provide quicker and higher returns per unit area on capital investment, as the crop is ready for harvesting within 6 months of planting. Presently, due to several technological advances in its cultivation, such as the introduction of day-neutral cultivars and protected cultivation, fresh strawberries remain available throughout the year (Sharma and Yamdgni 1999).

Fragaria comes from Latin *fragans*, meaning sweet-smelling, referring to the fragrant fleshy tissue of the fruit. Since ancient times, strawberry has seen several special uses other than as a food source (Sonsteby 2008). The exact origin of the common name *strawberry* is uncertain, but probably is an alteration of *strewnberry*. The latter is an old designation for the plant in reference to its berries being strewn about the ground (Trinklein 2012).

The genetics and breeding of strawberry is complicated due to its octoploid genome, originated through interspecific hybridization involving up to four species (Tennessen et al. 2014). Further studies reveal that one of the four sub-genomes originate from the diploid donor *Fragaria vesca*, one from the diploid *F. iinumae*, and the remaining two from an unidentified precursor close to *F. iinumae* (Sánchez-Sevilla et al. 2015).

A major problem with strawberries, however, is that they succumb to all manner of pests and pathogens. Bacterial and fungal diseases are a particular nuisance to strawberry growers. To eliminate pathogens and fungi which attack strawberries and are almost ubiquitous and endemic, farmers have been disinfecting soil used to grow strawberries for a decade. The major fumigants used are highly-toxic methylated halogens (Zhang et al. 2008).

Genetic transformation has initiated a new era in strawberry breeding and germplasm resourcefulness. It provides an exclusive system of generating varieties that selectively target a gene or a few heterologous traits for introduction into the strawberry genotypes. A strawberry has some 35,000 genes, which is about one and a half times more than humans.

Fragaria x *ananassa* was one of the pioneer species in applying in vitro culture techniques on a large scale (Jungnickel 1988; Popescu et al. 1997). Concerning the induction of adventitious-shoot formation, the first attempt was made to induce callus formation from meristems and regenerate plants (Nishi and Oosawa 1973). Strawberry plants regenerated from in vitro cell or tissue cultures are not always identical. Therefore, the authentication of genetic stability of the offspring with the aid of genetic engineering and molecular markers has become critical for research, while launching a protocol for the genetic improvement of strawberry (Zakaria et al. 2014) and conducive to human health improvement (Huang et al. 2016).

Flavor and sweetness of strawberry should be important targets of plant breeders. Mesifurane and γ-decalactone are two principal volatile compounds providing a sweet taste to strawberry fruits that are controlled by the FaOMT and FaFAD1 genes, respectively. Scientists have succeeded in optimizing a simple PCR test for combined analysis of these genes and determined prediction accuracy above 91%, using a set of 71 diverse strawberry accessions that could be implemented in current strawberry-breeding programs for the selection of new strawberry cultivars with superior flavor and taste (Cruz-Rus et al. 2017).

6.2 Taxonomy

Strawberry, genus *Fragaria* L., is a member of the family Rosaceae, subfamily Rosoideae (Potter et al. 2007). The history of the strawberry goes back to the Romans, and perhaps even the Greeks, but because the fruit was not a staple of agriculture, it is difficult to find its ancient references. However, it is evident from the literature that strawberry was cultivated in Europe in the thirteenth century, and then French began to transplant wood strawberry (*F. vesca*) from the wild to the garden. At the end of the fifteenth century, the two cultivated strawberries were the wood strawberry (*F. vesca*) and the musky-flavored strawberry (*F. moschata*), both characterized by their small and distinctly-flavored fruits. The only significant event of the sixteenth century to strawberry history was been the introduction of *F. virginiana* from eastern North America to Europe. Carrying the Chilean strawberry (*F. chiloensis*) to France was the most important event in the history of the modern strawberry, because the Chilean strawberries with attractive large-sized berries and had attracted the attention of French gardeners (Staudt et al. 2003). By the mid seventeenth century, Chilean strawberries were grown commercially in France. Its hybrids, which were accidental and the result of using *F. virginiana* as a pollinator for *F. chiloensis*, were more fertile and adaptable, and gardeners in England, Holland and France made careful selections for large-fruited varieties of *F.* x *ananassa* (Darrow 1966).

There are about 20 *Fragaria* species worldwide. Unlike most commercial and garden cultivars of strawberries, woodland strawberries (*F. vesca*) rarely form runners, and are usually propagated by seeds or division of the plants. Some cultivars have berries that are green, white, or yellow when fully ripe, in addition to the normal red. It is a perennial, herbaceous, and diminutive plant, growing low to the ground with 3 leaflets from a central crown (Davis 2015).

6.2.1 The Genus Fragaria

Fragaria species have been characteristically defined on the basis of morphological features, ploidy, cross-fertility and geographic distribution. The genus is comprised of at least 21 species, including some newly-described Asian species (Hummer et al.

Table 6.1 *Fragaria* species and their ploidy level

Taxon	Ploidy level
Vesca clade	
F. × *ananassa* Duchesnessp. *ananassa*	8x
F. × *ananassa* Duchesne ssp. *cuneifolia* (Nutt. ex Howell) Staudt	8x
F. bucharica Losinsk.	2x
F. cascadensis Hummer	10x
F. chiloensis (L.) Duchesne	8x
F. iturupensis Staudt	8x, 10x
F. mandshurica Staudt	2x
F. moschata Duchesne	6x
F. orientalis Losinsk.	4x
F. vesca L. ssp. *americana* (Porter) Staudt	2x
F. vesca L. ssp. *bracteata* (A. Heller) Staudt	2x
F. vesca L. ssp. *californica* (Cham. & Schldl.) Staudt	2x
F. vesca L. ssp.*vesca* L.	2x
F. virginiana Duchesne	8x
China clade	
F. chinensis Losinsk.	4x
F. daltoniana J. Gay	2x
F. gracilis Losinsk.	4x
F. moupinensis (Franch.) Cardot	4x
F. nipponica Makino	2x
F. nubicola Lindl.	2x
F. pentaphylla Losinsk.	2x
F. tibetica Staudt & Dickore	4x
Unresolved phylogenetic position	
F. hayatai Makino	2x
F. iinumae Makino	2x
F. nilgerrensis Schltdl. ex J. Gay	2x
F. viridis Duchesne	2x

Source Liston et al. (2014)

2009). Four levels of ploidy are represented among the recognized species (Table 6.1).

6.2.2 Origin and Spreading of the Cultivated Strawberry

Most garden berries are North American natives: blackberries, blueberries and cranberries. The Americas, North and South, have provided the garden strawberry: *Fragaria* x *ananassa* (Bailey 1894). Two species, one from the western coast of South America, and one from eastern North America, were hybridized by the French in the eighteenth century. Conventionally, a number of *Fragaria* species and novel hybrids have been brought into cultivation in different locations, including *F. chiloensis* (8x) in South America, *F. moschata* (6x) and *F. vesca* (2x) in Europe, and others (Table 6.2). Genomic studies of ploidy levels are instrumental to facilitate strawberry genetic improvement (Bonet and Monfort 2011; Liu et al. 2016). There are abundant wild species of *Fragaria* native to many parts of the world, and the fruits harvested in the wild (Guo et al. 2017).

6.3 Human Nutrition and Chemistry

Strawberries are consumed by millions of people in all climates, including temperate, Mediterranean, subtropical and taiga zones, as fresh fruit due to their pleasant taste, flavor and reputed medicinal value (Ulrich and Olbricht 2016). Strawberries are popular for their characteristic aroma, which is attributed to the presence of volatile esters (Sharma and Yamdagni 1999). The volatile components of woodland strawberry (*Fragaria vesca*) vary from that of the cultivated strawberry (Urrutia et al. 2017). The red fruit color is mainly due to the presence of anthocyanins (Mitra 1991). In addition to better taste and flavor, strawberries contain antioxidants and other nutritional compounds beneficial to certain chronic ailments (Giampieri et al. 2012, 2015). There has been substantial research in this field to identify the genes linked to the biosynthetic pathways of these compounds (Verma et al. 2017). The high-colored molecules (pigments) of strawberries are anthocyanins which act as antioxidants in the human diet (Wang and Zheng 2001). These reduce free-radical damage to DNA (a risk factor for cancer development) and decrease the chance of developing certain types of cancer. A strawberry-enriched diet has been related to a reduced risk of diseases, such as cardiovascular disorders and cancer (Block et al. 1992). Researchers have identified the SAAT (strawberry alcohol acyltransferase) gene which helps strawberries generate their aroma and flavor during ripening (Gasperotti et al. 2015).

Strawberry is cultivated throughout the world, not only for its digestive and tonic properties, but because of the nutritional value of its fruits, important source of folate, vitamin C, fiber, omega-3 fats, potassium, flavonoids, anthocyanins and antioxidants (Table 6.3).

Table 6.2 Worldwide distribution of native strawberries (*Fragaria* sp)

Species	Geographical distribution
F. bucarica Losink.	Asia (W. Himalayas)
F. daltonica J. Gay	Asia (Himalayas)
F. nubicola Lindl.	Asia (Himalayas)
F. gracilis Losink.	Asia (N. China)
F. mandshurica Staudt	Asia (N. China)
F. pentaphylla Losink.	Asia (N. China)
F. corymbosa Losink.	Asia (N. China)
F. moupinensis (French.) Card	Asia (N. China)
F. gracilis Losink.	Asia (N.W. China)
F. tibetica spec. nov. Staudt	Asia (China)
F. innumae Makino	Asia (Japan)
F. yezoensis Hara.	Asia (Japan)
F. nipponica Lindl.	Asia (Japan)
F. iturupensis Staudt.	Asia (Iturpu Island)
F. nilgerrensis Schlect.	Asia (S.E. Asia)
F. orientalis Losink. syn. = *F. corymbosa* Losink.	Asia (China, Far Eastern Russia)
F. Americana (Porter) Britton syn. = *F. vesca* (Porter) Staudt.	Europe, Asia, N. America
F. viridis Duch.	Europe, Asia
F. moschata Duch.	Europe, Asia
F. chilonensis (L.) Miller	N. America, S. America (Western N. America, Hawaii, Chile)
F. virginiana Miller	N. America

Source Hummer and Handcock (2009)

6.3.1 Total Antioxidant Capacity (TAC)

Research has determined that about 30% of the total antioxidant capacity (TAC) in strawberries is provided by their vitamin C content (Olsson et al. 2004). Antioxidant facilitates in reducing the risk of cardiovascular diseases. Medical research confirms that consumption of strawberries results in a lowering of platelet aggregation, fewer lipid peroxidations, less malondialdehyde formation, and scavenges the free radicals (Serrano et al. 2009). *Fragaria* x *ananassa* has been found to have a high content of phenolic compounds like hydroxybenzoic and hydroxycinnamic acid, anthocyanins, flavonols, ellagitannins, conjugated gallic acid, and antioxidant activity (Aaby et al. 2007).

Table 6.3 Strawberry (*Fragaria* × *ananassa*) nutritional values

Principle	Nutrient value (per 100 g)	Percentage (%) of RDA
Energy	32 kcal	1.5
Carbohydrates	7.7 g	6
Protein	0.67 g	0.1
Total fat	0.30 g	1
Cholesterol	0 mg	0
Dietary fiber	2.0 g	5
Vitamins		
Folates	24 µg	6
Niacin	0.386 mg	2.5
Pantothenic acid	0.125 mg	2.5
Pyridoxine	0.047 mg	3.5
Riboflavin	0.022 mg	2
Vitamin A	12 IU	0.5
Vitamin C	58.8 mg	98
Vitamin E	0.29 mg	2
Vitamin K	2.2 µg	2
Electrolytes		
Sodium	1 mg	0
Potassium	153 mg	3
Minerals		
Calcium	16 mg	1.6
Iron	0.41 mg	5
Magnesium	13 mg	3
Manganese	0.386 mg	17
Zinc	0.14 mg	1
Phyto-nutrients		
Carotene-ß	7 µg	–
Lutein-zeaxanthin	26 µg	–

Source USDA National Nutrient database (https://www.nutrition-and-you.com/strawberries.html)

6.3.2 Cardiovascular Benefits

The antioxidant and anti-inflammatory contents of strawberries provide cardiovascular benefits (Alvarez-Suarez et al. 2014). It has been recommended that, human consumption of berry fruits, including strawberries has advantageous effects against oxidative strain mediated syndromes. It has been revealed that strawberries contain several phenolic compounds and secondary metabolites that add to their biological

properties. Investigations on the antioxidant content of strawberries are providing additional confirmation about reduction level of lipid peroxidation in human blood vessel linings following strawberry consumption, and development of less malondialdehyde (Jenkins et al. 2008).

6.3.3 Antioxidant and Anti-inflammatory Benefits

Strawberry is not a critical component of the human diet, but its delicious flavor and taste make this fruit an excellent food from a medicinal point of view. The fruits are rich in phytochemical ingredients, mainly ellagic acid and flavonoids, which can lower the risk of cardiovascular disorders and tumorogenesis (Hannum 2004).

Strawberries are one of the best sources of antioxidants (Gianna et al. 2010). But the scope of strawberry antioxidants extends far beyond vitamin C and a collection of polyphenol antioxidants that includes flavonoids, phenolic acids, lignans, tannins and stilbenes. Each one of the polyphenol categories contains a broad range of antioxidants, most of which have also been shown to have anti-inflammatory properties (Reber et al. 2011).

Consumption of a phytochemical rich diet decreases the risk of certain chronic human illnesses such as cancer, heart, neurodegenerative and diabetes diseases (Pinto et al. 2010).

Folate is an important B-group vitamin that is critical for a range of biological functions in adults and children, including the production of DNA and other genetic material. It is also essential for healthy fetal development fetus in early pregnancy and can help to prevent neural tube defects such as spina bifida (Mike 2017).

6.3.4 Anticancer Effects

In cancer prevention, Wang and Stoner (2008) has been demonstrated the role of anthocyanins. Recently, Rengarajanm and Yaacob (2016) have also demonstrated the anticancer effects of individual phytochemical constituents of strawberries, as well as whole strawberry extracts. The anticancer properties of berries work in various ways in the human body including the antioxidant action of the berry's phenolic ingredients by protecting DNA damage (Seeram and Heber 2006). There have been many published reports on the anticancer effects of individual phenolics known to be present in the strawberry fruit (Seeram 2006). A series of studies revealed that phenolic compounds of strawberry can exert anti-inflammatory, anticarcinogenic, antiproliferative and antiatherosclerotic actions, thus creating defacing mechanism in the human body against common chronic diseases, such as metabolic syndrome, cardiovascular disease and cancer (Giampieri et al. 2017).

6.3.5 Anthocyanins

Scientists have detected up to 25 different anthocyanin pigments in different varieties of strawberry fruits. The most commonly found anthocyanins are cyanidin and pelargonidin. The main pigment in cultivated strawberries has been identified as pelargonidin 3-glucoside and the presence of cyanidin 3-glucoside is also recognized as well (Andersen et al. 2004). Anthocyanins are natural pigments providing scarlet to blue colors in flowers, fruits, leaves and storage organs (Huang et al. 2016). The recent interest in the field of anthocyanin chemistry has been generated by restriction and limitation of the use of synthetic food dyes (Cassidy et al. 2015). Because of their low toxicity anthocyanins have high potential as a food colorant as the substitute of synthetic red dyes. Recently, these anthocyanins have been thought to have pharmacological effects, such as lowering the atherogenic index (Igarashi et al. 1990) and decreasing triglyceride and free fatty acid levels (Igarashi and Inagaki 1991). Moreover, Cassidy et al. (2015) revealed that, anthocyanin more effectively inhibit the growth of tumor cells than other flavonoids. Studies concerned with anthocyanin production using plant tissue cultures have therefore become very important. Masayuki et al. (1999) demonstrated that *Fragaria* × *ananassa* callus produces high amounts of anthocyanin in the dark and accumulates more than 1000 μg of anthocyanin per g fresh cell. And Sato et al. (1996) reported that in the suspension cultures of *F.* × *ananassa* cells, anthocyanin content increased with the intensity of light irradiation of 2500–8000 lx. The significance of this result on clinical efficacy for humans requires research (Xiao et al. 2017).

6.4 Propagation

At present, strawberry is cultivation has extended to temperate, Mediterranean, subtropical and taiga climatic zones. Its cultivation is influenced by the specific regional adaptations related to critical photoperiod and temperature requirements, and thus its cultural systems are highly variable (Larson 1994). Strawberry can be propagated through sexual (seed) and asexual (vegetative) means. However, seed propagation is not considered viable for cultivated strawberries as progeny are not true-to-type. Thus, strawberry is usually propagated through runners.

Nowadays, large-scale commercial propagation by tissue culture is also widely used in the strawberry industry. Micropropagation using meristems (0.5–1 mm), shoot apex and axillary bud culture, adventitious shoot regeneration and somatic embryogenesis has been thoroughly studied. These types of plant regeneration are significantly governed by the culture medium composition, especially plant growth regulators (PGR) and on genotypes (Mahmoud et al. 2017).

6.4.1 Seed Propagation

Due to its heterozygous nature, propagation of strawberry by seed is not recommended as an appropriate method of propagation. However, hybrids are first raised by seed. A single berry produces numerous seeds, which is advantageous to strawberry breeders in the sense that a number of hybrid seedlings can be produced effortlessly. Strawberry seeds are very small and do not germinate properly unless they are subject to a definite period at a given temperature. The efficient chilling temperature (the temperature mandatory for breaking rest or dormancy) ranges from 2–6.5 °C (Durner et al. 1996). In fact, a chilling temperature of 9.5–10 °C is also effective. The extent of the chilling period is generally 2–4 weeks (Larson 1994). Treatment of seeds with H_2SO_4 or GA_3 and thiourea also break seed dormancy and improve seed germination and subsequent growth.

6.4.2 Vegetative Propagation

Strawberry produces stolons in the leaf axil of the plant which grow out from the parent plant during the summer. At the second node, a runner plant is formed and a new stolon arises on the runner plant to persist the runner train that often branches (Pırlak 2002). To begin with, runner plants produce a smaller number roots but subsequently puts forth excessive fibrous roots. Thus, the plant naturally propagates itself by the vegetative method of runner production. After the runner plant has acquired sufficient growth and roots, it can be separated from the mother-plant, and transplanted elsewhere (Savini et al. 2008). Viral diseases are frequently transmitted through runners by perpetuating all the characters of the mother plant. Therefore, separate beds should be used for runner production (Anna 2002).

Production of stolons is essential in strawberries for vegetative propagation at the expense of fruit yield, but the underlying molecular mechanisms are unknown. The stolon deficiency trait of the runner less (r) natural mutant in woodland diploid strawberry (*Fragaria vesca*) is due to a deletion in the active site of a gibberellins 20-oxidase (GA20ox) gene, which is expressed primarily in the axillary meristems dome, primordia and in developing stolons (Tenreira et al. 2017).

6.4.3 Micropropagation

Micropropagation refers to propagation from the smallest plant part under controlled nutrient, environmental and aseptic cultural conditions. Strawberry is perhaps the first fruit crop in which the micropropagation technique has been standardized (Sharma et al. 2001), and large-scale commercial propagation is now being done through tissue culture (Karim et al. 2015) (Fig. 6.1).

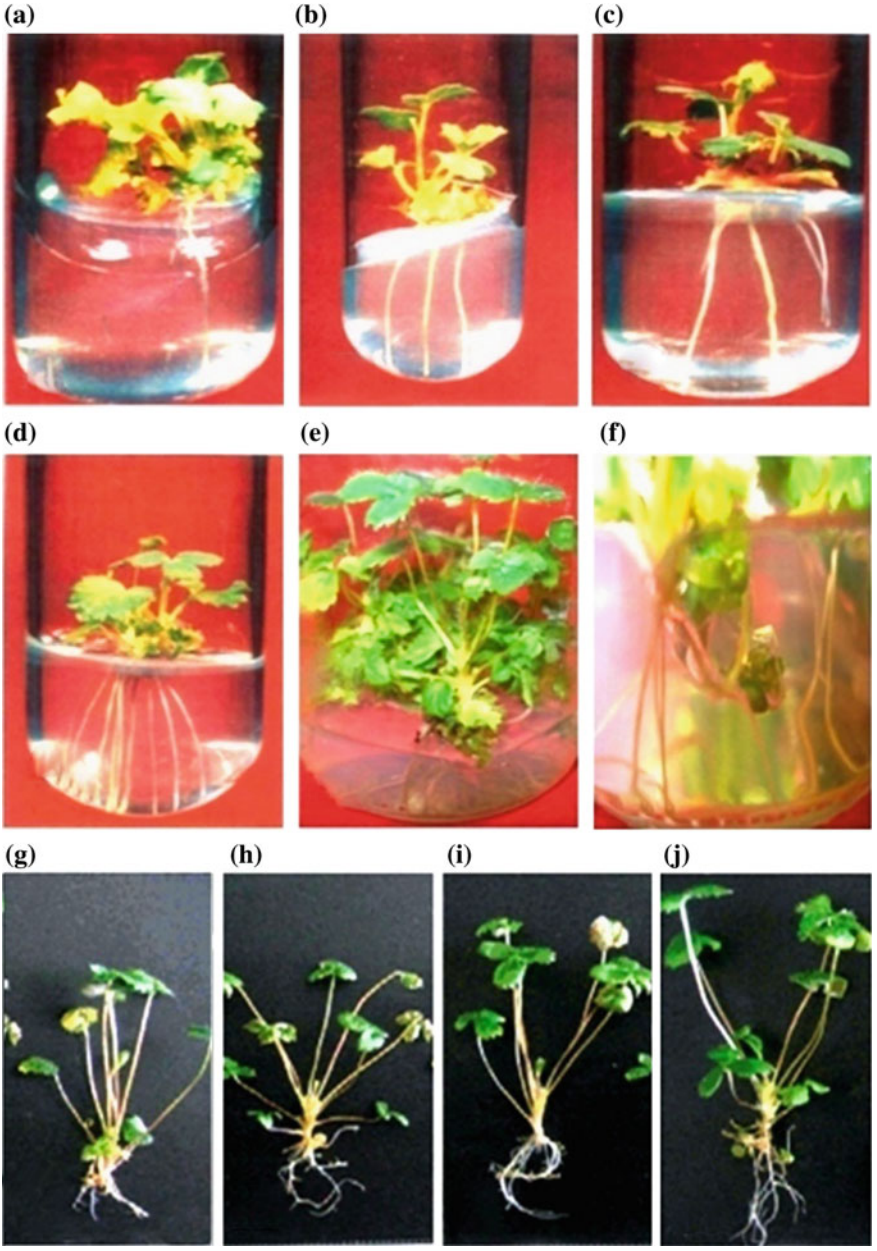

Fig. 6.1 Adventitious root formation from micro-cuttings of strawberry grown on **a** MS + 0.5 mg/l IBA, **b** MS + 0.5 mg/l NAA, **c** ½ MS0 and **d** MS0 after 15 days (**e**) and after 30 days (**f**). Resultant rooted plantlet obtained on **g** MS0, **h** ½ MS0, **i** MS + 0.5 mg/l IBA, and **j** MS + 0.5 mg/l NAA

Practical applications of strawberry micropropagation have significant impacts on breeding programs. This technology provides disease-free stocks throughout the year and can accelerate breeding series as: (i) massive production of elite genotypes irrespective of season, (ii) conservation of genetic resources, (iii) regeneration of haploids for accelerating breeding technologies and (iv) a source of genetic materials for molecular genetics research. Molecular-marker techniques are very conducive to the identification of somaclonal variants (Debnath et al. 2007).

Karim et al. (2015) found cv. Camrosa best for in vitro multiple shoot proliferation in MS medium fortified with BAP and for better root induction ½ MS without any hormones was sufficient. Different explants like meristem tips, anthers, immature embryos and first axillary buds of stolons have been used to produce millions of plants in a year (Boxus 1989). Many culture media have been standardized. Among the most widely used media are Knop's solution, and Murashige and Skoog (MS) and Linsmaier and Skoog (LS) supplemented with agar, sucrose and growth regulators (hormones) at the desired levels (Jones and Waller 1988). The addition of growth regulators into the culture media has shown precise effects on plant regeneration, growth and yield of tissue-cultured plants. The best shoot formation in Pajaro, Tioga and Tufts cvs. was obtained on solid MS medium, supplemented with 1 mg IBA + 1 mg GA_3 + 0.2 mg BA per liter (Sharma and Yamdagni 1999). Many investigators have proved the efficacy of the tissue-culture technique in ever-bearing strawberries using different explant sources (Table 6.4).

Micropropagation is a well-organized technique to produce quality plants from wilt-resistant varieties (Jain and Pehu 1992; Karhu and Hakala 2002) in a short span of time resulting in homogenous growth. Tissue-cultured plants have been reported to produce high yield as compared to conventionally-propagated (runner) cultivars. Badawi et al. (1990) had attempted different concentrations of sodium hypochlorite and mercuric chloride for disinfection of meristems in strawberry and had found that dipping them in 0.2% mercuric chloride for 20 min results in the lowest contamination (5–15%), and that addition of sodium chloride to the medium adversely affected shoot proliferation and percentage survival of explants (meristems). Their research highlighted that the best shoot regeneration in strawberry was found in MS medium supplemented with 1 mg IAA + 1 mg GA_3 + 0.2 mg BA/liter and plantlet survival was highest in a 2:1 peat: sand mixture in the greenhouse (Quiroz et al. 2017).

Nyman and Wallin (1991) developed protoplast technology for strawberry. They used isolated protoplasts for the production of transgenics also by the electroporation-mediated DNA uptake. Hammoudeh et al. (1998) regenerated plantlets of Honeoye cv. from axillary buds by culturing them for 4 weeks on MS medium supplemented with BAP (2.5 mg/l) or thidiazuron (5.5 mg/l) with vitamins for shoot proliferation (Haddadi and Aziz 2010). The technique of in vitro runner production was demonstrated by Mahmood et al. (1994) for Gorella and Hakras Romato cvs. by culturing sterile shoots on Boxus's medium supplemented with $AgNO_3$ and GA_3. For in vitro rooting of plantlets of Hofla and Tangi cvs., Boxus (1999) reported that (¾–½) strength salts of MS medium were better than full a concentration. Nevertheless, the enhancement of normal root development can be done if the rooting stage is per-

Table 6.4 In vitro propagation of strawberry from different explants sources

Explants	Response	Medium (PGR mg/l)	References
Leaf lamina, Petiole	Callus Somatic Embryo	MS + 5 μM 2,4-D 10.05 μM IAA + 105 μM BA	Green et al. (1990)
Apical meristem	Leaf rosette Multiple shoot Root	MS + 1 BA + 1 IBA + 0.1 GA3MS + 1 BA + 1 IBA MS + 0.5 IBA	Petrovic and Jacimovic (1990)
Anther	Callus, shoot Root	MS + 0.5–1 NAA + 0.5–10 BA ½ MS + 0.1 NAA	Xilin (1992)
Leaf disc	Shoot regeneration	MS + 0.1 IBA + 3BA + 600 CH	Sorvari et al. (1993)
Leaf disc	Multiple shoot Root	MS + 1.48–4.44 μM BA MS + 0.5 g/l Activated Charcoal	Lopez-Aranda et al. (1994)
Axillary bud	Multiple shoot, root	LS + 2 BA + 0.1 NAAMS	Kang et al. (1990)
Shoot tip	Multiple shoot Root	MS + 0.02 NAA + 2 BA ½ MS	Jeong et al. (1996)
Single shoot	Multiple shoot	MS + 0.1 IBA + 0.5 BA	Maodobry et al. (1997)
Leaf disc	Shoot regeneration	MS + 80 μM TDZ MS + 2 μM IBA	Sutter et al. (1997)
Stipule	Callus Shoot regeneration	MS + 5 2,4-D + 1 BAP + 9% Sucrose +MS + 1 BA	Damiano et al. (1997)
Axillary bud	Multiple shoot Root	MS + 1 BA MS + 0.1–0.2 NAA	Hammaudeh et al. (1998)
Leaf, petiole	Callus	MS + 0.1 NAA + BA 0.25	Infante et al. (1998)
Nodal segment	Multiple shoot	MS + 0.1 μM NAA + 4.0 μM BA½ MS + NAA 1.0 μM	Bhatt and Dhar (2000)
Meristem	Multiple shoot Root	MS + 1 IAA + 1 BA MS + 5% Activated Charcoal	Adak et al. (2001)
Axillary bud, runner tip	Root	½ MS + 1 IBA + 0.2 g/l AC	Mahajan et al. (2001)

(continued)

Table 6.4 (continued)

Explants	Response	Medium (PGR mg/l)	References
Leaf	Shoot regeneration	MS + 3.2 BAP	Zebrowska and Hortynski (2002)
Stipules	Shoot regeneration	MS + 1 BAP + 0.4 Thiamine	Palombi et al. (2003)
Runner tip	Multiple shoot Root	MS + 4 BAP ½ MS + 1 IBA	Lal et al. (2003)
Leaf, petiole	Callus Somatic embryo	MS + 0.75 NAA + 0.5 BAP MS + 0.25 NAA + 2 BAP	Kaushal et al. (2004)
Leaf disc	Callus Shoot regeneration	MS + 1 BA + 1 2,4-D MS + 2.25 BA + 0.18 NAA	Khan and Spoor (2004)
Shoot tip	Multiple shoot	MS + 1 BA + 1 IAA	Singh et al. (2004)
Vegetative bud	Multiple shoot	MS + 0.5 KIN + 1 BAP + 2 GA_3	Kaur et al. (2003)
Sepal	Multiple shoot Root	MS + 1–2 μM Zeatin	Debnath (2006)

CH—Casein hydrolysate, CW—Coconut water, AC—Activated charcoal. *Source* Gantait et al. (2011)

formed on micro-porous substrates, i.e. peat moss or a mixture of it with vermiculite in the greenhouse (Fekry and Wahdan 2017).

Usually, growth of tissue-cultured strawberry plants is very good, producing lust green foliage (Moradi et al. 2011). These plants produce high yield of good quality (Diengngan and Murthy 2014). However, Kinet and Parmentier (1989) reported the tendency of micropropagated plants to produce increased number of flowers/inflorescences and consequently large number of small fruits with a lower total yield. This was more common when the number of subcultures were high (more than 50), and it persisted only in the first progeny of runner plants derived from in vitro cultured plants (Ashrafuzzaman et al. 2013).

The advantages of micropropagation are shorter generation time and reduced exposure to insects and disease (Moisander and Herrington 2006). Nevertheless, during micropropagation of *Fragaria* plants, somaclonal variation may occur, which is dependent on the age of the culture and media composition (Mikiciuk and Rokosa 2017).

6.5 Flowering, Pollination and Fruit Set

6.5.1 Flowering

The small flowers borne by strawberries have 5 sepals, 5 petals, a very short hypanthium, many stamens, and an apocarpous gynoecium of many free carpels on an elevated receptacle (Fig. 6.2a). Light is the major environmental factor regulating growth and development of *Fragaria* spp. Increased irradiant levels result in increased leaf, root and crown dry weights, stamen development, fruit-set, yield, fruit size and stolon formation. Constant shading at 40% of the ambient sunlight reduced fruit yield and leaf and crown weights, but dry weight of the roots increased when shading was applied during stolon formation. Shading during spring and during the fruiting period may delay fruit maturity by 5–7 days (Doving and Mage 2001).Flowering in strawberry is influenced by photoperiod (Verheul et al. 2007), temperature and their interactions (Sønsteby and Heide 2006). Considerable improvement has been made in clarifying the genetic factors and molecular systems underlying the complex flowering response of strawberry (Heide et al. 2013).

6.5.2 Photoperiod

Environmental control of flowering of the cultivated strawberry (*Fragaria* x *ananassa* Duch.) has been extensively studied (Taylor 2002; Stewart and Folta 2010). However, because of a substantial interaction of photoperiod and temperature, flowering commences in most cultivars under long-day (LD) conditions, if the temperature is low (Verheul et al. 2007). Most strawberry cultivars are classified as short-day (SD), also known as single-cropping, June-bearing or non-ever bearing types, in which floral induction occurs with a photoperiod of less than 14 h (Larson 1994). Most short-day cultivars exhibit a facultative short-day response, in which floral induction occurs more or less continuously regardless of the day-length, provided the temperature is less than 16 °C. Thus, although, short-day cultivars may have different critical photoperiods, most of them bloom independent of the day length with moderately cool temperature. Photo-periodic groups comprise ever-bearing (EB) cultivars, also referred to as day-neutrals (DN); they generally flower continuously regardless of day-length. Although temperature also modifies the photo-periodic response of day-neutral cultivars, they are less sensitive than short-day cultivars. Some authors have recognized a third group as long-day (LD) types. This group is in between the ever-bearing and day-neutral types (Darrow and Waldo 1934; Durner et al. 1996). However, with the selection of several day-neutral cultivars, it is possible to grow strawberry throughout the year (Sharma et al. 2002).

For commercial production, day-neutral is a highly desirable trait in worldwide strawberry breeding programs. Day-neutral genotypes are not sensitive to photoperiod and will start flower initiation under any photoperiod condition as long as temper-

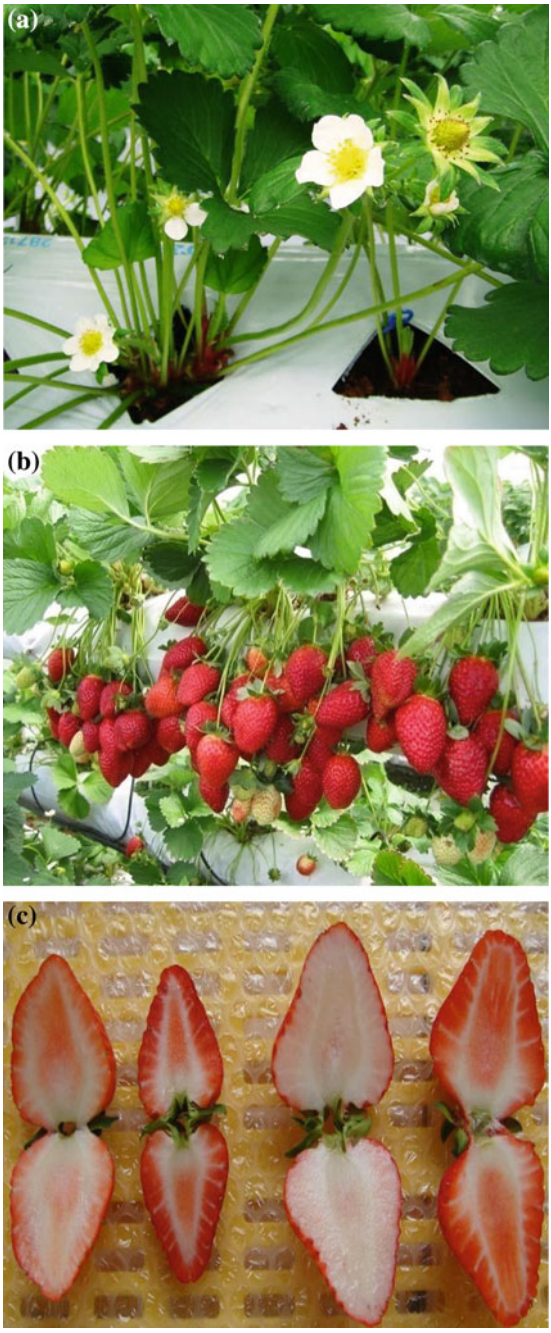

Fig. 6.2 Strawberry flowering and fruiting. **a** Flower and fruit set, **b** Ripened fruits, **c** Seedless strawberries

atures are moderate (below 30/26 °C day/night). It is understood that a single important gene or closely-linked group of genes in combination regulate day-neutrality in this population. Molecular biologists have found that day-neutrality can be qualitatively scored at least in locations where temperature does not permit short-day plants to behave as day-neutral plants (Castro et al. 2015).

6.5.3 Temperature

Temperature plays a vital role for flower induction under short-day conditions. The ideal temperature for short-day floral initiation is 15–18 °C, while below 10 °C and above 25 °C SD induction is rather ineffective (Verheul et al. 2007).

Strawberry is highly sensitive to variations in air and soil temperatures. The simple effects of temperature as well as its interactions with photoperiod often are cultivar and species dependent. An optimum growing season temperature of 15 °C has been reported for most strawberry cultivars and species, although a temperature range of 20–26 °C in ambient temperature for proper growth has also been recorded. Temperature effects on fruit-set may be cultivar-dependent (Ledesma et al. 2008). The soil temperature optimum for the vegetative growth of the strawberry appears to vary with cultivar. However; the optimum temperature for the period between the onset of flowering and the onset of the ripening is 13.8 °C for early, 14.4 °C for mid-season and 15.1 °C for the late cultivars (Bradford et al. 2010). Temperatures below 15.6 °C may inhibit pollen germination and pollen-tube growth, resulting in deformed fruits (Garren 1980). Temperature can also affect fruit-set and fruit quality due to an indirect effect on bee activity. Although, cultivated strawberry has perfect flowers and is self-fruitful, pollination is enhanced by bees. Bee activity decreases below 10 °C. Thus, cool temperature, rains or strong winds that inhibit bee flight, result in decreased pollination and an increased proportion of deformed fruits (Bradford et al. 2010).

Climate influences strawberry production has been well observed in various strawberry producing regions around the world. The prime climate parameters interrelated with strawberry yield includes temperature, precipitation, solar radiation, relative humidity and wind speed (Palencia et al. 2013).

6.5.4 Pollination

Commercial strawberry cultivars bear hermaphroditic flowers, which require pollination through the combined actions of the gravity and wind to generate maximum fruit size. Scientific reports available from different countries show that yield, size and shape of strawberry fruit depends upon adequate pollination of its flowers by different insects (Cruden and Hermann 1983).

Most strawberry hermaphrodite flowers are self-fertile. However, some also produce male or staminate, imperfect and female or pistillate flowers. A strawberry-

enriched diet has been related to reduce risk of diseases, such as cardiovascular disorders and cancer (Block et al. 1992). Although hermaphrodite flowers are self-fertile, pistillate flowers require cross-pollination for fruit production. In some cultivars, partial sterility has been reported (Darrow 1966). The first flush of flowers in some perfect flowered varieties open and set well, but the later flowers are set only partially or do not set at all. In these cultivars, natural sterility is the primary cause. Sometimes, the first flushes of flowers develop into nubbins and later flushes produce good berries; the poor development is probably due to partial pollination (Childers et al. 1995). Petals and sepals sometimes cover pistils and prevent pollination. Frost and insect injuries may also produce nubbins. Poor fruit development in some varieties may be due to the fact that they produce either pistillate flowers only, flowers with a few stamens and stamens that fail to produce sufficient pollen for fertilization (Sharma et al. 2002). Even the plants of the same cultivar vary in their capacity of pollen production in any one area as well as in different areas. The first to open flowers of many cultivars in the spring may develop good anthers with abundant pollens but almost none in the following season. Thus, a variety may be self-fertile in a particular locality but self-sterile in the other (Darrow 1966).

Honey bees are the chief insect pollinators of the strawberries in different countries. Other pollinators are blowflies and bumblebees (Partap 2000). Appropriate management and conservation of pollinators should be practiced in the vicinity of strawberry plantations (Ohishi 1999). Four strong honeybee colonies should be placed in the strawberry field/ha for proper pollination (Verma and Phogat 1995). This improves fruit-set, yield and quality and reduces the percentage of malformed fruits (Chang et al. 2001). For efficient working of bees, Butts (1991) has recommended the use of a commercial honeybee attractant. Similarly, in greenhouses, insect pollinators help in pollination and subsequent yield increases. A population of 10–15 thousand bee workers/l00 m^2 is required for adequate pollination in greenhouses but 60–100 bumblebees are sufficient for 100 m^2 as these are more active than bees in controlled conditions (Lieten 1993).

Numerous studies illustrate that bumblebees are a thriving alternative to honeybees in pollination, principally for the crops planted under greenhouse conditions, as they do not appear to become as easily disoriented as honeybees (Partap 2000).

Domesticated honeybees and commercially-reared bumble bees are ideal plant pollinators, particularly for strawberry, since its flowering period extends up to 3 months, which suggests that this crop can be a valuable source of nectar and pollen to bees (Chagnon et al. 1993).

Bee activity is hindered at temperature above 40 °C and below 4 °C; the optimum temperature being 10–15 °C. Similarly bee activities diminish in the rainy season and also during strong wind. Thus, climatic conditions also hinder pollination and fruit-set indirectly by interfering with bees activities (Childers et al. 1995). In the absence of insect-pollinators, wind also plays a vital role in strawberry pollination. Further studies are needed to explore the potential of breeding for increased herbivore resistance which could be a promising means to reduce damage and increase pollination in strawberries (Muola et al. 2017).

6.5.5 Fruit Set

Fruit set is influenced by many factors; especially by insect pollinators, but can also be increased by auxins. Pollen can be carried by wind, gravity and insects to the stigma surfaces at the tips of the pistils. Although honeybees are not essential to the pollination and fruit set process, wind is critical. How much honey bees aid in the pollination process is subject to question. Thus cross-pollination by insects is favored (Shaul 1986). Strawberries are usually ready for harvest 30 days after the flowers open. This may vary depending on the weather conditions and the cultivar of strawberry. However, maximum fruits are produced from tertiary flowers (Mitra 1991). Even under proper management, most of the varieties do not achieve 100% fruit set. Increased fruit set can be obtained with nitrogenous fertilizers, auxins (Mitra 1991) and with insect-pollinators, especially, honeybees, in the vicinity of the strawberry fields (Partap 2002).

With the aid of electronic fluorescent pictograph software (eFP), which facilitates the visual exploration of gene expression, researchers have established a *Fragaria vesca* eFP browser that has enabled visualization of both RNA-seq data sets on flower and early-stage fruit development and on ripening-stage receptacle fruit (Hawkins et al. 2017).

6.5.6 Fruit Growth and Development

After adequate pollination and subsequent fertilization, ovules develop rapidly and dry pulp around each fertile seed starts swelling. The fruit develops into an unusual structure as a false fruit, having true fruits or achenes on the outside of the fleshy receptacle and they are attached to receptacles through vascular connection. Strawberry is an aggregate fruit, which develops by simultaneous ripening of the number of separate berries of a single flower, adhering as the common unit on the common receptacle, botanically called an *etaerio of achenes*.

Fragaria vesca, the wild or woodland strawberry, is becoming an ideal species for the cultivated octoploid strawberry due to its small and sequenced genome, diploid ($2n = 14$), low height, easy growth and precise but simple life cycle (Shulaev et al. 2011). In the cultivated strawberry *Fragaria* x *ananassa*, fruit development from fruit set to ripened fruit (Fig. 6.2b) has been divided into seven stages: flower/anthesis, small green, medium green, large green, white, turning, and red (Fait et al. 2008).

High-quality strawberries can be produced by optimizing processing conditions for osmotic dehydration and dehydration time. Osmotic dehydration is a potential preservation technique for producing high-quality products and is widely used for partial removal of water from food materials by immersion in an osmotic solution. Osmotic dehydration provides many benefits in the food industry; this process features energy efficiency, reduced packaging and distribution cost, lack of chemical

treatments, and generates high-quality and stable products during storage (Bei and Bangzhu 2017).

6.5.7 Fruit Yield and Quality

Both yield and quality of strawberry are affected by the environment and genotype complex process. Therefore, selection of cultivars should be performed by exact recognition and present conditions.

Quality and quantity of strawberry fruits depend on genotypes, climatic conditions of the area and also agricultural practices (Gulsoy and Yilmaz 2004; Sharma et al. 2014). On the other hand, production under controlled conditions optimistically changes the earliness, yield and fruit quality traits in strawberry production (Gecer 2009). A low plastic tunnel, which is one of the most chosen applications in production, produces better results in these traits in comparison with the production in open-field conditions (Gulsoy and Yilmaz 2004). Organic growing being friendly to the environment and human health can be successful in strawberry production with a good management program.

Many factors including genotype, planting date, mulch type, temperature, fertilizer and production systems have been reported to affect strawberry yield and quality. Among these factors, production systems play an important role by enhancing berry size and marketable yield (Anttonen et al. 2006). Some cultivars of strawberry showed best results in terms of phosphorus and potassium when treated with black plastic mulch and a floating sheet along with organic planting media (Demirsoy et al. 2012). On the other hand results regarding yield and flower components were affected significantly by green polyethylene treatment (Sonkar et al. 2012).

The mite, *Tetranychus urticae* Koch, is considered to be the main pest of strawberries. Several factors can favor its development, among them the genotype susceptibility and cropping system. The degree of *T. urticae* attack on different cultivars is influenced by different field growing management systems. The genotype most suitable for growing under different managements is the Strawberry Festival cultivar; since it has tolerance to *T. urticae* has high fruit yield, and phenotypic stability (Costa et al. 2017).

6.5.8 Induced Parthenocarpy

Genomic research has greatly contributed to illuminate the function of phytohormones in fruit induction, providing simultaneously the genetic systems for introducing seedless fruits. Therefore, replacing the seeds and seed cavities with edible fruit tissue is an attractive offer to consumers and a challenge to the researchers (Yin et al. 2006). Strawberry fruits produce abundant seeds, and these are crunchy in nature. Some consumers do not desire large amounts of seeds. Therefore, production of

seedless fruits would be an added advantage. Past attempts to induce parthenocarpy in strawberries met with limited success.

However, some researchers produced Parthenocarpy strawberry fruits (Fig. 6.2c) using growth regulators. NAA at 0.05 and 0.10 mgl^{-1} in a lanolin emulsion to emasculate flowers resulted in 100% parthenocarpic fruit development. The induction of parthenocarpy in the hermaphrodite strawberry cv. Red Gauntlet was achieved by applying auxin and auxin analogues in a lanolin emulsion to emasculate unpollinated flowers.

Among the auxins, IAA, IBA, NAA, NOA and NAA have the potential power to induce parthenocarpy in most flowers with a concentration range of 0.05–1 mg/ml (Singh and Randhawa 1959).

6.6 Genetic Improvement

6.6.1 Breeding

Strawberries have become a favorite fruit in part because of their fragrance, which results from an amalgamation of sugars, acids and volatile organic compounds. However, breeding efforts have been changing this fruit for commercial production focused on important agronomic traits such as fruit size and shape, yield, pest resistance, fruit firmness, and postharvest life (Folta and Klee 2016; Ulrich and Olbricht 2016).

In the nineteenth century, formal strawberry breeding was initiated in England by Michael Keen and Thomas A. Knight (Darrow 1966). Their famous cultivars became the foundation of many modern European cultivars. Today the dessert strawberry, *Fragaria* × *ananassa*, dominates cultivation in the arable regions of the world (Hummer 2008).

The cultivated strawberry (*Fragaria* × *ananassa*) originates from the hybridization of the South American *F. chiloensis* and the North American *F. virginiana* (Hancock 1999). Research on the genetics and breeding of strawberry is complicated because of its octoploid genome ($2n = 8\times = 56$), (Rousseau-Gueutin et al. 2009; Tennessen et al. 2014). Strawberry breeders have identified one of the four sub-genomes developed from the diploid donor *F. vesca*, one from the diploid *F. iinumae*, and the remaining two from an unidentified precursor analogous to *F. iinumae* (Tennessen et al. 2014). Further research has shown that the genome of *F. vesca* (Shulaev et al. 2011) can be utilized as a reference for genomic studies.

The primary objective of a breeding program is to develop a cultivar that is especially well-matched to particular growing areas having different climate and environment and the hybrids must have superior traits when evaluated against existing strawberry cultivars (Lecerteau-Köhler et al. 2012). New strawberry cultivars should exhibit improved yield and other beneficial traits, and more importantly, nutritional values, without losing any established valuable traits. Moreover, new cultivars should

be bred to resolve ecological disadvantages and shortcomings for any given cultivable area (Galvão et al. 2017).

Preferred cultivar traits will likely remain in a state of flux. Demand of novel strawberry cultivars will be required to deal with climate change and with the amount of human consumption. The appearance of strawberries, their berry color, durability, shelf life, and shipping quality and flavor improvements are areas to be targeted (Sánchez-Sevilla et al. 2015). Developing new cultivars through breeding to combat common pests and diseases prevalent in a particular location is also important. Increasing the antioxidant levels in fruit through breeding and/or biotechnology is an important option to maintain a higher human antioxidant intake, particularly when fruit consumption is low. However, the success of both breeding and biotechnology approaches is dependent on detailed knowledge of the sources of the most useful wild and cultivated genetic diversity to be used in genetic and genomic studies (Mezzetti 2013).

6.6.1.1 Breeding Systems

Outcrossing has been used predominantly for improving strawberries (Darrow 1966). In this, most characters segregate in each generation and selection pressure can be exerted simultaneously on each of the characters. Seedlings with desirable combination of characters are selected. In general, a wide parentage base is necessary in outbreeding to avoid chances of inbreeding, which often results in loss of vigor and reduction in yield. However, inbreeding has also been used occasionally as a method for evolving new strawberry cultivars. Some investigators have tried to evolve inbred lines in strawberry but due to lanky and weak growth, lack of vigor and susceptibility to viral infection, it has become difficult to maintain them (Hancock et al. 1996).

6.6.1.2 Interspecific Hybridization

All octoploid *Fragaria* species are interfertile. *F. chiloensis* and *F. virginiana* have been used extensively in cultivated strawberry improvement. Traits that have been studied thoroughly including winter hardiness of *F. virginiana* ssp. *glauca* (Powers 1945), resistance of *F. chiloensis* to red-stele root rot *(Phytophtora fragariae* Hickman) (Waldo 1953), strawberry aphid (*Chaetosphon fragaefelli* Cockerell) (Crock et al. 1982), two spotted spider mite (*Tetranychus urticae* Koch) (Shanks and Barritt 1984), resistance of *F. chiloensis* and *F. virginiana* to *Verticillium* wilt (Arulsekar 1979), virus tolerance of *F. chiloensis* (Darrow 1966), the day-neutral character of *F. virginiana* ssp. *glauca* (Ahmadi et al. 1990), large fruit size and firmness of *F. chiloensis* (Bringhurst and Voth 1981) and high photosynthetic rate of *F. chiloensis* (Hancock et al. 1996). However, the incorporation of these characteristics into cultivated strawberry is not an easy task as some undesirable characters, small fruit size, partial fertility, soft pulp, black seeds, susceptibility to leaf diseases and dull fruit color are also inherited in the progeny. Thus, native octoploid species should be used

for strawberry breeding programs to raise at least 3–4 generations by outbreeding or backcrossing to obtain a progeny of the desired characteristics (Bringhurst and Voth 1981). Hence, a large population is required from which to select a seedling with the desired characters from the segregating population (Hancock et al. 1996).

6.6.1.3 Intergeneric Hybridization

Hybridization between *Fragaria* and its related genus *Potentilla* has been attempted but most hybrids died at an early age or were all sterile. Ellis (1962) and Jones (1955) also made several attempts to cross *F. vesca* with different species of *Potentilla*, but all hybrids died in early or late stages. However, two fertile colchicine induced amphidiploids have been produced, which could be used to transfer genes between the two genera.

The cultivated strawberry originated from two American octoploid well-matched wild species: *F. virginiana* and *F. chiloensis*; several breeders use *Fragaria* spp. of identical or lower ploidy level of different American, European or other origin to integrate some valuable characters. In many breeding programs, the successful inclusion of wild-derived traits have come through backcrossing, particularly the integration of day-neutrality from *F. virginiana* ssp. *glauca* into the cultivated strawberry. Achievements in strawberry breeding are directly associated with the availability of genetic resources.

Interspecific hybridization takes place at the ploidy level as well. Hybrids of diploid *Fragaria vesca* and *F. viridis* have been named *F* × *bifera* in Europe and most likely are capable of happening wherever the two parents are present (Staudt 2003, 2005, 2006; Staudt and Dickore 2001; Staudt and Olbrichr 2008). In northwestern North America, large zones of introgression occur among the octoploids *F. chiloensis* and *F. virginiana* (Luby et al. 1992; Salamone et al. 2013).

6.6.1.4 Targeted Breeding

Most plant and fruit characters of cultivated strawberries are inherited quantitatively (Galletta and Mass 1990). Additive, dominance and epistatic components may act in the expression of the particular character. This usually creates a complex breeding problem when maximum expression of a combination of characters is desired. Scientists have succeeded in developing strawberry lines resistant to the anamorphic fungi *Botrytis*. Cultivating such intragenic strawberries will be conducive in reducing fungicide applications and will benefit producers and consumers in addition to being favorable to the environment (Schaart et al. 2011).

6.6.1.5 Yield

Strawberry yield is related to the number and size of the fruits, plant vigor, hardiness, disease resistances and crown number per row. Fruit size differs within a fruit cluster, the primary berry being the largest and the others being relatively smaller. Fruit size is inherited quantitatively and is controlled by 6–8 allelic pairs. Crown number per row is often the factor most strongly associated with the fruit yield (Hancock et al. 1996). Crown production can be increased by stolon formation, branch-crown formation or by shifting to plants with the ever-bearing habit; *Fragaria virginiana* ssp. *glauca* is a rich source of both types of genes (Bringhurst and Voth 1984). For stolon production, Guardian and Honeoye are the best cultivars (Hancock et al. 1996). Lal and Seth (1982) have found that, heritability estimates were higher for a number of yield components, like berry weight, berries/flower, yield/flower-stem, flower-stem number and have suggested that, when several characters are combined in a breeding program, crosses should be designed to exploit all genetic variances, whether additive, dominant or epistatic.

The modern strawberry cultivars sold in most stores and markets, with their large fruits, belong to the species *Fragaria* × *ananassa*, an octoploid species with 56 chromosomes.

Molecular markers have been explored in strawberry since the 1980s but in recent years the development of high-throughput sequencing technologies has increased dramatically the amount of genomic data in this species. These advances in structural genomics in *Fragaria* have been reviewed by Chambers et al. (2014). A pragmatic strawberry breeding strategy should be focused on achieving new genotypes with superior nutritional value to become more acceptable to the consumers. Ideally, the progenies of a successful breeding program should have the following characters: (A) fruit yield should be increased and win farmer acceptance; (B) nutritional values should have tangible and substantial positive impact on human health; (C) micronutrient improvement qualities should be comparatively steady across various climatic conditions; (D) micronutrients in improved progenies must be tested in humans to detect any negative effect and (E) consumer acceptance should be recorded to ensure maximum impact on nutritional health (Mezzetti 2013). To achieve such objectives, research on the genetic diversity of strawberry is essential. Breeding programs help to assess the genetic diversity of strawberry accessions. Genotyping results will provide a molecular basis for future breeding programs and will facilitate the development of novel strawberry cultivars with increased genetic diversity (Soohwan et al. 2017).

6.6.2 Mutagenesis

Mutation is defined as any sudden and drastic heritable change in a gene that is not traceable or ascribable to segregation or recombination. Mutations play an important role in the evolution of new species when a major set of characters mutate. Mutation breeding has emerged as a major and practical tool of strawberry improvement in

recent decades and has revolutionized the concept of plant breeding (Rieger 2006). Mutations may be natural or induced and may occur at the chromosome level or at gene or molecular level or may take place involving the cytoplasm or cytoplasmic organelles like plastids. Mutations are induced by physical (e.g. gamma radiation) and chemical (e.g. ethyl methane sulfonate) mutagen treatment of either seed or vegetatively-propagated crops. Mutagen treatment breaks the nuclear DNA and during the process of DNA repair, new mutations are induced randomly and are heritable. The changes can occur also in cytoplasmic organelles, and result in chromosomal or genomic mutations that enable plant breeders to select useful mutants such as fruit color, fruit shape, disease resistance and early-fruiting types. Over the past 50 years, induced mutations have played a major role in the development of superior crop varieties, translating into a tremendous positive economic impact on agriculture and fruit production (Karim et al. 2015).

As an octoploid, cultivated strawberry represents a formidable obstacle in mutation breeding. The genetic structure of strawberry is composed of a small quantity of nuclear and cytoplasmic germplasm (Dale and Sjulin 1990). Somaclonal variations, like mutation breeding can be a valuable tool in exploiting random variation and somaclonal variation has been reported in strawberry (Biswas et al. 2009). The extent of somaclonal variation depends on the applications of plant growth regulator (PGR) (Anderson et al. 1982) and even applications of low concentrations of PGR have created variants in strawberry (Biswas et al. 2009).

There is no difference between artificially-induced and spontaneous mutants. Thus, mutation induction is a flexible, workable, unregulated, non-hazardous and low-cost alternative to genetically-modified organisms (GMOs) (Jain 2010).

It was anticipated that mutagens could enhance the genetic variation and change only one or a couple of definite traits of a selected cultivar to add value to a fruit improvement program (Predieri 2001). Mutagenesis has been applied in fruit crops to incorporate and establish many useful traits like plant size, blossoming, fruit ripening, fruit color, fruit quality, yield and resistance to different diseases (Kaushal et al. 2004). The incidence of variants depends on the physiology of genotypes and dosage of the mutagenic agent (Weimin et al. 2009). In strawberry, broad ranges (5–800 Gy) of gamma rays have been applied to different plant materials such as anther calli (Kasumi 2002), calli of leaves (Kaushal et al. 2004), axillary buds (Jain 1997), and runners (Weimin et al. 2009) to induce artificial mutation. Most received gamma rays for at a low concentration of PGR. Variants having dissimilar morphological characters were observed (Kaushal et al. 2004). For in vitro mutation induction in strawberry cv. Carmosa, the runner tips explants responded much better in comparison to shoot tips, leaf disc (abaxial) and leaf disc (adaxial). With the increase of EMS concentrations along with treatment duration there was a gradual decrease in the in vivo growth parameters of strawberry (Bhat et al. 2017).

6.7 Biotechnology

Increasing knowledge of plant genetics and, above all, improved methods of DNA analysis have led to an upgrade of existing techniques (such as crossbreeding) to arrive at marker-assisted selection. The genome sequence of *Fragaria vesca* was a scientific milestone, representing the first published plant genome obtained solely with next-generation sequencing technology. *Fragaria vesca* has several attributes that made it an attractive target for genome sequencing. The biological diversity of *Fragaria*, especially with respect to sexual system and polyploidy, also makes it a very attractive system for ecological and evolutionary genomics. The object of this review is to introduce *Fragaria* as a model genus and to provide a roadmap for future integrative research in these and other areas.

6.7.1 Somaclonal Variation

Tissue culture is particularly indispensable for the rapid multiplication of breeding material (Zimmerman 1981) and through micropropagation of strawberries (*Fragaria* × *ananassa*) a number of morphological aberrations can be observed as somaclonal variations which include leaf color variants and dwarf plants, among other (Morozova 2003). Callus derived strawberry plants differ considerably from typical runner plants with regard to several vegetative characters (Nehra et al. 1992). These variants create a problem for production of homogeneous, true-to-type plants. Nehra et al. (1992) noted that two cultivars of strawberry responded in different ways when they originated from callus-derived plantlets, but exhibited no sign of variation when derived from meristems or via direct leaf regeneration. A number of studies have revealed that modified characteristics are epigenetic and disappear with the passage of time (Koruza and Jeleska 1993). In vitro selection is one of the somaclonal variation methods. Its effectiveness and efficiency are due to its capacity to alter the plants to the preferred character, either by applying a selection agent on the culture media or by giving particular condition to change the somaclones with the required character. Induced mutation could boost genetic variation to the plant that ultimately could provide advantages and opportunities and furnish breeders with genetic material for plant selection.

A number of methods have been followed to determine the genetic reliability of in vitro produced progenies of strawberry. Consequently, a systematic evaluation of tissue culture derived plants becomes essential, especially for fruit species. Molecular marker tools have become capable of detecting somaclonal variation at an early stage of plant growth. These tools have become very favorable for the quick and precise identification of strawberry variants. Nevertheless, the morphological and cytological assays should remain as the key and indispensable methods for the prolonged success of fidelity tests linked with production of clonal plants (Krishna et al. 2016).

6.7.1.1 Application of Somaclonal Variation

Somaclonal variation is used to describe the variation seen in plants produced by plant tissue culture. Somaclonal variation may be used as a source of variation to obtain superior clones. In addition to the variants/mutants (cell lines and plants) obtained as a result of the application of a selective agent in the presence or absence of a mutagen, many variants have been obtained through the tissue culture cycle itself (Yusnita et al. 2005). These somaclonal variants, which depend on the natural variation in a population of cells, may be genetic or epigenetic, and are usually observed in the regenerated plantlets.

Somaclonal variation itself does not appear to be a simple phenomenon, and may reflect pre-existing cellular genetic differences or tissue culture-induced variability. The variation may be generated through several types of nuclear chromosomal rearrangements and losses, gene amplification or de-amplification: non-reciprocal mitotic recombination events, transposable element activation, apparent point mutations or re-activation of silent genes in multi-gene families, as well as alternations in maternally-inherited characteristics.

Many of the changes observed in plants regenerated in vitro have potential agricultural and horticultural significance (Sahijram et al. 2003). These include alterations in plant pigmentation, seed yield, plant vigor and size, leaf and flower morphology, essential oils, fruit solids, and disease tolerance or resistance. The same types of variation obtained from somatic cells and protoplasts can also be obtained from gamete tissue. One of the most important prospective sides of somaclonal variation is the formation of auxiliary genetic variability in agronomically-useful cultivars, without the need to undertake hybridization which is comparatively time consuming. This technique could be precious if the identification is possible in vitro followed by micropropagation. Somaclonal variants can be improved for a few characters, while in vitro culture is important for resistance to biotic and abiotic stresses (Karp 1992).

Although somaclonal variation can be used as a source for variation to obtain superior clones, it can be also a very serious problem in the plant tissue culture industry resulting in the production of undesirable plant off-types. RAPD approach is suitable, speedy and reproducible to identify the presence of genetic variation associated with tissue culture of strawberry (Mohamed 2007). Somaclones may themselves have numerous applications in plant breeding and genetic improvements and recovery of such novel variants can be enhanced by applying suitable in vitro selection pressure (Krishna et al. 2016; Lestari 2006).

6.7.1.2 Callogenesis

Plant tissue cultures isolated from even a single cell can show a variation after repeated subculture. Distinct lines can be selected with their own particular morphology and physiology. This suggests that the tissue culture contains a population of genotypes whose proportion can be altered by imposing an appropriate selection pressure. This variation can be transmitted to plants regenerated from the tissue cul-

tures. It provides an additional source of novel variation for exploitation by plant breeders. Callus-derived plants usually show signs of great genetic variability in agronomic traits that is known as somaclonal variation (Larkin and Scowcroft 1981). Later it became known that a wide array of alterations in nuclear and cytoplasmic genetic elements contributed to the observed phenotypic variation, and that many of them were of epigenetic nature. Somaclonal variation can expand the genetic deviation in strawberry such as in plant height, yield, and number of flower per plant, early flowering, grain quality, and resistance to diseases, insects, cold, drought and salt (Patnaik et al. 1999). These somaclonal variations have been successfully employed in strawberry improvement.

A reproducible protocol for the regeneration of complete plantlets from callus cultures using leaf and petiole explants was standardized for strawberry cv. Chandler (Kaushal et al. 2004). Tissue-culture induced (somaclonal) variation generated strawberry germplasm such as cvs. Chandler, Delmarvel, Honeoye, Latestar, Pelican and Sweet Charlie, to produce somaclones with increased levels of resistance to anthracnose (caused by the fungus *Colletotrichum acuatatum*). The greatest increases in disease resistance were observed for somaclones of cv. Pelican and Chandler exhibiting 17.5-fold and 6.2-fold increases in resistance, respectively. The highest levels of anthracnose resistance (2–6% leaf necrosis) were exhibited by somaclones of cv. Pelican and Sweet Charlie (Hammerschlag 2006).

Rejuvenations of strawberry plants via callus culture from explants have been investigated by a number of scientists. Leaf and petiole tissue has been studied and shown to have the significant regeneration capacity of strawberry plants (Passey et al. 2003; Popescu et al. 1997). Nehra and Stushnoff (1989) found that leaf disks from immature plants had a higher regeneration rate than from adult plants (James et al. 1990). Passey et al. (2003) considered seven commercial cultivars of strawberry using leaf disks, petioles, roots and stipules as explants materials in one of their tissue culture based research. Successful procedures were to establish and grow runner tips in vitro and sub-culture them every 3 weeks until enough material was produced to begin the experiments. Leaf disks showed the highest regeneration rates for all cultivars with greater than 90% of explants regenerating shoots. A research team at department of Botany, University of Rajshahi, Bangladesh also investigated seven strawberry genotypes to evaluate somaclonal variations through callus culture (unpublished data). They found that, cv. Giant mountain (Fig. 6.3g) and Sweet Charlie (Fig. 6.3h) performed better in the field for ex vitro establishment as compared to others tested where bushy canopies were observed (Fig. 6.3b, d). The characteristics of better performances were perpetuated and reflected in fruits also in cv. Giant Mountain and Sweet Charlie. These cultivars yielded well-shaped and colorful fruits (Fig. 6.4a, b, d, e, f). However, the remaining cultivars developed fruits but they were deformed and unattractive as a result of somaclonal variation (Fig. 6.4c, g, h, i, j).

Throughout the micropropagation of strawberries (*Fragaria* × *ananassa*), quite a few morphological aberrations were detected as somaclonal variations. Changes included leaf color variants and dwarf plants, among others (Morozova 2003). Genetic integrity in plants regenerated by organogenesis from callus culture of strawberry has also been reported (Anuradha et al. 2017).

Fig. 6.3 Strawberry genotypes showing normal plants exhibiting better canopy and leaf morphology (**a**, **c**, **e**, **f**, **g**, **h**) as well as abnormal growths, due to somaclonal variation, developing bushy appearance (**b**, **d**)

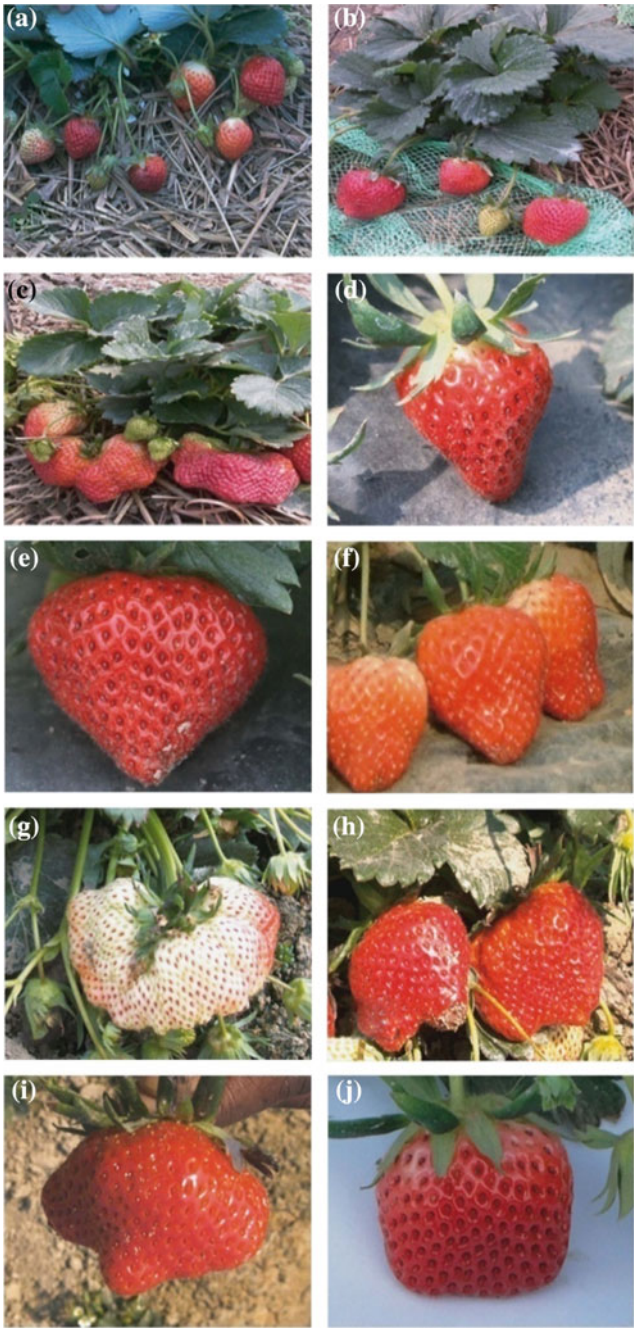

Fig. 6.4 Somaclonal variation in fruit morphology. Normal shaped fruits (**a**, **b**, **d**, **e**, **f**) and abnormal shaped (somaclonal variation) are distinctly visible (**c**, **g**, **h**, **i**, **j**)

6.7.1.3 Evaluation of Variants to Biotic and Abiotic Stress Condition

Abiotic stress is defined as the negative impact of non-living factors on organisms in a specific environment. Abiotic stress is essentially unavoidable. The non-living variable must influence the environment beyond its normal range of variation to adversely affect the population performance or individual physiology of the organism in a significant way. Bio-stimulants can defend strawberry plants against external adverse factors and control specifically the plant growth, development and metabolism followed by the flower induction and formation of quality fruit.

Foliar treatment with a complex mineral fertilizer and growth stimulants at different phases of strawberry cultivation enhances the plant tolerance to weather stresses due to activating defense mechanisms (Prichko et al. 2014). Abiotic stress is one of the most detrimental factors in relation to the growth and yield of all kinds of crops (Hall and Jin 2017).

6.7.1.4 Biotic Stress

Harmful living organisms are responsible for the biotic stress causing damage to strawberry plants. Biotic stress occurs as a result of damage to strawberry plants by different organisms, such as bacteria, viruses, fungi, parasites, insects and weeds.

Strawberries are attacked by numerous diseases and pests that vary widely in their destructiveness and distribution. The following diseases and pests affect the strawberry:

(a) ***Foliage disease***

Powdery mildew (*Sphaerotheca humuli* and *Phytophthora parasitica*). Leaf spot (*Alternaria alternate* sp. *Fragariae*, *Colletotrichum fragariae*). Slime molds (*Selerotinia sclerotiorum, Pythium* sp.).

(b) ***Rootand crown diseases***

Red stele (*Phytophthora fragariae*), Verticillium wilt (*Verticillium albo-atrum*, *V. dahliae*), Black root rot (*Rhizoctonia fragariae, Ceratobasidium, Pythium* spp.), Nematodes.

(c) ***Fruit diseases***

Gray mold (*Botrytis cinerae, Botryotinia fuckelian*). Rhizopus rots (*Rhizopus stolonifer*). Leather rots (*Phytophthora parasitica, P. citricola*).

(d) ***Phytophthora crown rot***

Phytophthora cactorum produces a kind of leathery rot on the surface of the fruits, crown rot and wilting of plants. Leather rot evidence on green fruit is distinguished by dark brown contaminated areas or natural green outlined with a brown margin; the entire berry ultimately turns brown, with a rough surface and is leathery in appearance. Infection of mature fruit may cause a slight change in color or can cause

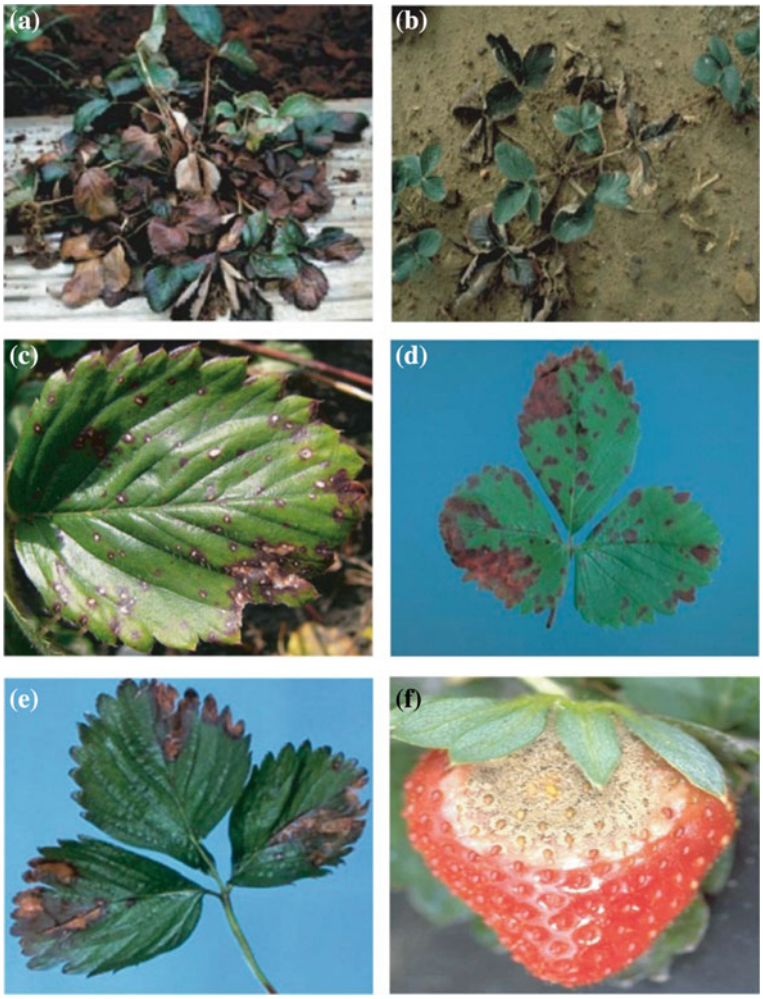

Fig. 6.5 **a** *Phytophthora* crown rots disease of strawberry. **b** Strawberry plant dying from *Verticillium* wilt. **c** Strawberry leaf spot symptoms on leaflet. **d** Leaf scotch on strawberry. **e** *Phomopsis* leaf blight on strawberry. **f** *Botrytis* fruit rot on mature strawberry fruit

discoloration, making the fruit dull. The initial crowns are brown with crumbled vascular tissue (Fig. 6.5a).

(e) ***Verticillium* wilt**

Verticillium wilt, caused by the soil-borne fungus *Verticillium alboatrum* can be a major factor limiting production. The first symptoms in new strawberry plantings often appear about the time runners begin to form. When a plant is severely infected, the probability of it surviving to produce a crop is greatly reduced. On infected

strawberry plants, the outer and older leaves drop, wilt, turn dry, and become reddish-yellow or dark brown at the margins and between the veins (Fig. 6.5b).

(f) **Leaf spot**

Leaf spot is caused by the fungus *Mycosphaerella fragariae*. The fungus overwinters in purple spots on infected plants. This disease first appears on leaves as purplish spots that resemble leaf scorch. Later these spots enlarge to a diameter of 3–6 mm. The centers become gray or white with reddish to purplish borders. Leaf petioles or stalks, stolons, runners and, to some extent, calyxes (caps or hulls) may be attacked (Fig. 6.5c).

(g) **Leaf scorch**

Leaf scorch is caused by the fungus *Diplocarpon earliana*. It can transmit this disease to leaves, petioles, runners, fruit stalks and caps of strawberry plants by primary and secondary infections. Leaf scorch symptoms are very similar to the early stages of leaf spot. Round to angular or uneven dark-purple spots up to 6.4 mm in diameter are scattered over the upper leaf surface (Fig. 6.5d). As spots expand, they resemble small drops of tar. The centers of the spots remain dark purple. This differentiates the disease from leaf spot where the center turns off-white. If several infections occur on the same leaf, the entire leaf becomes reddish or light purple.

(h) ***Phomopsis* leaf blight**

Leaf blight is caused by the fungus *Phomopsis obscurans.* Leaf blight is found most commonly on plants after harvest. The disease is distinctively different from either leaf spot or leaf scorch. The enlarging leaf spots of this disease are round to elliptical or angular and 6.4 mm in diameter (Fig. 6.5e). Spots are initially reddish-purple. Later, they develop a darker brown or reddish brown center surrounded by a light brown area with a purple border.

(i) ***Botrytis* Fruit Rot** (Gray mold)

One of the most serious and common fruit rot diseases of strawberry is gray mold. It is caused by the fungus *Botrytis cinerea*. The disease is most severe during prolonged rainy and cloudy periods during bloom and harvest. Abundant gray brown, fluffy, fungal growth on infected tissue gives the disease its name. Fruit infections usually appear as soft, light brown, rapidly enlarging areas on the fruit (Fig. 6.5f). Disease incidence percentage of seven strawberry varieties and their somaclones are presented in Table 6.5.

Verticillium wilt, *Phytophthora* crown rot, leaf scotch, leaf spot, leaf blight and botrytis fruit rot disease are more or less common in strawberry fields. Among the six strawberry diseases, disease incidence percentages *Verticillium* wilt and *Phytophthora* crown rot is high (60%) in seven donor parent's viz. AOG, JP-2, JP-3, Camarosa, Sweet Charlie, Giant Mountain and Festival but in somaclones the incidence was 15%. In other diseases viz. leaf scotch, leaf spot, leaf blight and botrytis fruit rot, disease incidence was high (45–50%) in donor plants but in their somaclones

Table 6.5 Disease incidence (%) of seven strawberry cultivars and their somaclones. Data recorded 90–120 days after plantation in the field

Cultivars/Somaclones	Disease incidence (%)					
	Verticillium wilt	*Phytophthora* crown rot	Leaf scorch	Leaf spot	Leaf blight	*Botrytis* fruit rot
AOG	60	60	50	50	50	45
AOG SC 1	15	10	10	10	10	10
AOG SC 2	15	15	10	10	10	10
AOG SC 3	10	10	00	00	00	00
AOG SC 4	15	15	10	10	10	10
JP-2	60	60	50	50	50	50
JP-2 SC 1	15	15	10	10	10	10
JP-2 SC 2	15	15	10	10	10	10
JP-3	60	60	50	50	50	50
JP-3 SC 1	15	15	10	10	10	10
JP-3 SC 2	15	15	10	10	10	10
Camarosa	60	60	50	50	50	50
Camarosa SC 1	15	15	10	10	10	10
Camarosa SC 2	15	15	10	10	10	10
Sweet Charlie	60	60	50	50	50	50
Sweet Charlie SC 1	15	15	10	10	10	10
Sweet Charlie SC 2	15	15	10	10	10	10
Giant Mountain	60	60	50	50	50	50
Giant Mountain SC 1	15	15	10	10	10	10
Giant Mountain SC 2	15	15	10	10	10	10
Festival	60	60	50	50	50	50
Festival SC 1	15	15	10	10	10	10
Festival SC 2	15	10	10	10	10	10

it was low (10%). Most of the plants were severally affected with these diseases during the summer months and perished. There were no plants found resistant to fungal diseases (Fig. 6.5a–f).

6.7.1.5 Abiotic Factors

Abiotic stresses play a vital role in controlling growth, development and fruiting of strawberry by interacting with the physiological system of the plant.

Hot, dry weather (>38 °C) and soil pH are major abiotic factor affecting strawberry cultivation all over the world, especially in tropical and subtropical countries. During the summer months April–May temperatures become elevated (above 38 °C) and the plants fail to thrive in the field. The knowledge and experience of growing factors

that affect the generative and vegetative behavior of the strawberry plants is therefore essential to improve the yield and fruit quality by anticipating or delaying flower induction and determining inflorescence numbers and dimensions (Massetani et al. 2011).

6.7.1.6 Somaclonal Variation Potential

Somaclonal variation describes the variation observed in plants that have been produced by tissue culture. It can broaden the genetic variation in strawberry plants; a number of traits can be altered, such as plant height, yield, and number of flower per plant, early flowering and fruit quality and resistant to biotic and abiotic stresses. A single cell can demonstrate a variation after repeated subculture in tissue-culture media. Specific lines can be chosen with their own particular morphology and physiology. Tissue cultures contain a population of genotypes whose proportion can be altered by imposing appropriate selection pressure. This variation can be passed on to plants regenerated from the tissue cultures. It provides an extra source of new genotypes for the plant breeders for further genetic improvement.

This technology is very conducive to the improvement of horticultural crops, because somaclonal variations do not require much space and time for the viewing of desirable traits (Krishna et al. 2016). Rather it provides a comprehensive tool for breeders to obtain genetic variability in a short time for the strawberry which would be difficult through conventional breeding. Nevertheless, there are disadvantages of somaclonal variation which make it reasonably difficult to implement in many cases. Although this technology can bring about some changes in the offspring, it is still not possible to forecast the outcome of a somaclonal program as it is random and lacks reproducibility (Karp 1992). Somaclonal variations can become a component of plant breeding provided they are genetically transmissible and stable. A restricted number of promising varieties so far have been released using somaclonal variations. The revival of somaclones can be amplified by blending micropropagation with induced mutagenesis in vitro (Afrasiab and Iqbal 2010).

Somaclonal variation is used to clarify the deviation observed in progenies that have been produced by tissue culture and may be a source of variation to obtain superior clones. Molecular-marker techniques are very conducive to the identification of somaclonal variants and ensure quality control of the micropropagated plants (Debnath et al. 2007). Although variants could be obtained through the application of mutagenesis, a number of variants may also be achieved through the tissue culture cycles. These somaclonal variants may be genetic or epigenetic and are usually observed in regenerated plantlets (Krishna et al. 2016).

6.7.2 Genetic Engineering

In strawberry, the first genetic alteration procedures were standardized in the early 1990s and the technologies have developed tremendously over the last decade. In principle, genetic modification reveals a comparatively quick improvement of existing elite strawberry cultivars, for example, by the insertion of disease-resistance genes. The implementation of plant tissue culture and genetic engineering are potentially significant for genetic improvement of the strawberry. Octoploid strawberry cultivars are extremely unpredictable from genotype to genotype as far as the gene transformation and regeneration abilities are concerned. Moreover, phenotypic traits of different genotypes are variable depending on cultivation practices and more especially on agro climatic conditions.

The responses to factors influencing genotype-specific rejuvenation create consistency of an efficient regeneration method for each strawberry genotype, a necessary precondition for successful development of transgenic plants using *Agrobacterium*-mediated transformation. The complicated genetic structure of the strawberry genome has somewhat shackled traditional plant breeders investigating the intrinsic DNA structure, thus making a sort of barrier for the genetic improvement of this fruit. Nevertheless, a series of comprehensive research studies are essential to emphasize different stages of fruit growth and development, the effect of geographical site, ecological conditions and varying agronomic practices to acquire crystal-clear information of the secondary metabolites content of the fruits and other genetic and epigenetic changes for molecular research.

Generally biotic and abiotic stresses influence strawberry production. The trouble-free growing system of strawberry and its compatibility to *Agrobacterium* infection make strawberry quite appropriate for *Agrobacterium*-mediated genetic transformation. This permits insertion of a novel gene(s) with new preferred traits into strawberry plants. Exploitations of in vitro techniques and molecular biology could be applied to eliminate the inadequate prospective of strawberry improvisation through conventional breeding. Introductions of novel traits into selected plants can be accomplished by clonal multiplication, germplasm improvement and gene conservation of strawberry which would improvise elite genotypes in a short span of time. An introduction of molecular markers facilitates the production of genetically-engineered plants of desired traits. Establishment of a competent rejuvenation system for each strawberry cultivar is an indispensable prerequisite for the successful development of transgenic plants. Strawberry genetic transformation has made significant progress in launching gene banks. Selection and transformation of desired genes would help breeders screen genotypes for resistance to different biotic and abiotic stresses under different climatic conditions.

The complicated genetic constitution of the strawberry genome has kept most researchers from investing in the development of methods that could improve breeding of strawberry. Only a few years ago, the first results towards the production of a genetic map for strawberry were published (Haymes et al. 2000), opening up possibilities for molecular marker-assisted breeding.

In strawberry, the first genetic modification protocols were developed in the early 1990s (James et al. 1990; Nehra et al. 1990, 1992) and this approach has gained increasing attention over the last decade (Debnath et al. 2008). In strawberry, certain genes are responsible for creating valuable compounds for human consumption. For example, strawberry (*Fragaria* × *ananassa*) is rich in flavonoids and proanthocyanins. These compounds are beneficial to human health. The anthocyanidin reductase (ANR) is the particular gene within the flavonoid which diverts the anthocyanin pathway to flavonol synthesis (Mandave et al. 2017).

In the eighteenth century the widely cultivated strawberry (*Fragaria* × *ananassa*) was developed in Europe through hybridization (Luby et al.1992; Salamone et al. 2013), and provide sample opportunity to study the genetic relationship of the domesticated strawberry in different ecological niches. The sequencing and annotation of a genome offers access to the genes, their recognized functions, and their genomic positions, as well as a reference that can be used to evaluate nucleotide variation and gene expression across the individuals and conditions (Bevan and Uauy 2013). The genome sequence of *F. vesca* was a scientific milestone, representing the first available plant genome (Shulaev et al. 2011). A proficient transformation system for *F. vesca* has been devised (Oosumi et al. 2006) and transposon-tagged mutants are obtainable for reverse genetics applications (Veilleux et al. 2012). The genome chain of *F. vesca* has already contributed to in functional genomics of flowering (Hollender et al. 2014; Mouhu et al. 2013) and fruiting (Kang et al. 2013), and these achievements will eventually be applied to the genetic improvement of crops (Folta 2013; Longhi et al. 2014). More immediate progress is expected from the vast numbers of genetic markers developed from the genome sequence of *F. vesca* that can be used in genetic-linkage mapping (Tennessen et al. 2013) and high-throughput assays for marker-assisted breeding (Chambers et al. 2013). Accordingly, a kind of *cross-fertilization* can occur with the development of genomic resources across the Rosaceae family (Longhi et al. 2014; Shulaev et al. 2008). The small genome size of *Fragaria* and the availability of a reference sequence also facilitate comparative genomics, and a relevant research has included whole genome sequencing of the cultivated strawberry and four wild relatives (Hirakawa et al. 2014). The biological diversity of wild *Fragaria* has been regularly used in breeding efforts as a source of novel genetic variation that can be introgressed into the cultivated strawberry (Chambers et al. 2013; Hancock 1999).

Genotyping-by-sequencing (GBS) was applied to review genome-wide single-nucleotide polymorphisms (SNPs) in three bi-parental strawberry (*Fragaria* × *ananassa*) populations (Kelly et al. 2017). *Fragaria vesca* has numerous characteristics that make it an important objective for genome sequencing.

DNA marker technology in breeding is being applied to improve the genetic structure of strawberry. The scientists are endeavoring to develop techniques to alter genes in strawberry by the recent advanced technology of gene-editing. This technology will be advantageous to develop elite varieties in a quick span of time without performing traditional breeding thus releasing resistant strawberry varieties suitable to cultivation that have better qualities and medicinal values for the consumers (Lee et al. 2016).

6.7.2.1 Application of Molecular Markers

One of the most vital and promising tools for strawberry research is that of molecular markers. The advance and application of DNA markers and biotechnology in strawberry has been addressed by several researchers (Hokanson and Maas 2001; Sargent et al. 2009). Research in this area is moving ahead rapidly and markers are being used in strawberry for diversity and taxonomic categorization, scholar assets security, mapping and genome analyses, and marker-assisted breeding. The ever-bearing trait of strawberry is regulated by a single dominant gene. The locus regulating the ever-bearing or June-bearing flowering habit is the same in long-day and day-neutral types (Honjo et al. 2016).

6.7.2.2 Genetic Assortment

The assessment of cultivars through genetic characters can directly influence the superiority and yield of strawberry plants in the field. The application of inter simple sequence repeat (ISSR) markers to assess strawberry genotypes generates a series of scientific information on genetic polymorphisms. For genetic improvement of strawberries, molecular markers have been very useful. To follow the clonal reliability of micro-propagated strawberry plants Debnath (2009) used ISSR markers. For the assessment of strawberry genotypes, the use of ISSR markers has been found to be the most accurate and successful, contributing a great deal of data on genetic polymorphism.

Arnau et al. (2002) demonstrated that the ISSR technique needs only a few markers to attain the correct fingerprinting pattern of strawberry cultivars, signifying the prospective of this technique for genetic research. The studies carried out by Kuras et al. (2004) and Debnath et al. (2008), with the goals of evaluating the genetic similarity and genetic diversity, respectively, of strawberry cultivars confirmed the technical feasibility of ISSR markers.

The ISSR markers help detect genetic variability among strawberry genotypes. These genetic diverse genotypes may be useful to breeders for the selection of new varieties with improved yield. The strawberry has a reasonable level of genetic variability, which can be exploited by breeders to develop elite genotypes adaptable to different climatic conditions. Recent research to expand collection of elite genotypes is to incorporate the genes responsible for the expression of adjusted characters; for example, resistance to biotic and abiotic stresses and adaptation to various conditions of nutrition. In modern molecular breeding, emphasis should be given to develop improved genotypes with the characters responsible for high yield and qualitative fruit traits (Kulikov and Marchenko 2017).

Molecular marker statistics can make it possible to decide appropriate sampling methods for collection, and ensuring that adequate genetic diversity is present to conserve desirable traits. This is essential; as it has been established that genetic structures of *Fragaria virginiana* populations can differ entirely due to spatial difference in clonal vs. sexual reproduction (Wilk et al. 2009). Molecular marker techniques, as

well as ISSR markers, are conducive in the varietal identification of strawberry genotypes preferred for breeding and simultaneously be applied to ascertain the genetic reliability of the stages throughout the clonal multiplication of genotypes. With the knowledge of molecular genetics, scientists have succeeded in characterizing the perpetual flowering pattern and identified new genetic controls of this pattern in the cultivated strawberry (Perrotte et al. 2016).

6.7.2.3 Cultivar Identification

Currently the major and most prevalent use of molecular markers in strawberry is to discover genotypes. This is principally important for preventing erroneous identification of varieties during nursery propagation, clearly identifying accessions in germplasm, and the security of intellectual property (IP) by strawberry breeders. Primarily, random marker systems such as random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) are used for this purpose. Simple sequence repeat (SSR) markers as well cleaved amplified polymorphic sequence (CAPS) markers and their derivatives were later developed from DNA sequence information. Over time, SSR markers have become the favored and convenient marker type for genotype identification in the research, academic and private sectors. Random marker systems have all been used to differentiate among genotypes (Kuras et al. 2004). Gene pair markers could be considered a type of CAPS marker that targets regions between genes of a known sequence. The technique was demonstrated in diploid *Fragaria* by designing primer pairs from exons of the adjacent CDPK1 and BHLH1 genes (Davis et al. 2008). There is also potential for use in the octoploid strawberry by identifying multiple polymorphisms in each intergenic region that may be associated into haplo-types. The near-isogenic line (NIL) compilation of *F. vesca* would make easier to read the genetics of many traits and offer insight into the more complex *F.* × *ananassa* genome. Agronomically-interesting traits such as flowering, germination, fruit size and shape, and nutritional content are generally inherited by progenies quantitatively and they need to be studied utilizing molecular markers (Urrutia et al. 2015).

6.7.2.4 Linkage Mapping

Linkage maps provide a combined structure for the genetic account of a species. It is conducive to theoretical and applied plant genetic research. The strong connection among the molecular markers and loci controlling character of beneficial interest offers a foundation for marker-assisted selection (Stuber 1992) and for map-based positional cloning (Wing et al. 1994). Comparative mapping can be engaged into identify linkage group and hence chromosome rearrangement among related taxa (Moore et al. 1993).

Linkage mapping in strawberry has been much more than an intellectual exercise. Research on genetic mapping has raised significant questions about the configuration

and performance of diploid and octoploid strawberry genomes and their performance in the improvement of many useful markers for other applications. The first available linkage map in strawberry was constructed using an F2 population from an intra-specific cross of diploid *Fragaria vesca.* The projected seven linkage groups were determined using RAPD markers (Davis and Yu 1997). A unique feature of this initial map was that it contained a large number of co-dominant RAPD markers. The first linkage map in cultivated strawberry was developed by Lerceteau-Kohler et al. (2003) by analyzing single-dose markers in a population of different pedigree. In addition, a number of single-dose markers are obtainable to establish individual sub-genomes, to serve in finding the allelic prosperity of the octoploid genotypes. It is anticipated that these mapping resources will ultimately result in markers linked to traits of interest (Tennessen et al. 2014).

6.7.2.5 Markers Linked to Traits of Interest

Strawberry is considered a valued horticultural crop for fundamental genomics and recombinant DNA studies because of its fast growth and small genome compared to other woody species of the same family. A marker link is an alternative efficient strategy to implement strawberry improvement. In order for markers to be workable for genetic upgrading and marker linkages to important characters, whether major genes or quantitative trait loci (QTL) must be established. In strawberry, a number of such links have been identified for traits such as resistance to biotic and abiotic stresses, photoperiodic flowering, and male and female infertility. These markers have been originated and improvised by means of a variety of methods, including conventional mapping, bulked-segregant analysis (BSA), and candidate gene approaches (Hancock et al. 2016).

6.7.2.6 Marker-Assisted Breeding

Marker-assisted selection (MAS), also called marker-assisted breeding (MAB), is a combined product of traditional genetics and molecular biology. MAS allows for the selection of genes that control traits of interest. Combined with traditional selection techniques, MAS has become a valuable tool in selecting organisms for traits of interest, such as fruit color, meat quality and disease resistance. In traditional breeding, individual plants expressing desired fresh traits, such as sweeter strawberries are selected from crosses of a wide range of strawberries and simple traits such as sugar content or size can easily be measured; on the other hand, more complex traits such as disease or drought resistance become more problematic for breeders to identify when choosing individuals expressing those traits from among a large group of plants. MAS can avoid this problem by using genetic markers that are linked to the desired trait.

MAS could be more resourceful, valuable and dependable than phenotypic selection. In addition, MAS can cut down the varietal improvement time significantly

and in some cases it will be more cost effective than selection based on phenotypes. It is very conducive for the genetics and breeding of complex traits not practicable through previous traditional methods. It is a promising approach to traditional plant breeding. MAS can be more efficient, effective and reliable than phenotypic selection.

Methods based on knowledge provided by advances in molecular genetics, notably molecular markers, promise faster and more efficient approaches to strawberry cultivar improvement (Rai and Shekhawat 2014). Early selection with molecular markers permits a precise screening of offspring many years before the traits can be assessed in the field and it makes possible the building of a number of resistance features in an important genotype, or reduce the number of generations required to retrieve the genotypes of the cultivated strawberry after hybridizing with either foreign genotypes or uncultivated genotypes (Gezan et al. 2017).

MAS methods could be applicable to strawberry breeding programs while maintaining the following conditions: (A) the trait (s) is purely inherited in the offspring; (B) the trait is expressed in the mature phase of long-lasting species with lengthy juvenile periods; (C) MAS is accomplished very early in the immature and infantile stage; (D) the trait(s) needs more additional expenses for traditional screening in the target population of different areas, environment and climatic conditions; (E) locus detection and marker genotyping are cost-effective due to low-cost marker technology and strong marker-locus associations in a germplasm collection and lastly; and (F) MAS will present a significantly better chance of choosing better-quality individuals in comparison better traditional breeding and assessment practice.

MAS can be more efficient, effective and reliable than phenotypic selection. Furthermore, MAS can shorten the development time of varieties significantly and also allows the breeding of complex traits not feasible through previous conventional methods. Perpetual-flowering (PF) is an extremely advantageous characteristic within cultivated strawberries (*Fragaria* × *ananassa*) making this cultivar more valuable for commercial purpose. Recently, biotechnologists have successfully introgressed the PF trait from a wild *F. virginiana* ssp. *glauca* (Salinas et al. 2017).

Plant breeders mostly use MAS for the detection of appropriate dominant or recessive alleles across a generation and for the identification of the most encouraging individuals across the segregating offspring. It is very necessary to correlate genotype with phenotype more precisely. To assemble the correct information about the traits of interest, a high-throughput phenotyping platform (HTPP) is being applied using molecular markers (Nadeem et al. 2017).

6.7.3 Gene Banks

The ideal procedures and management to standardize strawberry gene banks are complicated and require experience, and knowledge of botany and climate. Elite genotypes must be maintained vegetatively for clonal selection and the species diversity also should be represented through seed and pollen storage. Although the conserva-

tion of strawberry plants in the field is not difficult, ex vitro genotype banks run the risk of attack by viruses, bacteria, fungi and insects. Strategies for field collections of plant germplasm have been developed by IPGRI, now known as Bioversity International (Reed 2007). A few countries (e.g. Denmark) conserve mother stocks of strawberries in the field, protected by insect-proof netting. On the other hand, Chile, Canada, France, Germany and the United States maintain mother germplasm in small earthen or plastic pots under greenhouse conditions to protect the plants from insects and pests. Greenhouses constructed of glass, polycarbonate or insect-proof screen are capable of protecting the plants from biotic stresses. A primary clonal collection of strawberries is the foundation of a gene bank and should be preserved for future breeding and genetic engineering research activities.

Ex vitro strawberry germplasm is resource consuming and labor intensive as well. To conserve genotypic uniqueness, runners from adjacent containers or plots must be prohibited or trimmed and flowers and fruit must be roughed out to prevent growing unwanted seedlings. Care should be taken during cultural activities such as watering, fertilizing, repotting, and pruning. Mother stocks of strawberry should allow growing in cross ventilated place to prevent foliar fungal diseases. Soil disinfection is also important to protect the plants from soil borne diseases. Pathogen testing based on Enzyme-Linked Immunosorbent Assay (ELISA) must be done regularly to ensure the plants are free from systemic pathogens especially viruses. Intense vigilance is necessary to prevent insect vectors from entering the area. Recording morphological data of the plants is also important for future breeding program.

Germplasm conservation through seed preservation is another natural, cost- effective method, but in this case storage facilities, temperature, humidity and other microclimatic conditions have to be maintained to ensure seed viability. For most seeds, viability is maintained at −20 °C. Even seed which has totally lost viability can be a valuable genetic (DNA) resource.

Germplasm maintenance through alternative storage techniques such as in vitro culture or cryogenic preservation of meristems are also components of a gene bank. Genetic resources can stored and kept disease-free in a tissue culture laboratory for 3–5 years, while meristems maintained cryogenically can survive for decades. Cryopreservation techniques (such as slow cooling, vitrification or encapsulation of meristematic tissue) are applicable for strawberries with good success rates have been successfully developed. Presently, more than 12,000 accessions are being maintained in different gene banks in about 30 countries. A great number of these represent highly-developed breeding lines of the cultivated hybrid strawberry, *Fragaria* × *ananassa*.

A steady refinement of gene banks should be ensured by government or donor agencies. If gene banks are not supported and sustained, their expansion will be stagnated. Skilled, well-trained personnel, modern equipment, adequate storage facilities and financial backup are the fundamentals of a scientific gene bank. Coordination among the proprietors/investigators of gene banks, preservation of data electronically, exchange of experience through websites is very conducive to maintaining a healthy strawberry gene bank. The primary accessions in gene banks consist of living plants grown in containers within greenhouses or in the insect-protected fields. Secondar-

ily, germplasm is maintained in vitro under refrigerated temperatures and in growth chambers of a tissue-culture laboratory. In long-term preservation, meristematic tissues or living plant parts are placed in cryogenic storage for decades. Ex vitro gene bank development is also possible by preserving the seeds at −18 °C or backed up in cryogenics. Maintenance of genetic resources by vegetatively propagated material is more complicated and expensive as compared to seed preservation technologies. The physiological and rejuvenation stage of in vitro or ex vitro germplasm is a vital platform for the biotechnologists and indispensable to the meet plant quarantine conventions.

6.8 Conclusions and Prospects

The strawberry is a delicious and very popular berry fruit in the world because of its sweet-sour taste, pleasant flavor, vivid color and soft texture. Although it is a commercial fruit of temperate areas, with the introduction of day-neutral varieties, its farming and demand have expanded into tropical and subtropical regions. Strawberries are also an excellent source of vitamins, minerals, dietary fiber, iodine, omega-3 fatty acids, Anthocyanins and a rich source of phenols. Although the secondary metabolites of strawberries are substantial, they are unstable from organ to organ and even from cell to cell of the plants and fruits. On-going research is required to understand the role of these metabolite assortments in plant growth and in human health improvement.

In spite of these valuable ingredients in strawberries, there are certain unusual problems associated with this fascinating fruit. Short shelf-life, delicate nature, susceptibility to rot and sensitivity to salinity stress have made this fruit quite vulnerable to different stressful situations. Genetic limitations associated with high heterozygosity and different polyploidy levels represent a biological barrier to the improvement strawberry. Poor cultivation and management practices such as harvesting the fruits before full ripening, the short postharvest life or the extensive use of hazardous fumigants and pesticides to control diseases and pests are among the major troubles of this crop.

To refrain from residual effects of chemicals in strawberry, researchers should strive for organic agriculture and biotechnology to produce elite varieties of quality traits. Under increasing global warming conditions, the importance of growing strawberries needs to be addressed in parallel with projected changes. This depends on generating premium varieties suited to different growing conditions and/or cultivation systems all over the world.

Consumers are now aware of the fact that, consumption of strawberries is beneficial to health if they are properly grown. Thus, the nutritional quality of strawberry should be of concern to plant breeders and biotechnologists; endeavoring to produce novel high-yielding varieties with improved nutritional quality which improve the defense mechanism in the human body. The development of efficient molecular markers for accurate prediction of desired aroma compounds would greatly accel-

erate the selection of new cultivars with improved characteristics. The execution of biotechnological practices within traditional breeding programs can facilitate reaching these objectives and will help to overcome existing obstacles. At present, the significant use of molecular markers in strawberry research is in cultivar classification, which has become a vital means for the nursery industries, germplasm curators and breeders for IPM protection.

One goal of strawberry breeders, geneticists and molecular biologists should be to create an information highway between researchers and farmers/producers to generate tangible genetic improvement and ultimately to produce an improved fruit for the human consumption, only then we can achieve the desideratum in the near future. Reviewing the increasing global warming and greenhouse effects, identification of genotypes resistant to biotic and abiotic stresses is vital for strawberry improvement programs through conventional breeding and biotechnology.

Appendix 1

Research Institutes concerned with strawberry

Institute	Research activities	Contact information
California Strawberry Commission	1. Weed Management 2. *Fusarium, Verticillium* and *Macrophomina*	California Strawberry Commission P.O. Box 269 Watsonville, CA 95077 Phone: (831) 724-1301 Fax: (831) 724-5973 www.calstrawberry.com
Davis Strawberry Breeding Research	'Setting the Benchmark' for Quality, Yield and Sustainability	University of California, Davis, One Shields Avenue, Davis, CA 95616|530-752-1011. https://www.ucdavis.edu
The James Hutton Institute	Working on soft fruit particularly strawberry and raspberries	The James Hutton Institute Craigiebuckler, Aberdeen, AB15 8QH, Scotland. Telephone +44(0)844 928 5428, http://www.fruitbreeding.co.uk/FruitVarieties
The Weizmann Institute of Science	Sequence the Full Woodland Strawberry Genome	http://www.eurekalert.org
International Strawberry Congress	Offers combination of commercial strategy and scientific research	2323 Hoogstraten,Belgium congress@hoogstraten.eu http://www.iscbelgium.com
Hoogstraten Research Centre	Storage of strawberry plants	http://www.hoogstraten.eu/en/fruit-vegetables/strawberries

Institute	Research activities	Contact information
Shanghai Strawberry Research Institute- Organic Strawberry	Organic strawberry production	http://www.ofrcc.com/c18/HTML/1014.html
Taiwan Agricultural Research Institute	TARI conducts research for horticulturecrops in breeding and biotechnology	189, Zhongzheng Rd., Wufeng Dist., Taichung City 41362, Taiwan https://www.tari.gov.tw/english/content/index
Bangladesh Agriculture Research Institute (BARI)	BARI conducts applied research for horticultural crops	Dhaka, Bangladesh www.bari.gov.bd
University of Guelph's Simcoe Research Station	Developing HYV of Strawberry through breeding research	185 Robinson St, Ste 200, Simcoe, ON N3Y 5L6 Canada, Phone: 519-426-9497 Email: business@norfolkcounty.ca http://www.norfolkbusiness.ca
National Agriculture and Food Research Organization (NARO)	High yielding Strawberry production technologies	E-mail q_info@ml.affrc.go.jp Fax. +81-96-249-1002 www.naro.affrc.go.jp/org/karc/Eng/index.htm
Centre for Environmental and Climate Research (CEC)	Pollination, Production and Strawberry Harvesting	Sölvegatan 37, 223 62 Lund, Sweden https://www.cec.lu.se/

Appendix 2

Genetic resources: Fruit uniqueness of elite strawberry cultivars

Cultivar	Hardiness	Season	Fruit size	Texture	Flavor
Early season					
Veestar	Very good	Early	Small	Soft	Excellent
Annapolis	Very good	Early	Med	Firm	Very good
Itasca	Very good	Early	Med	Mod soft	Good
Earliglow	Fair	Early	Small	Mod firm	Excellent
Evangeline	Good	Mid	Med	Mod firm	Very good
Sable	Very good	Early	Small-med	Soft	Excellent
Honeoye	Excellent	Early	Med	Soft	Good
Wendy	Good	Early	Med	Mod firm	Very good
Northeaster	Fair	Early	Small-med	Mod firm	Excellent

Cultivar	Hardiness	Season	Fruit size	Texture	Flavor
Mid Season					
Glooscap	Excellent	Mid	Med	Mod Firm	Good
Mesabi	Excellent	Mid	Med-large	Mod Firm	Very good
Cavendish	Very good	Mid	Large	Firm	Very good
Darselect	Good	Mid	Large	Mod firm	Very good
Kent	Very good	Mid	Med	Mod firm	Good
Brunswick	Good	Mid	Med-large	Firm	Very good
Mira	Fair	Mid	Med-large	Firm	Fair
Cabot	Fair	Mid-Late	Very large	Very firm	Fair
L'Amour	Good	Mid	Med-large	Firm	Very good
Redchief	Fair	Mid	Med	Firm	Good
Seneca	Good	Mid	Med	Firm	Good
Delmarvel	Fair	Mid	Med	Firm	Very good
Allstar	Fair	Mid	Med	Firm	Very good
Primetime	Fair	Mid	Med	Firm	Very good
Jewel	Fair	Mid-late	Large	Very firm	Excellent
Late season					
Winona	Very good	Late	Large	Mod Firm	Good
Clancy	Fair	Late	Med-Large	Very firm	Good
Bounty	Very good	Late	Med	Mod firm	Very good
Sparkle	Good	Late	Med	Mod firm	Very good
Latestar	Fair	Late	Med	Firm	Very good
Lateglow	Fair	Late	Med	Mod firm	Very good
Ovation	Fair	Late	Med-Large	Mod firm	Good
Valley Sunset	Good	Late	Large	Mod firm	Excellent

Source Regents of the University of Minnesota (2017)
http://fruit.cfans.umn.edu/strawberries/production/varieties/characteristics/

Appendix 3

Genetic resources of strawberry: Important traits of privileged cultivars

Cultivars	Season	Developed by	Released	Important traits
Albion	Day-neutral	University of California	2006	This strawberry plant is known for its large, conical, red fruit. Its flavor is very good, taste is sweet and pleasant. It is a high yielding cultivar with robust runners and stalks. It is resistant to *Verticillium* wilt, P*hytophthora* crown rot and has some resistance to anthracnose crown rot

Cultivars	Season	Developed by	Released	Important traits
Altess	Ever bearing	Flevo Berry Holding B.V.	2015	Variety which combines a good taste, yield and fruit size. It brings beautiful good tasting berries which maintain their gloss after storage. Plants are easy to grow and tolerate root and leaf diseases. The variety can stand flower thrips and botrytis relatively well. The variety can also stand some rain
Archer	Midseason	Cornell/NYSAES	2016	Very aromatic, delicious flavor; high yield; cold-hardy; Tolerant to root rots. Holds large fruit size through multiple harvests for 2–3 weeks. Fruit maximum size 45 g+
Camarosa	Early-season, short-day	University of California	1992	This is an early-season short day cultivar. Fruit is larger and firmer than Chandler, very flat conic, productive, has good appearance. Widely adapted producing fruit over an extended period at low latitudes
Chandler		University of California	1983	Strawberry plant is a high yielding variety, produces very large fruit, and appears to be well adapted to southern regions. It is adaptable to the eastern US, and in many different production systems including matted rows
Faith	Late	Flevo Berry Holding B.V.	2014	Faith is a late variety with beautiful, bright fruit and an excellent flavor
Favori	Ever bearing	Flevo Berry Holding B.V.	2013	The quality and yield of Favori are very good. Favori is a fast grower. The fruit are conical and elongated in shape and have the Elsanta color, which, just as the shine, is also preserved during storage
Flair	Early	Flevo Berry Holding B.V.	2008	Flair is a variety that comes into production quickly and gives a concentrated early harvest. Even under cold conditions, Flair continues growing and maintains its earliness. The excellent quality of the pollen ensures that the Flair flowers produce beautifully shaped fruit

Cultivars	Season	Developed by	Released	Important traits
Fleurette	Early	Flevo Berry Holding B.V.	2013	Fleurette is the variety which combines earliness, productivity and excellent fruit quality
Florentina	Ever bearing	Flevo Berry Holding B.V.	2011	Florentina is an ever bearing variety with excellent characteristics. The fruit are firm in texture, conical in shape
Florina	Ever bearing	Flevo Berry Holding B.V.	2010	It is an ever bearer with excellent characteristics having vigorous growth and high yield. It develops quickly and starts production early. The fruit are conical in shape and the brightness of the color remains even after harvesting
Furore	Ever bearing	Flevo Berry Holding B.V.	2014	The quality and yield are very good and can be used in many forms of cultivation. The variety comes into production early. The fruit are conical and elongated in shape and have a bright red color, which, just as the shine, also remains well preserved during storage
L'Amour	Midseason	Cornell/NYSAES	2003	Heart shaped berries with bright red color and a fancy calyx. Berries are bright red and firm but not hard, with excellent eating quality and flavor. The plants are vigorous and disease resistant
Mesabi	Midseason	University of Minnesota and the USDA-ARS	2000	Mesabi produces large, firm berries that are red all the way through with excellent flavor. Plants are very winter hardy and show excellent disease and red stele root rot resistance
Monterey	Day-neutral	Univ. of California	2009	Vigorous plant may require slightly more space than Albion. Fruit is slightly larger than Albion, but less firm. Outstanding flavor, good disease resistance profile, although it is susceptible to powdery mildew

Cultivars	Season	Developed by	Released	Important traits
Portola	Day-neutral	University of California	2009	It is a strong day-neutral cultivar. Fruit is similar in size to Albion. It has excellent flavor and a slightly earlier ripening season than Albion. It is a vigorous plant and may require a slightly lower planting density than Albion. It is somewhat less tolerant to rain than Albion
San Andreas	Day-neutral	Univ. of California	2009	It has high quality fruit, outstanding flavor, exceptional appearance, and is especially superior to Albion in the early season. Fruit color is slightly lighter
Stellarossa	Late Season	Cincinnati	2005	Small plants that develop numerous runners. Medium, deep-red berries with a wedge shape. Flavor is sweet. Cold hardy. Ripens in August and bears well into fall. Self-pollinating
Festival	Short Day	Florida Agr. Expt. Station	2000	Distinguished by the numerous runners it produces in the fruiting field, the long pedicels attached to its fruit, and the production of fruit that are flavorful, firm fleshed, deep red on the outside, bright red on the inside, and conically shaped
Sussette	Late	Flevo Berry Holding B.V.	2013	This variety is a late, short-day variety with light red colored fruit and an excellent flavor
Tillamook	Early mid-season	USDA-ARS, Oregon Agr. Expt. Station, Washington State University	2002	High yielding cultivar, with an extremely large fruit size that is maintained throughout the season. Fruit are extremely firm, cap easily, and have excellent flavor
Valley Red	Early mid-season	USDA-ARS, Oregon	2010	Processing cultivar

Source https://en.wikipedia.org/wiki/List_of_strawberry_cultivars

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Chapter 7
Quince (*Cydonia oblonga* Mill.) Breeding

Salih Kafkas, Burhanettin Imrak, Nesibe Ebru Kafkas, Abdulkadir Sarıer and Ali Kuden

Abstract Quince, *Cydonia oblonga* Mill. is one of the most important pome fruit species in the Rosaceae family. The genus *Cydonia* is monospecific and includes only a single species. Quince is grown for both fruit production and rootstock, and pear scion cultivars. It is cultivated for fruit production all over the world but most of the production is around its origin. Its fruits are used both for fresh consumption and for industry such as jam, jelly, marmalade, canning etc. Quince rootstocks such as Quince A and Quince C provide dwarfing to pear scion cultivars. The cultivar breeding by intraspecific crossing is very limited, and mostly all the cultivars in the production are selections from either nature or backyards. There are a few quince germplasm resources in the world characterized phenotypically and by different molecular marker systems. There are also studies aiming to produce quince x apple and pear x quince intergeneric crosses for rootstock. Quince is highly sensitive to fire blight (*Erwinia amylovora* Burril) which is the most important problem in fruit and rootstock production. Although genomic resources are very limited for *Cydonia*, apple and pear genomic resources in genebanks can be used for genetic and molecular studies in quince. It is necessary to have a large germplasm collection from diverse countries and with segregating populations for economically-important characters to start genetic studies in quince. This will allow use of molecular tools in the future breeding programs in quince.

S. Kafkas (✉) · B. Imrak · N. E. Kafkas · A. Sarıer · A. Kuden
Department of Horticulture, Faculty of Agriculture, University of Cukurova, 01330 Adana, Turkey
e-mail: skafkas@cu.edu.tr

B. Imrak
e-mail: bimrak@cu.edu.tr

N. E. Kafkas
e-mail: ebru@cu.edu.tr

A. Sarıer
e-mail: kasarier@cu.edu.tr

A. Kuden
e-mail: akuden@cu.edu.tr

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7.1 Introduction

Quince, *Cydonia oblonga* Mill. (syn. *Cydonia vulgaris* Pers.) is one of the oldest pome fruit species which has been grown for over 4000 years and belongs to the family Rosaceae (rose), subfamily Maloideae (Pomoidae). Maloideae has about 1000 species and 30 genera including *Cydonia*, which has only the species *Cydonia oblonga* Mill., with a large number of varieties. The name is derived from the name of a Greek city on the island of Crete, Cydonea, now called Canea. Quince is closely related to apples (*Malus*), pears (*Pyrus*), and Japanese quince (*Chaenomeles*). There are various botanical varieties and subspecies in *Cydonia* (Table 7.1). Many of them should probably not be considered as formal taxa but merely represent genetic variation within the species (Bell and Leitao 2011; Sykes 1972).

Quince is native to western Asia, and the center of origin is considered to be the Trans-Caucasus region including Armenia, Azerbaijan, Iran, southwestern Russia, and Turkmenistan (USDA 2009). During ancient times, quince spread from its wild center of origin to the countries bordering the Himalaya Mountains to the east, then throughout Europe to the west (Postman 2009).

Worldwide, there are about 82,376 ha of quince in production with a total crop of 649,364 mt in 2014. Turkey is the largest producer with about 16% of world production, followed by China, Uzbekistan, Iran, Morocco, Argentina and Azerbaijan (Faostat 2017).

Quince is not only cultivated commonly for fruit production but also has been used as a rootstock for European pear (*Pyrus communis* L.). Fruit production is mostly in backyards or on the borders of fields and some in commercial orchards (Fig. 7.1). Almost all genetic resources are to be found in existing wild populations and in cultivar forms due there being only one species in the genus *Cydonia*. The exceptions are artificial intergenetic hybrids with apple (*Malus* × *domestica* Borkh.) and Japanese pear (*Pyrus pyrifolia* [Burm. F.] Nakai). Diploid, triploid and tetraploid F_2 progenies have been produced and are thought to be valuable in breeding for increasing cold hardiness, presumably in a crop with more quince-like fruit. Pear × quince hybrids may have use as rootstocks for pear (Bell and Leitao 2011). The discussion in this chapter will emphasize the breeding of quince both for rootstock and fruit production.

7.2 Morphology

Quince grows as a multi-stemmed shrub or small tree (Fig. 7.2) and has pubescent to tomentose buds, petioles, leaves and fruit. The fruits are globular (Fig. 7.3), oblong

Table 7.1 Varieties, subspecies and forma of *Cydonia oblonga* Mills

Cydonia oblonga f. *lusitanica* (Mill.) Rehder
Cydonia oblonga f. *marmorata* (Dippel) C.K. Schneid.
Cydonia oblonga f. *pyramidalis* (Dippel) Rehder
Cydonia oblonga f. *pyriformis* (Dierb.) Rehder
Cydonia oblonga subsp. *integerrima* Lobachev
Cydonia oblonga subsp. *lusitanica* (Mill.) D. Rivera
Cydonia oblonga subsp. *maliformis* (Mill.) Thell.
Cydonia oblonga subsp. *oblonga*
Cydonia oblonga subsp. *pyriformis* Medik. ex Thell.
Cydonia oblonga var. *biserrulata* Kakhadze, nom. *illegit*.
Cydonia oblonga var. *integerrima*
Cydonia oblonga var. *integerrimo-sepala* Lobachev
Cydonia oblonga var. *integerrimosepala* Kakhadze, nom. *illegit*.
Cydonia *oblonga* var. *lusitanica* (Mill.) C.K. Schneid.
Cydonia *oblonga* var. *maliformis* (Mill.) D. Rivera, comb. superfl.
Cydonia oblonga var. *maliformis* (Mill.) Rehder
Cydonia oblonga var. *oblonga*
Cydonia oblonga var. *obpyricarpa* Lobachev
Cydonia oblonga var. *obpyriformis* Lobachev
Cydonia oblonga var. *orbiculato-complanata* Lobachev
Cydonia oblonga var. *ovalicarpa* Lobachev
Cydonia oblonga var. *ovalis* Lobachev
Cydonia oblonga var. *plano-cyclocarpa* Lobachev
Cydonia oblonga var. *pomiformis* Lobachev
Cydonia oblonga var. *pyriformis* (hort. ex Petz. & G. Kirchn.) Buia
Cydonia oblonga var. *rotundata* Kakhadze
Cydonia *oblonga* var. *serrulata* C.K. Schneid.
Cydonia oblonga var. *typica* Kakhadze, comb. inval.
Cydonia oblonga var. *urceolata* Lobachev

Source Encyclopedia of Life (2013)

or pear-shaped in form with a rich yellow or orange color when ripe, an austere taste and emitting a peculiar but pleasant fragrance.

The seeds are in wide use medicinally and highly valued as a demulcent tonic. Leaves are ovate to oblong, about 5 cm across and 10 cm long. Quince has hermaphroditic flowers. The solitary white flowers are 4–5 cm across, have 5 petals, 20 or more stamens, 5 styles, an inferior ovary with many ovules, and are borne on current season growth. Stamens are composed of a large number of filaments

Fig. 7.1 A commercial quince orchard in Turkey

Fig. 7.2 A quince tree

(15–20) and light-yellow anthers, which are arranged in three circular rows. Bloom time overlaps with that of apples, usually beginning mid-April in the central latitudes of the northern hemisphere (Nagy-Dèri 2011).

Fig. 7.3 A quince fruit

7.3 Health Benefits

Quince fruit has many benefits for human health and is regarded to be a health-promoting fruit and an excellent source of pectin for the jelly and jam industries (Moradi et al. 2017). The nutritional value of the quince fruit is high (Bucsek et al. 1996; Kopec and Balík 2008) and 100 g fresh weight of quince provides energy (176 kJ), protein (0.6 g), acid (0.9 g), carbohydrate (9.1 g), water (86.9 g), ash (0.6 g), fiber (1.9 g) and total lipid (0.10 g) (Rodriguez-Guisado et al. 2009).

Quince fruit shows antioxidant effects (Silva et al. 2002), which result from the presence of a number of polyphenolic substances (Fattouch et al. 2007), e.g. flavonoids quercetin, rutin, kaemp ferol, etc. (Silva et al. 2005), and also high levels of vitamin C (Tetera 2006). Hegedus et al. (2013) reported that there is a genetic variation among quince cultivars based on their polyphenolic profiles, which are responsible for the major part of beneficial health-effects.

Due to its beneficial properties it is suitable for application in nutritional/pharmaceutical fields, in the prevention and treatment of free radical-mediated human chronic pathologies, such as cardiovascular diseases and cancer (Golubev et al. 1990; Mir et al. 2015; Mushtaq and Wani 2013; Oliveira et al. 2007). Quince is reported to have beneficial effects against several diseases including as a hypoglycemic, anti-inflammatory, anticarcinogenic, antimicrobial, antiallergic, antiulcerative and atopic dermatitis (Hamauzu et al. 2006; Hegedus et al. 2013). Alesiani et al. (2010) identified several compounds with antiproliferative effects from quince peels whereas Carvalho et al. (2010) could not show inhibitory effects of quince fruit flesh and peel on human kidney and colon cancer cells. The author implied that the seed extracts exhibited no effect on colon cancer cell growth, whereas strong antiproliferative efficiency was observed against renal cancer cells for the highest concentration

tested (500 μg/mL). The antimicrobial activity of quince extracts against different microorganism strains was reported by Fattouch et al. (2007) and quince peel extract was found as the most active for inhibiting bacterial growth. At present, quince fruit is recognized as an important dietary source of health promoting compounds, due to its antioxidant, antimicrobial and antiulcerative properties (Fattouch et al. 2007; Fiorentino et al. 2007, 2008; García-Alonso et al. 2004, Hamauzu et al. 2005, 2006; Silva et al. 2004, 2008; Wang et al. 2006).

Quinces are consumed either fresh or processed by the canning industry, due being rich in pectins (Forni et al. 1994; Kyzlink 1990). Pectins are a naturally-occurring carbohydrate in quince fruit and due to their gelling properties, pectins are often used as additives in the food industry. Rop et al. (2011) reported the greatest pectin amount ranging between 1.75 and 3.51 g/100 g FW. Quince pectin has a high galacturonic content (78%), and a degree of methoxylation of about 60% corresponding to a medium-high methoxyl pectin (Forni et al. 1994).

Quince fruits are less suitable for direct consumption (Hričovský et al. 2003) and for that reason they are mostly used either cooked (Alvarenga et al. 2008) or preserved (Kyzlink 1990). From the sensory point of view, an intensive quince-like fragrance represents an important qualitative trait (Lutz and Winterhalter 1992); this is due to an increased content of aromatic substances. For this purpose, quince fruit can also be used for the production of an aromatic distillate (Naf et al. 1991) commercially (Adler 2001).

Quince fruit is not appreciated fresh due to its pulp hardness, bitterness and astringency. The phenolic compounds are not evenly distributed in different tissues and the peel usually contains at least two times more phenolic compounds than the flesh. The peel also possesses the higher antioxidant activity and antiproliferation activity against cancer cell lines (Sun et al. 2002).

Costa et al. (2009) reported on a comparison of the phenolic profile and antioxidant potential of quince leaf and green tea (*Camellia sinensis*); quince leaf may be applied as a preventive or therapeutic agent in diseases associated with free radical damage (Costa et al. 2009). A total of 26 polyphenolic compounds found in quince tissues were identified by Wojdylo et al. (2013). Fattouch et al. (2007) reported that total phenolic contents of the flesh and peel parts ranged from 37 to 47 and 105 to 157 mg/100 g of fresh weight, respectively in Tunisian quinces; chlorogenic acid (5-*O*-caffeoylquinic acid) was the most abundant phenolic compound in the flesh (37%), whereas rutin was the main one in the peel (36%).

7.4 Growing Conditions and Cultivation Problems

Cydonia is well adapted to hot, dry climates and to acid soils. Under favorable conditions ripe fruit can become quite fragrant, juicy and flavorful. When grown in high pH soils, however, trees can become stunted and chlorotic due to iron deficiency. Quince is grown either for fruit production or for use as a dwarfing pear rootstock in the world.

In the region around Angers, France, quince has been used as a pear rootstock since 1500. The French were growing quince plants from cuttings and layering in stool beds by the early 1600s and France became an important source of rootstock around the world. East Malling in England also developed several quince rootstocks for pear. Quince A, Quince C, BA-29C, Quince Adams, Quince Eline are rootstocks in cultivation for both quince and pear. Quince rootstocks provide good vigor control, but there are still key management challenges associated with their use. One major issue is the incompatibility of quince with many important European pear scion cultivars such as Williams, Beurre Bosc and Packhams. This can be overcome with the use of interstems of compatible cultivars such as Comice, Old Home, or Beurre Hardy (Francescatto et al. 2010; Postman 2009; Roach 1985). Quince rootstocks are also susceptible to lime-induced chlorosis, which is associated with alkaline soils. Limited winter hardiness is an issue with quince rootstocks as well, and this has limited their use in areas that suffer severe winters.

Quince has several disease problems in cultivation: fire blight (*Erwinia amylovora* (Burrill) limits the cultivation of quince for either fruit or rootstock, especially in regions with warm and humid summers. The genus *Cydonia* is one of the most susceptible to fire blight in the Rosaceae family, which includes many genera that are hosts to the disease (Postman 2008). Powdery mildew caused by *Podosphaera leucotricha* (Ell. & Ev.) Salmon and various rust diseases can impact quince production. Leaf and fruit spot caused by *Fabraea maculata* (anamorph = *Entomosporium mespili*) can result in tree defoliation and production of disfigured, unmarketable fruits if not controlled. Genetic improvements are necessary for expanding the use of quince as a dwarfing pear rootstock with resistance to fire blight for warm and humid summer climates, and increased winter cold hardiness for northern climates. Adaptation to alkaline soils can allow quince production to expand to more diverse soil conditions both as a rootstock for pear and for production of quince fruit (Postman 2009).

Quince for fruit production may benefit from earlier ripening, and elimination of summer *rat-tail* blooms, which predispose a tree to attack by fire blight. Most quince genotypes are adapted to regions with long, hot growing seasons and will not ripen properly without adequate heat units. Fruits that are picked too green may never ripen in storage (McCabe 1996). Resistance to fungal rusts and mildews will allow quince to be produced with fewer pesticide applications. For nearly a century, quince has been ignored for fruit production in North America, while many improvements have been made in the Middle East and central Asia (Postman 2009).

7.5 Germplasm Resources

In order to initiate a quince breeding program, the first step is to establish a germplasm collection, especially from countries of origin. Germplasm collections are important tools for conservation and providing a large genetic variability for breeding programs. *Cydonia* germplasm collections resulting from plant exploration and exchange have

been established in several countries, including Italy (Ianni and Mariotti 2005), USA (Postman 2008; USDA 2009), and UK (University of Reading 2009). Coordinated documentation of 11 collections in Italy, France, Spain, and Greece has been initiated (European Cooperative for Plant Genetic Resources 2009) as part of a European project on underutilized fruit crop species (Bellini and Giordani 2000; European Commission 2007). There are also quince germplasm collections from native populations in different countries such as Turkey (Kuden and Kuden 2008; Sahin and Misirli 2016; Sykes 1972), Iran (Abdollahi 2013; Amiri 2008; Gharaghani et al. 2016), Turkmenistan (Vitkovskii and Denisov 1991) and Ukraine (Yezhov et al. 2005).

In Turkey, a quince germplasm collection was established in Izmir in 1964 (Sykes 1972). A survey was performed in the Aegean region and 31 selections collected for the Aegean Agricultural Research Institute in Izmir. Three of them (Ege-2, Ege-22 and Ege-25) were superior and assigned as registered cultivars due to their high yield and fruit characters (Ercan et al. 1992). Currently, there are 61 accessions in the germplasm collection in the Aegean Agricultural Research Institute. A survey study was also performed for rootstock purposes in 1969 in Ankara University and 17 clones were selected and tested for graft-compatibility (Celik 1982, 1988). There are currently 26 registered quince cultivars in Turkey: 17 are for rootstock and 9 are for fruit production. There are quince germplasm collections with those cultivars together with new local selections at the Yalova Atatürk Horticultural Central Research Institute (Fig. 7.4) and in the Egirdir Fruit Research Institute in Turkey.

In Iran, the earliest identification of quince cultivars and genotypes was undertaken in Isfahan and Khorasan provinces in the last decade. These genotypes along with the genotypes of Guilan, Ardebil, Azerbaijan and Tehran provinces were acquired and conserved in a collection in Karaj where 50 *Cydonia* accessions are maintained, including both cultivated and wild types (Abdollahi 2013; Amiri 2008; Gharaghani et al. 2016).

Fig. 7.4 A view of the quince germplasm collection of Atatürk Horticultural Central Research Institute, Turkey

A large quince fruit tree collection in Kara Kala, Turkmenistan was once a part of the Vavilov Institutes during Soviet times. A dozen quince accessions from that collection are now growing at the USDA genebank at the National Clonal Germplasm Repository (NCGR), in Corvallis Oregon. The NCGR *Cydonia* collection includes more than 100 clones with origins from 15 countries maintained as self-rooted trees in a field collection (Postman 2008). About half of this collection represents cultivars for fruit production, and the other half are pear rootstock selections, wild types and seedlings. Observations made at the genebank have revealed a wide diversity of genotype resistance to Fabraea leaf and fruit spot [*Fabraea maculata* Atk. (anamorph = *Entomosporium mespili* (DC.) Sacc.)], and a range of ripening seasons that may make it possible to produce quince fruit in short-season production areas (Postman 2009).

In Ukraine, the Nikita Botanical Garden (NBG) initiated an in vivo quince germplasm collection in 1940. The NBG currently has more than 200 *Cydonia* accessions in the germplasm collection, and six commercial cultivars have been released. Unique precocious and early-maturing genotypes with high yield and resistant to pest and disease were developed at NBG for both fresh consumption and food processing (Yezhov et al. 2005).

7.6 Use of Molecular Markers in Quince

The use of molecular markers for identification and characterization of germplasm collections is a reliable experimental approach because it is difficult to distinguish cultivars phenotypically due to the environmental influence on phenotypes. In addition, tree and fruit morphological properties of quince genotypes are very similar to each other, which makes difficult distinguishing closely-related genotypes.

Sanchez et al. (1988) was the first researcher to use molecular markers in quince by employing isoenzymes. Eleven groups of quince and two groups of x*Pyronia* (quince-pear crosses) were distinguished successfully by isozyme patterns such as acid phosphatase, esterase, peroxidase and phenol oxidase. Peroxidases was studied in graft incompatibility between quince rootstocks and certain pear scion cultivars. Gulen et al. (2002) speculated that matching of isoperoxidase 'A' in quince rootstocks in the graft compatible Beurre Hardy scion may be associated with a compatible graft combination, and that the presence of isoperoxidase 'A' and 'B' in graft union tissues predicts compatibility between quince and Bartlett scions. Later, Gulen et al. (2005) identified an association between rootstock/cultivar compatibility and a specific anodal isoperoxidase marker. Esterases and malate dehydrogenases were used by Hudina et al. (1999) to discriminate between eight European pear cultivars and the quince rootstock cultivars Quince A and BA-29C. The authors speculate that an esterase band, common to both quince varieties and to the pear cultivars used as interstocks, Beurre Hardy and Vicar of Winkfield, could be associated with rootstock/scion cultivar compatibility.

Among the DNA-based molecular markers, simple sequence repeats (SSRs) are known for being codominant, highly polymorphic and having a large number of alleles per locus (Schlotterer and Tautz 1992). One more advantage of SSRs is that these markers can be easily transferred between species of the same subfamily and between closely related genera. Unlike other pome fruit species including apple and pear, information about evaluation of genetic diversity of cultivated quinces is limited. In all investigations related to quince, SSR markers derived from pear (Bao et al. 2007; Kimura et al. 2002; Miranda et al. 2010; Tian et al. 2012; Yamamoto et al. 2002) and apple (Galli et al. 2005; Gianfranceschi et al. 1998; Liebhard et al. 2002; Zhang et al. 2012) have been employed, because markers from quince have not yet been developed.

The first genetic diversity study with SSRs for quince was undertaken with 20 quince cultivars by Yamamoto et al. (2004) using SSR markers developed from apple and pear. The authors found that 50% of the SSR markers derived from Japanese and European pears and 73% of the SSR markers derived from apple were successful in the amplification of quince. Quince cultivars used as rootstock appeared as genetically distinct from varieties cultivated for fruit production. Although most of the fruit cultivars were discriminated, some of them amplified identical molecular patterns.

Cydonia germplasm collections from 15 diverse countries at the USDA-ARS National Clonal Germplasm Repository in Corvallis, Oregon were characterized by Bassil et al. (2011) with nine polymorphic SSR markers in quince developed from apple and pear. Among the analyzed 92 quince accessions, 12 clones could not be differentiated. Bassil et al. (2015) performed another study to differentiate those 12 clones by replacing four of the less-informative SSRs with four SSRs amplifying at least four alleles. Although several pairs of accessions were identical, the new SSR fingerprinting set improved the power of differentiation. Dumanoglu et al. (2009) used SSR markers to differentiate six clones from Kalecik cultivars. The authors used seven SSR primer pairs to fingerprint six cv. Kalecik clones and one clone was found different suggesting clonal variation within cv. Kalecik. SSR markers from pear and apple were also used to characterize quince cultivars at the Technical University Munich by Xuan et al. (2013). The authors analyzed 22 cultivars using 17 SSR markers, but two pairs of cultivars could not be separated. Yüksel et al. (2013) characterized 15 quince cultivars along with several pear and apple ones by eight SSR loci. All cutivars were clearly separated and quince was much closer to apple than pear. An Iranian national quince collection containing 40 genotypes, originating from six distinct geographic areas, was screened using 15 SSR markers developed originally from apple and pear. All the genotypes were separated from each other and cluster analysis divided them into five major clusters based on their origins (Azad et al. 2013).

As a dominant marker, randomly amplified polymorphic DNA (RAPD) was used by Bayazit et al. (2011) to assess genetic relationships among 13 traditional quince accessions selected from different parts of Turkey. The authors used 16 polymorphic RAPD primers that distinguished 13 quince accessions based upon their geographical origin. Orhan et al. (2014) also used RAPD technique to characterize 14 quince genotypes collected from the Coruh Valley in Northeast Anatolia of Turkey, and the

genotypes were well separated with 10 polymorphic primers. Amplified fragment length polymorphism (AFLP) is another dominant marker system which is highly polymorphic and may have potential to produce hundreds of markers with one primer pair combination. Mnaica-Berto et al. (2013) used AFLP technique to assess genetic similarity between quince cultivars in Brazil. The authors obtained two groups in characterizing 21 cultivars with high genetic variability. Topcu et al. (2015) characterized quince germplasm collection in the Ataturk Horticultural Research Institute located in Yolava Province, Turkey by AFLP markers. The authors analyzed 40 quince accessions which were previously selected for their fruit and yield characteristics from different parts of Turkey. Six AFLP primer combinations separated closely related accessions successfully, and three main clusters were obtained. Ganopoulos et al. (2011) used inter-simple sequence repeat (ISSR) markers to characterize quince germplasm collections located at the Pomology Institute, Department of the National Agriculture Research Foundation Naoussai, Greece. The authors analyzed 49 quince accessions with 18 ISSR markers, and five groups were obtained in the dendrogram by discriminating all accessions. Sequence-related amplified polymorphism (SRAP) is another dominant molecular marker technique producing multiple bands in one reaction. SRAP was used to characterize quince germplasm collection in the Egirdir Horticulture Research Station, Isparta, southwestern Turkey, by Pinar et al. (2016). Seventeen quince cultivars were distinguished successfully by using 23 SRAP primer combinations with a low level of genetic variation.

Restriction fragment length polymorphisms (RFLPs) analysis in chloroplast DNA showed that *Cydonia* was more closely related to *Pyrus* than to *Malus* and *Chaenomeles* (Iketani 1993). Campbell et al. (1995) studied the relationships among 19 Maloideae genera by analysis of sequences of internal transcribed spacers (ITS) and a part of the 5.8S nuclear ribosomal gene, and the authors indicated that the Maloideae are not monophyletic, that there is considerable homoplasy, and that there is strong support for a close relationship between *Cydonia* and *Pseudocydonia*. Kaneko et al. (2000) confirmed the closer genetic relationship between quince (*Cydonia*) and Chinese quince (*Pseudocydonia*) and their genetic distinctness from flowering quince (*Chaenomeles*) by random amplified polymorphic DNA (RAPD) markers.

7.7 Traditional and Molecular Breeding in Quince

Quince has 2n = 34 chromosome numbers like apple and pear. The nuclear DNA content is 0.73 pg (Dickson et al. 1992). Selection and cross-breeding methods have been used to develop new cultivars for quality of the quince fruit, yield, and resistance to diseases such as fire blight, which are very important properties for breeding focus. Breeding in quince for both fruit and rootstocks has been conducted mostly by selection from nature or backyards, thereby intraspecific crosses are very limited. There is no currently breeding program in quince using molecular tools.

Intraspecific crosses for fruit breeding were done in 1968 between Leskovacka, Bereczki, Reans Mammoth, Vranjska, Pazardzijska and Ceska cultivars in

Yugoslavia, and a total of 646 seedlings were produced. Morova cultivar (Leskovacka x Reans Mammoth) was released in 1987 with high yield and good fruit characteristics suitable for both fresh consumption and for the food industry (Stancevic and Nikolic 1992). Bobev et al. (2009) performed screening of 274 crosses against fire blight during the epidemic years of 2003 and 2005. The authors identified 18 progenies exhibiting significant resistant to fire blight.

Several survey studies in the different regions of Turkey were conducted to select quince genes with high yield and good fruit characteristics. The first survey study was done in the Aegean region by the Aegean Agricultural Research Institute and three cultivars (Ege-2, Ege-22 and Ege-25) were released (Ercan et al. 1992, Sahin and Misirli 2016). Another survey study was performed by Gungor (1989) during 1985–1987 in the Central Anatolia region of Turkey, and three genotypes were found superior, among 82, for both fresh consumption and industry. Çil (2014) did a survey study in the Kayseri province of Turkey during 2011–2013, and five genotypes were found to be promising for further testing. The Aegean Agricultural Research Institute started a new breeding project in quince to develop cultivars for both fruit production and rootstock resistance to fire blight. Another aim of the breeding program is to breed red-fleshed quince cultivars for their high antioxidant contents that may have greater health benefits.

Rootstock breeding in quince for pear was also started in Turkey by Sebahattin Ozbek in the 1970s and 17 clones (SO clones) were selected for further studies. Different pear cultivars were grafted on them, and several of them were promising rootstock cultivars (Celik 1982, 1988; Dumanoglu et al. 1993). A new quince rootstock (B-35) was also released by the Aegean Agricultural Research Institute in 2015.

In the early 1900s, researchers at East Malling in England collected rootstocks from a number of nurseries and designated clones with letters of the alphabet. Quince rootstock clones now available include Quince A and Quince C, which came from East Malling-Long Ashton (EMLA); and Provence Quince (=Quince BA 29-C) from France. A pear tree grafted onto Quince A becomes about half the size, is more precocious and fruit size is larger than a tree grafted onto pear seedling rootstock. Quince C produces a tree slightly smaller and more precocious. BA 29-C quince rootstock produces a pear tree slightly larger than Quince A or C (Postman 2009; Roach 1985). Later, Quince Adams was released in France, which is quite resistant to moist soil and iron chlorosis with high compatibility with pear scion cultivars. Quince EMH, Quince C132 and Quince Eline are new rootstocks as well. Quince EMH developed at East Malling, Quince C132 is a selection from the Caucasus region of Russia, and Quince Eline is a Romanian selection sourced from the Fleuren Nurseries in The Netherlands.

Intergeneric crossings have occurred naturally on occasion in the Rosaceae. *Cydonia* has had a number of matings with related genera that resulted in intergeneric offspring. The first *Pyrus* x *Cydonia* hybrid, *xPyronia veitchii* (Trabut) Guill was produced before 1916 (Rudenko 1985) and presently is used as an indicator of virus diseases. An artificial hybrid between the Japanese pear *Pyrus serotina* and *C. oblonga* has been produced by Shimura et al. (1983). An attempt was done to

produce a rootstock for pears by backcrossing a clone of x*Pyronia veitchii* var. *luxemburgiana* to pear (Rogers 1955).

In addition, an artificial hybrid genus, x*Cydomalus*, was produced in Russia by crossing apple (*Malus pumila*) and quince (*Cydonia oblonga*) (Rudenko and Rudenko 1994). The hybrids tended to have fruit similar to quince but with the flavor of apples (Rudenko 1983). One triploid F2 had large (230 g) fruit. Backcrossing an allotetraploid F2 with apple produced 50 mostly tetraploid hybrids with many traits of apple (Rudenko 1984). The allotetraploid F3 hybrids were thought to be valuable in breeding for increased frost resistance and fruit quality (Rudenko 1987), presumably in a crop with quince-like fruit. Similar production of F1 hybrids was obtained in Bulgaria, where the quince cultivar Champion was pollinated with a mixture of five apple cultivars; two hybrids had fruit and other anatomical traits intermediate between the two parental species. Irradiation of pollen, embryo culture and in vitro micropropagation have been used to improve the recovery and survival of *Cydomalus* intergeneric hybrids (Bell and Leitao 2011; Papikhin et al. 2007).

7.8 In Vitro Tissue Culture in Quince

Traditional methods of propagation have certain disadvantages because they are unable to meet the increasing demand for planting materials of desired genotypes. The usual methods of quince propagation by the rooting of cuttings or layering have some disadvantages such as producing limited quantities of planting materials, are seasonal dependence and take a long period of time. Like other fruit species, micropropagation is the only alternative technique to increase the supply of high-quality planting material of quince. Micropropagation was first achieved in quince in 1986 (Maarri et al. 1986). Since then, significant progress has been made in different areas of in vitro culture of quince and published by several authors (Anirudh and Kanwar 2008; Giorgota et al. 2009).

Very efficient in vitro adventitious shoot regeneration from cultured leaves of *Cydonia oblonga* cv. Quince A was achieved by Dolcet-Sanjuan et al. (1991). Protocols for callus induction and maintenance and subsequent shoot regeneration were reported by Chartier-Hollis (1993), and in vitro adventitious shoot regeneration, environmental influence in regeneration ability, low concentrations of NaCl in somatic embryo formation and adventitious shoot and root regeneration, effects of various macroelement and plant growth regulators of quince were also studied by various researchers (Antonelli 1995; Aygun and Dumanoglu 2007; Baker and Bhatia 1993; D'Onofrio and Morini 2002a, b, 2005; Dolcet-Sanjuan et al. 1991; Erig and Schuch 2005; Fisichella and Morini 2003; Marino and Berardi 2004; Marino et al. 2008; Mingozzi and Morini 2009; Staniene and Stanys 2004; Zalunskaite et al. 2007). Isolation of protoplasts from in vitro grown quince (BA-29) was successfully performed by D'Onofrio et al. (1999). Grimaldi et al. (2016) reported a protocol for micropropagation of Quince BA-29 in semi-solid media. For rapid commercial production,

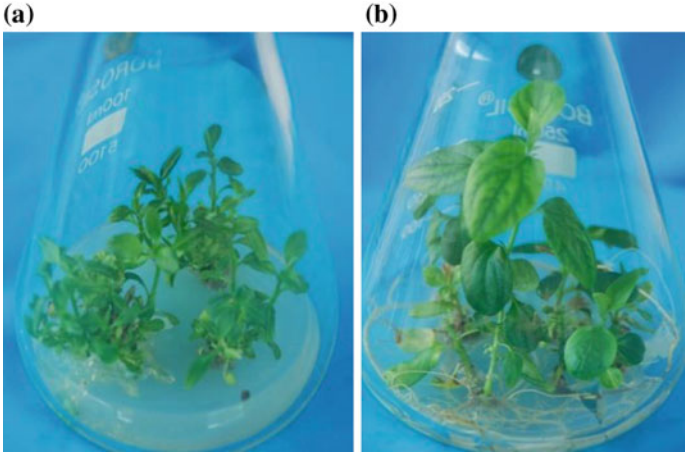

Fig. 7.5 Quince in vitro culture. **a** Proliferating buds induced from axillary bud explants. **b** Shoot elongation and rooting

Basu et al. (2017) established a protocol for the micropropagation of SKAU-016 (Fig. 7.5).

7.9 Genomic Resources

Although genetic linkage maps are powerful tools in plant research, there is no current genetic linkage map for quince. However, high density genetic maps have already been constructed for apple (Di Pierro et al. 2016; Motalebipour et al. 2015; Naik et al. 2006) and pear (Chen et al. 2015; Yamamoto et al. 2007). Nevertheless, a high transferability of DNA markers from apple and pear to *Cydonia* (Bassil et al. 2015; Yamamoto et al. 2004; Yüksel et al. 2013), as well as a high level of genome synteny were found between genera of the same subfamily, such as between apple (*Malus*) and pear (*Pyrus*) (Pierantoni et al. 2004; Yamamoto et al. 2007). It is very important to note that whole genome sequences of apple (Velasco et al. 2010) and pear (Wu et al. 2013) are available and they may help to develop markers and rapidly construct a reference genetic map in *Cydonia*.

There are 104 cloned partial or complete DNA and 146 protein sequences in Genbank isolated from *Cydonia*. Some of the genes and other DNA sequences isolated from *Cydonia* are given by Bell and Leitao (2011); the rest are listed in Table 7.2. There are also two genomic SRA (sequence read archive) data in Genbank: one is 42.1 Gb obtained from short insert pair-end libraries, and another is 26.4 Gb from mate-pair libraries (National Center for Biotechnology Information 2017). There are currently adequate DNA sequences in Genbank to start marker development and to construct linkage maps in quince.

Table 7.2 *Cydonia* genomic resources

Function	Gene	Genbank accession
NPR1-like protein	*NPR1-like*	DQ149931
NPR1-like protein	*NPR1-like*	DQ149933
	–	JQ390654
	–	JQ390655
	–	JQ390697
	–	JQ390836
	–	JQ390837
	–	JQ390994
	–	JQ390995
	–	JQ390996
	–	JQ391146
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*rbcL*	JQ391147
	–	JQ391148
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*rbcL*	JQ391332
ribulose-1,5-bisphosphate carboxylase/oxygenas large subunit	*rbcL*	JQ391333
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*rbcL*	JQ391334
	rpl16	JQ391472
	rpl16	JQ391473
	rpl16	JQ391474
	rps16	JQ391640
	rps16	JQ391641
	rps16	JQ391642
	–	JQ391791
	–	JQ391792
	–	JQ391953
	–	JQ391954
	–	JQ391955
	–	JQ392265
	–	JQ392266
	–	JQ392267
	–	JQ392420
	–	JQ392421
	–	JQ392422

(continued)

Table 7.2 (continued)

Function	Gene	Genbank accession
pathogenesis related protein 1a	*PR1a*	JQ917106
pathogenesis related protein 5	*PR5*	JQ965004
pathogenesis related protein 5	*PR5*	JQ965007
pathogenesis related protein 3	*PR3-Ch3*	JQ965008
pathogenesis related protein 3	*PR3-Ch3*	JQ965009
	–	AB636344
pathogenesis related protein 8	*PR8*	JX174172
pathogenesis related protein 8	*PR8*	JX174174
pathogenesis-related protein 3	*PR3-Ch5*	JX524193
pathogenesis-related protein 3	*PR3-Ch5*	JX524194
pathogenesis-related protein 3	*PR3-Ch1*	KC619534
28S ribosomal RNA	–	KF861967
28S ribosomal RNA	–	KF861968
beta-1,3-glucanase	*PR2*	KF981878
actin	–	KF981879
Ycf1	*ycf1*	KP088090
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*rbcL*	KP088562
maturase K	*matK*	KP089040
ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*rbcL*	KM360743
maturase K	*matK*	KM456210
maturase K	*matK*	KM456211
tRNA-His	*tRNA-His*	KM456212
tRNA-His	*tRNA-His*	KM456213
starch branching enzyme I	*SbeI*	KU760443
ribosomal protein S16	*rps16*	KU760511
WD repeat-containing protein	–	KU760626
sorbitol-6-phosphate dehydrogenase	*S6PDH*	KU985065
photosystem II protein D1	*trnH-GUG*	KX499857
	nad1	KX683226
	–	GR420429
pathogenesis related protein 1a	*PR1a*	JQ794874

Source National Center for Biotechnology Information (2017)

7.10 Conclusions and Prospects

Quince has economic importance whether grown for fruit production or for use as a dwarfing pear rootstock throughout the world. Quince fruit has many human health benefits and prevents many diseases; it is regarded to be a health-promoting fruit and represents an excellent resource of the food industry. However, genomic resources are very limited for *Cydonia*, when compared with apple and pear. Selection studies from nature for fruit and rootstock characters have been the primary breeding approaches and there are a very limited number of cross-breeding studies in quince. Further research is necessary to expand germplasm collections, especially with international collaboration. Then, those collections may help to create different segregating populations for various economically-important characters. Segregating populations are necessary not only for breeding purpose, but they are also needed for further genetic studies in quince that may help to develop markers linked to both fruit and rootstock production. There are inadequate data in Genbank for quince. Whole genome sequencing studies have been performed in many major fruit tree species due to development of next generation sequencing instruments over the last decade. Genome sequencing in quince can be another approach to provide genetic tools in breeding programs in a shorter period. In conclusion, diverse germplasm, full-sib segregating populations and genomic tools can facilitate new cultivars with desired characters to assure the future of quince.

Acknowledgements We thank to Dr. Adnan Dogan and Mehmet Emin Akçay from Atatürk Horticultural Central Research Institute in Yalova provinces of Turkey for providing several photos.

Appendix 1

Research institutes and germplasm resources and contact person.

Germplasm location	Country	Number of accessions	Contact address
National Clonal Germplasm Repository (NCGR) Corvallis, Oregon	United States	100	jpostman@ars-grin.gov.
Nikita Botanical Gardens	Ukraine	219	p.lapshin@mail.ru
Department of National Agriculture Research Foundation Naoussa	Greece	49	palexios(at)agro.auth.gr
Atatürk Horticultural Research Institute Yalova	Turkey	40	Skafkas@cu.edu.tr
Çukurova University Pozantı Agriculture and Research Center	Turkey	13	bimrak@cu.edu.tr
Aegean Agricultural Research İnstitute	Turkey	31	mugesahin67@hotmail.com
Horticultural Research İnstitute of Eğridir-İsparta	Turkey	17	ergul@agri.ankara.edu.tr
Faculty of Agriculture and Natural Resources, Azad University	Iran	40	Khorramdel.mahsa@gmail.com
Kompetenzzentrum obstbau-Bodensee (KOB) Research station	Germany	22	haibo@kob-bavendorf.de
Institute of Botany, Armenian National Academy of Sciences Department of Plant Taxonomy, Yerevan	Armenia	18	vancat@freenet.am
The Schroeder Institute Breeding and Germplasm Collections	Uzbekistan	4	zaurov@aesop. rutgers.edu
Agricultural University, Department of Phytopathology, Poldiv	Bulgaria	10	svetoslavbobev@abv.bg
East Malling Research Station	United Kingdom	22	www.emr.ac.uk/
N.I. Vavilov Research Institute of Plant Industry, St. Petersberg	Russian Federation	16	n.dzyubenko@vir.nw.ru
Plant Systematics, Institute of Botany Georgian Academy of Sciences	Georgia	10	mosulish@usa.net
INRA	France	4	http://institut.inra.fr
Turkmenian Experiment Station Kara-Kala	Turkmenistan	16	n.dzyubenko@vir.nw.ru

Appendix 2

A list of cultivars, their important traits and cultivation location.

Name	Origin	Type	Fabraea		Mildew		Rust	
Aiva from Gebeseud	Turkmenistan	Wild	1.0	(3)	1.7	(3)	3.7	(3)
Aiva from Kara-Kala no. 9	Turkmenistan	Fruit	3.8	(5)	4.3	(3)	1.4	(5)
Akhtubinskaya O.P. sdlg 2	Russia	Seedling	2.8	(5)	5.7	(3)	1.4	(5)
Akhtubinskaya O.P. sdlg 3	Russia	Seedling	3.6	(5)	1.7	(3)	3.0	(5)
Akhtubinskaya O.P. sdlg 4	Russia	Seedling	3.4	(5)	1.0	(3)	3.0	(5)
Aromatnaya	Russia	Seedling	1.8	(5)	3.0	(3)	1.4	(5)
Bereczki (=Vrajna)	Serbia	Seedling	5.0	(5)	3.0	(3)	1.8	(5)
C. oblonga sdlg 1—Alema	Armenia	Wild	6.4	(5)	3.7	(3)	1.8	(5)
C. oblonga sdlg 2—Alema	Armenia	Wild	6.8	(5)	1.7	(3)	3.0	(5)
C. oblonga sdlg 3—Alema	Armenia	Wild	7.4	(5)	4.3	(3)	2.6	(5)
C. oblonga sdlg—Arakseni	Armenia	Wild	6.2	(5)	4.3	(3)	3.0	(5)
C. oblonga sdlg—Arakseni	Armenia	Wild	7.6	(5)	4.3	(3)	2.6	(5)
C. oblonga sdlg—Arakseni	Armenia	Wild	6.0	(5)	4.3	(3)	3.0	(5)
C. oblonga sdlg—Babaneuri	Georgia	Wild	4.2	(5)	4.3	(3)	3.0	(5)
C. oblonga sdlg—Babaneuri	Georgia	Wild	4.2	(5)	2.3	(3)	2.6	(5)
C. oblonga sdlg—Babaneuri	Georgia	Wild	6.0	(5)	3.0	(3)	2.6	(5)
C. oblonga sdlg—Dusheti	Georgia	Wild	4.6	(5)	3.0	(3)	3.0	(5)
C. oblonga sdlg—Dusheti	Georgia	Wild	3.4	(5)	4.3	(3)	3.0	(5)
C. oblonga sdlg—Dusheti	Georgia	Wild	4.4	(5)	5.7	(3)	3.0	(5)
C. oblonga sdlg—Megri	Armenia	Wild	5.8	(5)	2.3	(3)	2.4	(5)
C. oblonga sdlg—Megri	Armenia	Wild	2.6	(5)	3.0	(3)	2.6	(5)
C. oblonga sdlg—Megri	Armenia	Wild	2.6	(5)	3.7	(3)	4.2	(5)
C. oblonga sdlg—Seghani	Armenia	Wild	5.8	(5)	3.7	(3)	3.0	(5)

Name	Origin	Type	Fabraea		Mildew		Rust	
C. oblonga sdlg—Seghani	Armenia	Wild	7.4	(5)	3.7	(3)	3.8	(5)
C. oblonga sdlg—Seghani	Armenia	Wild	7.2	(5)	4.3	(3)	3.4	(5)
Champion	United States	Fruit	3.0	(3)	1.7	(3)	1.7	(3)
Coburg	United States	Fruit	3.0	(3)	2.3	(3)	2.3	(3)
Chaenomels Cathayensis	United States	Ornamental	4.2	(5)	4.3	(3)	1.0	(5)
Cooke's Jumbo	California	Fruit	4.2	(5)	1.7	(3)	1.8	(5)
Ekmek	Turkey	Fruit	2.8	(5)	1.0	(3)	1.0	(5)
Fontenay	France	Rootstock	4.3	(3)	3.0	(3)	1.7	(3)
Gourton of Esfahan	Iran	Fruit	4.0	(2)	3.0	(2)	1.0	(2)
Hand Quince sdlg 1	Armenia	Seedling	4.0	(2)	2.0	(2)	2.0	(2)
Hand Quince sdlg 2	Armenia	Seedling	5.0	(2)	1.0	(2)	1.0	(2)
Hasardagskaya	Turkmenistan	Fruit	7.0	(5)	5.0	(3)	3.0	(5)
Havran	Turkey	Fruit	4.2	(5)	1.0	(3)	1.4	(5)
Isfahan	Iran	Fruit	1.8	(5)	3.0	(3)	2.2	(5)
Karakalinskaya no. 6	Turkmenistan	Fruit	2.2	(5)	1.0	(3)	4.2	(5)
Karakelskaya no. 5	Turkmenistan	Fruit	1.7	(3)	2.3	(3)	2.3	(3)
Karp's Sweet—Majes	Peru	Fruit	6.0	(5)	5.0	(3)	1.0	(5)
Valley, Araquipa		Fruit						
Kashenko no. 8	Ukraine	Fruit	3.0	(5)	1.7	(3)	1.0	(5)
Kaunching	United States	Fruit	2.3	(3)	3.7	(3)	3.0	(3)
Khrimskaya Aromatnaya	Ukraine	Fruit	1.6	(5)	2.3	(3)	2.0	(4)
Kichikara Dede 88-1	Turkmenistan	Fruit	1.0	(3)	1.0	(3)	1.7	(3)
Kichikara Dede 88-2	Turkmenistan	Fruit	6.2	(5)	5.7	(3)	1.4	(5)
Krimskaya	Ukraine	Fruit	2.2	(5)	3.0	(3)	3.0	(5)
Krukovskaya O.P. sdlg2 2	Russia	Seedling	4.6	(5)	3.7	(3)	3.0	(5)
Krukovskaya O.P. sdlg3 3	Russia	Seedling	2.2	(5)	1.7	(3)	2.6	(5)
Krukovskaya O.P. sdlg4 4	Russia	Seedling	3.6	(5)	3.0	(3)	3.4	(5)
Kuganskaya	Ukraine	Fruit	2.3	(3)	2.3	(3)	1.7	(3)
Le Borgeot	France	Fruit	4.2	(5)	1.0	(3)	4.2	(5)
Limon	Turkey	Fruit	1.4	(5)	1.0	(3)	1.0	(5)
Maslenka Rannaya O.P.1	Russia	Seedling	3.0	(5)	1.0	(3)	1.8	(5)
Maslenka Rannaya O.P.2	Russia	Seedling	2.4	(5)	3.7	(3)	1.4	(5)
Maslenka Rannaya O.P.3	Russia	Seedling	3.4	(5)	3.0	(3)	3.0	(5)

Name	Origin	Type	Fabraea		Mildew		Rust	
Meech's Prolific	United States	Fruit	4.2	(5)	1.7	(3)	2.8	(5)
Miradzhi 88-2	Turkmenistan	Fruit	1.4	(5)	3.7	(3)	1.4	(5)
Myakoplodnaya	Ukraine	Fruit	3.0	(2)	3.0	(2)	2.0	(2)
Pigwa S-1	Poland	Rootstock	7.2	(5)	3.0	(3)	1.8	(5)
Pigwa S-1	Poland	Rootstock	6.0	(5)	5.0	(3)	1.4	(5)
Pigwa S-2	Poland	Rootstock	2.6	(5)	3.0	(3)	1.0	(5)
Pigwa S-3	Poland	Rootstock	5.8	(5)	2.3	(3)	1.6	(5)
Pigwa S-3	Poland	Rootstock	2.3	(3)	3.0	(3)	1.0	(3)
Pillnitz 1	Germany	Rootstock	4.6	(5)	3.0	(3)	2.2	(5)
Pillnitz 2	Germany	Rootstock	5.0	(5)	3.0	(3)	1.4	(5)
Pillnitz 3	Germany	Rootstock	5.0	(5)	3.0	(3)	1.4	(5)
Pillnitz 5	Germany	Rootstock	5.4	(5)	2.3	(3)	1.8	(5)
Pineapple	California	Fruit	3.2	(5)	2.3	(3)	1.4	(5)
Portugiesische	Portugal	Fruit	4.2	(5)	2.3	(3)		
BA-29-C	France	Rootstock	4.2	(5)	4.3	(3)	1.0	(5)
Provence (BA-29)	France	Ornamental	4.0	(5)	2.3	(3)	1.4	(5)
Pseudocydonia	Chinese	Ornamental	4.0	(5)	2.3	(3)	1.4	(5)
Quince—Angers, France	France	Rootstock	6.4	(5)	2.3	(3)	1.4	(5)
Quince—OSU Medford	France	Rootstock	5.2	(5)	4.3	(3)	1.8	(5)
Quince A Quince A	United Kingdom	Rootstock	5.0 3.6	(5) (5)	3.0 4.3	(3) (3)	1.8 1.0	(5) (5)
Quince C7/1	United Kingdom	Rootstock	5.0	(5)	3.7	(3)	1.8	(5)
Quince E	United Kingdom	Rootstock	4.4	(5)	4.3	(3)	1.8	(5)
Quince S (=Pigwa S-1)	United Kingdom	Rootstock	7.0	(5)	3.0	(3)	1.4	(5)
Quince W	United Kingdom	Rootstock	4.4	(5)	2.3	(3)	1.6	(5)
Rannyaya from Tange	United Kingdom Turkmenistan	Fruit	2.3	(3)	1.7	(3)	1.7	(3)
Rich	Oregon		2.3	(3)	1.0	(3)	1.7	(3)
Seker Gevrek	Turkey	Fruit	2.8	(5)	1.0	(3)	1.4	(5)
Shams	Iran	Fruit	2.2	(5)	3.7	(3)	1.8	(5)
Shevlan	Turkmenistan	Fruit	5.0	(5)	4.3	(3)	1.6	(5)
Skorospelka O.P. sdlg 1	Russia	Seedling	3.4	(5)	3.7	(3)	4.2	(5)
Skorospelka O.P. sdlg 2	Russia	Seedling	5.0	(5)	3.7	(3)	1.4	(5)
Skorospelka O.P. sdlg 3	Russia	Seedling	6.2	(5)	2.3	(3)	2.2	(5)
Tashkent AR-232 sdlg 2	Uzbekistan	Fruit	3.8	(5)	6.3	(3)	1.8	(5)

Name	Origin	Type	Fabraea		Mildew		Rust	
Tashkent AR-232 sdlg 3	Uzbekistan	Fruit	2.6	(5)	6.3	(3)	2.6	(5)
Tashkent AR-232 sdlg 4	Uzbekistan	Fruit	3.8	(5)	5.7	(3)	2.6	(5)
TE-2-73	Turkmenistan	Fruit	2.3	(3)	1.0	(3)	1.7	(3)
Tekes	Turkey	Fruit	4.6	(5)	1.0	(3)	1.8	(5)
Tencara Pink	United States	Fruit	2.2	(5)	5.7	(3)	1.4	(5)
Teplovskaya O.P. sdlg	Russia	Seedling	5.0	(5)	3.7	(3)	1.4	(5)
Teplovskaya O.P. sdlg	Russia	Seedling	3.0	(2)	2.0	(2)	3.0	(2)
Trentholm	Oregon	Seedling	4.6	(5)	3.0	(3)	1.6	(5)
V-46.O.P	Bulgaria	Fruit	2.2	(5)	5.7	(3)	1.4	(5)
W-4	France	Rootstock	5.0	(5)	4.3	(3)	1.4	(5)
WF-17	France	Rootstock	4.4	(5)	2.3	(3)	1.2	(5)
Yuz-Begi 83-4	Turkmenistan	Fruit	3.0	(5)	1.0	(3)	2.6	(5)
Yuz-Begi 89-1	Turkmenistan	Fruit	6.2	(5)	3.7	(3)	1.2	(5)
Zeakli 89-1	Turkmenistan	Fruit	1.7	(3)	3.0	(3)	2.3	(3)

Source Adopted from Postman (2008)
Mean disease rating for number of years in parentheses

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Part II
Subtropical Fruits

Chapter 8
Cactus Pear (*Opuntia* spp.) Breeding

Mouaad Amine Mazri

Abstract Cactus pear (*Opuntia* spp.) belongs to the family Cactaceae which is composed of about 130 genera and 2000 species. It is native to the tropical and subtropical regions of America but can be found in different regions of the world with various climate conditions. The fruit is an ovoid-spherical berry that, at maturity, may vary in color. *Opuntia ficus indica* is the most economically important and widely cultivated species of this genus. Cactus pear is an economically and ecologically important crop with various uses. It is cultivated for human consumption since its fruits are rich in carbohydrates, vitamins, proteins, minerals and antioxidants and have anti-cancer, anti-inflammatory, anti-viral, anti-diabetic and neuroprotective properties. The juice is rich in glucose and fructose and has a potent antioxidant activity. The cladodes of some species are edible as vegetables in salads. They are also used to feed cattle in arid and semiarid lands. The plants are well adapted to drought, high temperatures and low water availability and are able to absorb and hold CO_2 excess from the atmosphere. Cactus pear is mainly propagated by cladodes. The breeding programs of this genus aim at the development of spineless cultivars with cold tolerance, high yield and fruit quality, and resistance to diseases and pests. To date, biotechnological tools have been scarcely used for cactus pear improvement. In the present chapter, the main problems, challenges, strategies and objectives of cactus pear breeding are described, and the recent achievements in terms of biotechnology and molecular biology are reported.

Keywords Biotechnology · Breeding · Characterization · Fruit · *Opuntia* spp.

M. A. Mazri (✉)
Institut National de la Recherche Agronomique, CRRA de Marrakech, UR Agro-Biotechnologie, BP 533 Marrakech, Morocco
e-mail: m.a.mazri@gmail.com

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8.1 Introduction

8.1.1 Botanical Classification and Distribution

The genus *Opuntia* belongs to the family Cactaceae and order Caryophyllales. The family contains approximately 130 genera and 2000 species with different ploidy levels (Pinkava and Mcleod 1971; Segura et al. 2007; Shedbalkar et al. 2010). The *Opuntia* species have fleshy stems (cladodes) with spines or glochids arranged in areoles. The leaves are mostly small, sometimes absent, since the stems function in photosynthesis. The flower is usually solitary and has numerous sepals and petals. The fruit is an ovoid-spherical berry that consists of pulp, peel and seeds, and that varies in color (yellow, orange, green, red or purple; Fig. 8.1). The root system is usually extensive and close to the soil surface, and the species are characterized by a thick and waxy outer surface cuticle that prevents water loss in dry climate conditions (Glimn-Lacy and Kaufman 2006; Kuti 2004). The plants of this genus are referred to as bunny ears, prickly pear and cactus pear. The fruits and the edible cladodes of the domesticated cultivars are known as tunas and nopales, respectively (Segura et al. 2007). *Opuntia ficus indica* is probably the most economically important and widely cultivated species (Caruso et al. 2010).

The genus *Opuntia* is native to the tropical and subtropical regions of America (Sáenz 2013a). Its cultivation dates back to the ancient Mesoamerican civilizations and specifically to the Aztec culture (Sáenz 2013a). It is believed that the plants of this genus were brought to Spain by Christopher Columbus then spread throughout the European and African countries of the Mediterranean basin (Russell and Felker 1987). In the seventeenth century, cactus pear was brought to Australia from Brazil to produce natural red dyes, contained in the cochineal scale insect (*Dactylopius opuntiae*) living on the cladodes, which was believed to be suitable for the Australian

Fig. 8.1 Fruits of cactus pear with different colors

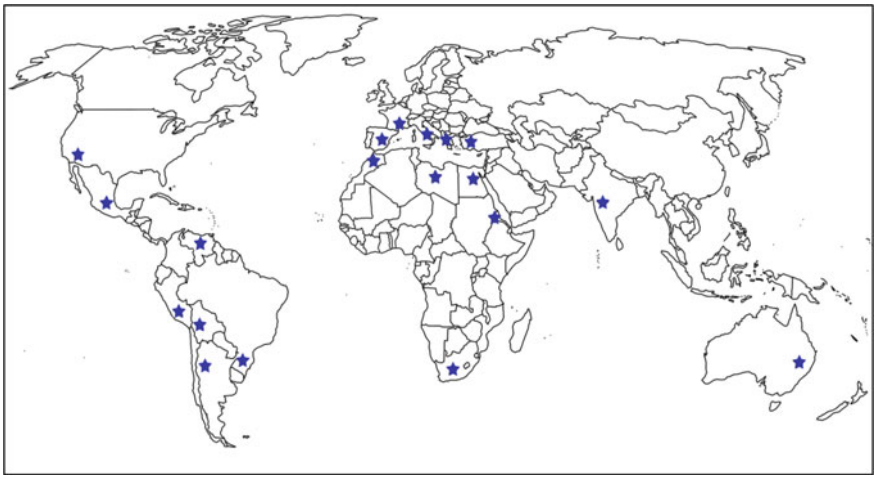

Fig. 8.2 Geographic distribution of cactus pear. *Source* Sáenz (2013a)

economy (Mondragón-Jacobo 2001b). The species *O. ficus indica* was introduced to South Africa more than 250 years ago (Zimmermann and Moran 1991).

Cactus pear species are mostly grown in arid and semiarid regions, but can be found in many areas in the world with different climate conditions (Fig. 8.2). They are cultivated in more than 30 countries (Jiménez-Aguilar et al. 2014), including Argentina, Bolivia, Colombia, Peru, Venezuela, Italy, Morocco, Tunisia, Algeria, and South Africa among others. The most cultivated species for fruit production are *Opuntia ficus indica*, *O. amyclaea*, *O. xoconostle*, *O. megacantha*, *O. streptacantha*, *O. robusta*, and *O. hyptiacantha* (Sáenz 2013a; Yahia and Sáenz 2017). Mexico produces approximately 44% (428,300 mt per year) of the world cactus pear production, estimated at 973,000 mt per year. Other producing countries include Tunisia (12.8%), Argentina (7.7%), Algeria (7.7%) and Italy (6.6%) (Jiménez-Aguilar et al. 2014, 2015). Italy is today the world largest exporter of cactus pear fruits to the European Union (Timpanaro and Foti 2014).

8.1.2 Importance and Uses

Cactus pear is an economically and ecologically important crop with various uses. It is cultivated for human consumption and can be consumed as a fresh fruit or in the form of beverage. It can also be used as a nutritional supplement or additive in food. The fruit, which is a berry, is delicious, healthy and nutritious. The cladodes of some species are also edible. Cactus pear plants are also used for pharmaceutical and cosmetic purposes and as forage.

In arid and semiarid regions, *Opuntia* species can play a major role in sustaining livelihood, generating employment and reducing poverty. They are also an essential source of income for producers and exporters. The fruits of cactus pear are highly nutritious (Table 8.1). They are rich in carbohydrates, vitamins, proteins, minerals, dietary fiber, pigments, phenolic compounds and amino acids and form an important part of dietary requirements of people in many countries (Shetty et al. 2012). A detailed review of the phytochemical content of cactus pear fruits has been recently published by Yahia and Sáenz (2017), who reported that the chemical composition of fruits and cladodes varies depending on species and climatic conditions. The fruit juice is very rich in glucose and fructose and has a potent antioxidant activity (Abdel-Hameed et al. 2014). El Kharrassi et al. (2016) investigated the fruit and juice characteristics of various *Opuntia* genotypes grown in Morocco. They found major differences between Moroccan genotypes and cactus pears grown in other countries in terms of fruit weight, juice pH and titratable acidity, and concluded that the Moroccan accessions seem particularly suitable for juice production. The immature fruit is also edible as mock-gherkin (Shetty et al. 2012). In addition to fresh consumption, a number of food products can be derived from cactus pear fruit including jams, syrups and alcoholic drinks among others. The cladodes are a source of dietary fiber. They are used as natural ingredient in foods after dehydration and grinding to powder and, in some countries, they are consumed as a vegetable in salads (Shetty et al. 2012; Yahia and Sáenz 2017).

Species of the genus *Opuntia* are of enormous agronomic and ecologic importance. Cactus pear is easy to establish and maintain, and produces fruits of high nutritional quality for local and international markets, with up to 25 tons/ha (Nefzaoui et al. 2014). It can be cultivated in arid and semiarid regions with no or little irrigation, since it has a wide range of anatomical and physiological characteristics to conserve water, which allow it to reach acceptable productivity levels even in years of severe drought. Besides, cactus pears play significant roles in revegetating arid and semiarid lands, and in slowing down deforestation, environmental destruction, biodiversity loss, soil degradation and erosion (Nefzaoui et al. 2014; Paiva et al. 2016; Sáenz 2013a; Shetty et al. 2012). Cactus pear species can be used as an alternative food in extremely arid and hot environments, and as a live fence to protect agricultural fields (Shetty et al. 2012). In addition, farmers can cultivate *O. ficus indica* as an emergency forage under drought conditions (Paiva et al. 2016). Sáenz (2013a) reported that cactus pear plants can be valuable against the increase of atmospheric CO_2 caused by deforestation and large-scale use of fossil, since they are able to absorb and hold CO_2 excess in areas where they can be established but where nothing else will grow.

Cactus pear is also used in pharmaceutical industry and medicine since it has anticancer, anti-inflammatory, antiviral, anti-diabetic and neuroprotective properties, and can prevent human degenerative diseases due to its antioxidant activity. Shetty et al. (2012) and Yahia and Sáenz (2017) reported that fruits and other parts of cactus pear can be used to treat burn wounds, edema and indigestion. They can also be used to enhance antioxidant defense, inhibit cancer cell growth and proliferation and

Table 8.1 Chemical and phytochemical composition of the fruit (pulp, pulp juice or pulp oil) of some cactus pear species

Species	Country	Compound	Quantity	Edible product	References
O. dillenii	Spain	Sodium (mg/kg)	153	Pulp	Medina et al. (2007)
O. dillenii	Spain	Potassium (mg/kg)	908	Pulp	Medina et al. (2007)
O. dillenii	Spain	Calcium (mg/kg)	535	Pulp	Medina et al. (2007)
O. dillenii	Spain	Magnesium (mg/kg)	454	Pulp	Medina et al. (2007)
O. dillenii	Spain	Iron (mg/kg)	1.53	Pulp	Medina et al. (2007)
O. dillenii	Spain	Copper (mg/kg)	0.33	Pulp	Medina et al. (2007)
O. ficus indica	KSA	Glucose (g/100 ml juice)	1.45–3.17	Pulp juice	Abdel-Hameed et al. (2014)
O. ficus indica	KSA	Fructose (g/100 ml juice)	0.51–3.04	Pulp juice	Abdel-Hameed et al. (2014)
O. ficus indica	KSA	Total phenolics (mg gallic acid equivalent/ 100 ml juice)	667.82–1065.15	Pulp juice	Abdel-Hameed et al. (2014)
O. ficus indica	KSA	Total flavonoids (mg rutin equivalent/ 100 ml juice)	80.35–159.49	Pulp juice	Abdel-Hameed et al. (2014)
O. ficus indica	KSA	Total flavonols (mg rutin equivalent/100 ml juice)	160.02–341.81	Pulp juice	Abdel-Hameed et al. (2014)
O. megacantha	Morocco	Total carotenoids (μg/L)	4.8–18.6	Pulp juice	El Kharrassi et al. (2016)
O. megacantha	Argentina	Total betalains (μg/g)	15.1–131.4	Pulp	Coria Cayupàn et al. (2011)
O. megacantha	Argentina	Total chlorophylls (μg/g)	15.8–56.5	Pulp	Coria Cayupàn et al. (2011)
O. ficus indica	Morocco	Vitamin C (mg/L)	11.1–29.2	Pulp juice	El Kharrassi et al. (2016)

(continued)

Table 8.1 (continued)

Species	Country	Compound	Quantity	Edible product	References
O. ficus indica	Germany	Vitamin K1 (g/kg)	0.532	Pulp oil	Ramadan and Mörsel (2003)
O. ficus indica	Italy	Total vitamin E (μg/100 g)	111.5–115	Pulp	Tesoriere et al. (2005)
O. ficus indica	Italy	α-Tocopherol (μg/100 g)	67–69	Pulp	Tesoriere et al. (2005)
O. ficus indica	Italy	γ-Tocopherol (μg/100 g)	29–32	Pulp	Tesoriere et al. (2005)
O. ficus indica	Italy	σ-Tocopherol (μg/100 g)	14–16	Pulp	Tesoriere et al. (2005)
O. ficus indica	Germany	Campesterol (g/kg)	8.74	Pulp oil	Ramadan and Mörsel (2003)
O. ficus indica	Germany	Stigmasterol (g/kg)	0.73	Pulp oil	Ramadan and Mörsel (2003)
O. ficus indica	Germany	Lanosterol (g/kg)	0.76	Pulp oil	Ramadan and Mörsel (2003)
O. ficus indica	Germany	β-Sitosterol (g/kg)	11.2	Pulp oil	Ramadan and Mörsel (2003)
O. ficus indica	Germany	Δ^5-Avenasterol (g/kg)	1.43	Pulp oil	Ramadan and Mörsel (2003)

reduce the serum cholesterol level. In addition, they are useful for patients with lipid metabolism or gastrointestinal disorders.

Cactus pear is used as animal forage. The nutritional and good rumen fermentation characteristics of *Opuntia* species as well as their capacity to grow under drought conditions makes them a good food for animals raised in dry regions (Andrade-Montemayor et al. 2011; Nefzaoui et al. 2014; Vazquez-Mendoza et al. 2017). Cladodes of cactus pear have a high water, fiber and ash content. Accordingly, many researchers have recommended their use to feed cattle in arid and semiarid lands. For example, Andrade-Montemayor et al. (2011) suggested their use to feed goats in semiarid regions since they can provide a large proportion of their water requirements. Likewise, Costa et al. (2012) suggested to use cactus pear cladodes as a part of the diet of Santa Inês lambs since they favor high digestibility of nutrients and reduce the voluntary intake of water. Generally, spineless cladodes are preferred. However, the spines can be burned with propane torches before feeding the cattle (Shetty et al. 2012).

Cactus pear plants are also used in cosmetic industry since many products such as soaps, shampoo, creams, lotions and face masks are made from them (Sáenz 2013b).

8.1.3 Domestication, Selection and Early Improvements

Cactus pear has been grown for thousands of years and is one of the oldest plants cultivated in Mexico (Mondragón-Jacobo 2001b). According to Nobel (1994), there are evidences that cactus pear has been cultivated and used by people for many purposes, including consumption, for more than 9000 years. Hoffman (1995) indicated that cactus pear domestication started before 6000 BC. The best wild species were selected by the ancient growers and cultivated in family gardens (Mondragón-Jacobo 2001b). It is believed that spineless cactus pear species are the results of domestication. In fact, spineless species do not exist among wild populations. The ancient growers aimed to cultivate spineless cactus pear species since the presence of spines hampers the management and utilization of the plants and their fruits (Mondragón-Jacobo 2001b; Mondragón-Jacobo and Pérez-Gonzalez 2001). As regards to *Opuntia ficus indica*, the most commercially-important cactus pear species, it was reported that it was domesticated by the ancient Mexicans and dispersed throughout the world by European travelers in the fifteenth and the sixteenth centuries (Griffith 2004; Kiesling 1998). Labra et al. (2003) suggested that *O. ficus indica* is a domesticated form of *O. megacantha* (Fig. 8.3). According to Zimmermann and Moran (1991), *O. ficus indica* was introduced to South Africa more than 250 years ago. Reyes-Agüero et al. (2005) classified some cactus pear species according to their domestication gradient, and reported that *O. ficus indica* and *O. albicarpa* are the most domesticated of all, whereas *O. streptacantha*, *O. hyptiacantha*, *O. joconostle* and *O. leucotricha* are wild in the extreme.

This chapter presents the main problems, challenges and objectives of cactus pear (*Opuntia* spp.) breeding, and highlights the latest advances made in this field. Accordingly, recent achievements in areas such as micropropagation, genetic diversity assessment and species characterization through molecular markers and other descriptors, germplasm conservation, functional genomics and genetic engineering are reported.

8.2 Cultivation and Traditional Breeding

8.2.1 Current Cultivation Practices

Cactus pear can be propagated through different methods: by seeds or vegetatively either through in vitro culture techniques or by cladodes. Propagation by seeds is not preferred since it results in genetic segregation, long juvenility and slow

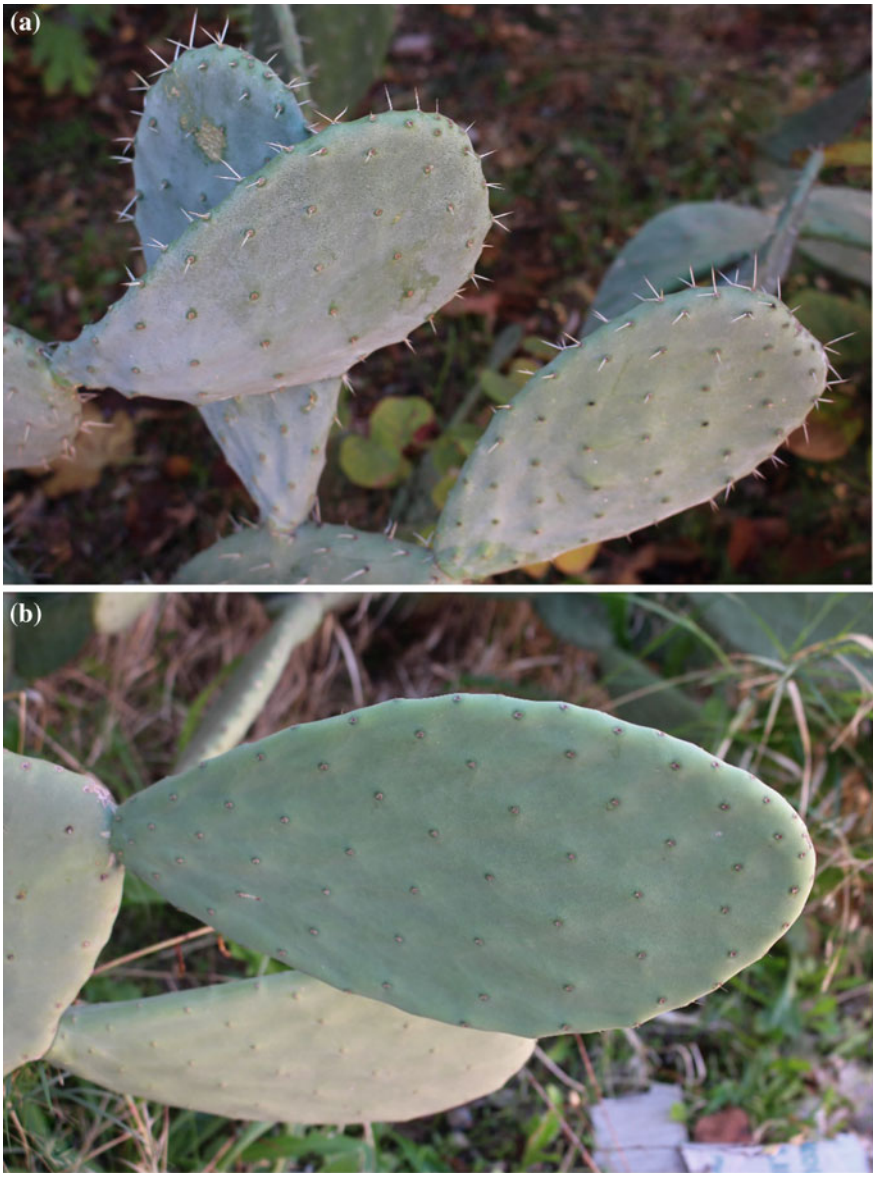

Fig. 8.3 Cladodes of young plants of **a** *Opuntia megacantha*, **b** *O. ficus indica* widely suggested as a domesticated form of *O. megacantha*

growth (Mohamed-Yasseen et al. 1996). In vitro propagation can be done through either somatic embryogenesis or organogenesis, but the most common propagation method is through the use of cladodes. In this case, segments or whole cladodes are

used (Mondragón-Jacobo and Bordelon 1996). The cladode contains areoles which are the basic meristematic unit in cactus pear, and from which a new shoot can be developed. Briefly, cladodes are dried in a shaded and aerated place for 2 weeks then they are planted in the soil. The planting density depends on soil and climate conditions as well as crop management practices. Generally, the rooting process is initiated soon after plantation (Mondragón-Jacobo et al. 2001), while the size of the cladodes does not affect the rooting ability (Mondragón-Jacobo and Pimienta-Barrios 1995). Soil moisture is important for rooting (Mondragón-Jacobo et al. 2001), and a plant growth regulator, for example 1-naphthaleneacetic acid (NAA), may be used to improve rooting ability (Mulas et al. 1992). Cactus pear plants have shown great adaptability to various soil conditions and are present in different types of soils, such as vertisols and luvisols in Mexico, and regosols, lithosols, cambisols and fluvisols in Italy (Paiva et al. 2016). Dubeux et al. (2006) reported that increasing *Opuntia ficus indica* plant population, as well as fertilizing with N and P, resulted in greater productivity. Singh and Singh (2003) suggested to plant in spring. A whole cladode is able to produce at least one layer of new shoots per year, which contains 2–6 cladodes (Mondragón-Jacobo et al. 2001).

Fruit production starts 2–3 years after planting while full production is reached after 7 years (Mohamed-Yasseen et al. 1996). Flowering and fruit growth and maturity varied considerably depending on the species and environmental conditions (El Kharrassi et al. 2015). The marketability, crunchiness and shelf life of the harvested fruit depends on the harvest season (seasonal or late) and the ripeness stage at harvest (Allegra et al. 2015).

8.2.2 Current Agricultural Problems and Challenges

One of the main problems affecting cactus pear cultivation is the high perishability of the fruits. It is well-known that cactus pear fruits are highly perishable, with only few days of shelf life under marketing conditions (20 °C, 60–70% RH), while they are susceptible to chilling injury when stored at temperatures below 9–10 °C (Rodriguez-Felix 2002). Moreover, the composition of cactus pear fruit and its consistency make it highly susceptible to decay caused by pathogenic fungi, yeast and bacteria (Potgieter and D'Aquino 2017).

The development of spineless cultivars is one of the main objectives of cactus pear breeding. In fact, spines in cactus pear plants and fruits hamper harvest operations and postharvest processing and management. Another objective of cactus pear breeding is the development of cold-tolerant genotypes. This is necessary for cactus pear expansion in cold regions.

Disease and insect susceptibility is another issue in cactus pear cultivation. In Morocco, the major problem currently affecting cactus pear is the cochineal (*Dactylopius opuntiae*). This insect has been responsible for killing young and old cactus pear plants in Rhamna and Doukkala regions since 2015. Consequently, infested plants (Fig. 8.4) have been removed and burned while chemical treatment has also

been in use. In addition, researchers of the National Institute of Agronomic Research of Morocco (INRA-Morocco) are currently carrying out investigations to control this pest through the selection of resistant genotypes of high fruit yield and quality and by developing biological means of control.

8.2.3 Improvement of Strategies

The high agronomic, economic, ecological and nutritional values of cactus pear and the recent progress in genetics and biotechnology have led scientists and researchers to carry out investigations using modern research strategies. The current strategies to improve cactus pear cultivation involve the use of biotechnological tools. Tissue culture and micropropagation techniques such as somatic embryogenesis and organogenesis have been used for many purposes, including rapid and large-scale propagation of selected genotypes, to combat desertification, for production of secondary metabolites, preservation of endangered genotypes, in vitro conservation of germplasm and genetic transformation, among others. Various plant growth regulators and culture conditions were evaluated, and reliable and reproducible regeneration systems were established. Protocols for genetic transformation have also been developed for cactus pear through either *Agrobacterium tumefaciens* or biolistics, which can be used as powerful tools to introduce agronomically-interesting genes to achieve the breeding objectives of this genus.

Although DNA isolation from cactus pear plants is quite difficult, molecular marker analyses have been widely performed by researchers from different countries. Molecular markers such as random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) and simple sequence repeat (SSR) have been used to assess the genetic diversity within and among cactus pear species, to evaluate the genetic relationship among them, to elucidate their origin, to contribute to germplasm evaluation, characterization and conservation, and for selection and breeding programs.

Since the nutritional and medicinal values of the fruits and cladodes of cactus pear are established, phytochemical studies of plants of this genus have attracted much attention in the recent years. Such strategies aim to characterize species and to identify compounds with nutritional and medicinal properties. The phytochemical analysis of cactus pear fruits and cladodes revealed the presence of many nutrients and pharmaceutical compounds.

8.2.4 Traditional Breeding Methodologies and Limitations

The main objectives of cactus pear breeding are as follows: out-of-season production; cold tolerance; disease and pest resistance; developing spineless and glochid-free cultivars or at least reducing the number of spines and glochids; production of yellow-

Fig. 8.4 Cactus pear attacked by the cochineal insect (*Dactylopius opuntiae*)

orange and red-purple large fruits with high nutritional value, high soluble-sugar content, improved flavor, low number of seeds and juicy pulp; reducing the mucilage content; reducing the cuticle; improving postharvest life and resistance to handling and packing (Cruz-Hernández and Paredes-López 2010; Mondragón-Jacobo and Bordelon 1996).

Since natural hybridization is common in the genus *Opuntia*, the ancient breeders of cactus pear selected cladodes from the most vigorous plants and used them in their orchards for natural cross pollination, which was followed by selection from the new generated genetic material (Flores-Valdés and Olvera 1996; Pimienta-Barrios 1990). Traditional breeding programs by crossing and selection are based on the exploration of the cultivated and wild gene pool. The conventional technique has been fully described in the literature (e.g. Mondragón-Jacobo 2001b). However, the breeding programs using seeds are hampered by long selection and juvenile periods, genetic segregation and slow growth (Cruz-Hernández and Paredes-López 2010). Moreover, apomixis, which is a widespread phenomenon in cactus pear, complicates the genetic studies and the screening of plants obtained from crosses (Mondragón-Jacobo 2001a).

8.2.5 Role of Biotechnology

Considering the high importance of cactus pear, biotechnological tools such as in vitro culture and molecular markers would be of great interests for different improvement programs. In the recent years, many researchers have attempted to develop in vitro culture systems for cactus pear, and that was mainly through somatic embryogenesis and organogenesis. Somatic embryogenesis is the in vitro process by which somatic cells develop into somatic embryos, which, after maturation, will be converted into fully developed plants (Mazri and Meziani 2015; Mazri et al. 2017). Somatic embryos can be formed directly on the explant or indirectly after callus induction. Organogenesis is the in vitro process by which adventitious organs such as buds, shoots and/or roots are formed on the explant. The formed organs have a vascular connection with the mother tissue and their isolation required the use of a blade (Mazri and Meziani 2015; Mazri et al. 2013). These two in vitro regeneration pathways have been used for various purposes, e.g. rapid and large-scale propagation, genetic transformation, in vitro conservation of genetic resources and metabolite production. In addition, molecular markers have been used to assess genetic diversity, relationships, and origins of cactus pear species and accessions. This latter point will be addressed in Sect. 8.3.1 of this chapter.

Khalafalla et al. (2007) reported a successful micropropagation system for *Opuntia ficus indica* through organogenesis, using young cladode explants containing one areole. High shoot multiplication and rooting were reported and plants were successfully established in soil. The developed protocol was suggested for massive propagation of *O. ficus indica* as a tool to combat desertification. Angulo-Bejarano and Paredes-López (2011) developed a reliable regeneration system by indirect organo-

genesis in *O. ficus indica* cv. Blanco sin Espinas which may be used for genetic transformation of this cultivar. They reported that the regenerated plants did not show any phenotypic differences with normal plants in terms of height, cladode size and shape. Zoghlami et al. (2012) used the axillary branch micropropagation technique for the proliferation of *O. ficus indica* cv. Gialla. The reported regeneration pathway resulted in a very low level of genetic variability even after more than 5 years of in vitro culture, suggesting that this multiplication method can be used for the safe in vitro conservation of the interesting genetic resources of cactus pear. Gomes et al. (2006) reported an efficient regeneration protocol for *O. ficus indica* cv. Gigante through direct somatic embryogenesis. Shoot apices isolated from in vitro grown shoots were used as explants and it was reported that the type, age, physiological and developmental stage of the explants, as well as the different culture conditions, affect somatic embryogenesis. The regenerated plants rooted well and no apparent differences were observed between them and the mother plants. Jedidi et al. (2015) reported somatic embryogenesis from ovules of *O. ficus indica*, extracted from flowers 10 days after anthesis. Somatic embryos were formed indirectly after callus induction while secondary somatic embryogenesis was also observed during the development of primary somatic embryos. The conversion of somatic embryos into plantlets was achieved successfully. Robles-Martínez et al. (2016) established callus and cell suspension from seeds of *O. streptacantha* cv. Tuna Loca, *O. megacantha* cv. Rubi Reina and *O. ficus indica* cv. Rojo Vigor and evaluated their metabolite production potential. It has been found that the concentration of metabolites in callus and cellular suspensions was higher compared with cladodes. Accordingly, these authors suggested that in vitro culture of cactus pear species is a practical alternative for the production of metabolites under controlled conditions. Camarena-Rangel et al. (2017) reported that metabolite content of calli from these same cultivars can be improved after exposure to jasmonic acid, drought or UV light.

In addition to these in vitro culture techniques, micrografting was also reported in cactus pear. Estrada-Luna et al. (2002) used 5 cactus pear species: *Opuntia streptacantha*, *O. robusta*, *O. cochinera*, *O. leucotricha* and *O. ficus indica* as rootstocks and combined them with *O. ficus indica* to produce homografts (grafts between same species) and heterografts (grafts between different species). It has been found that horizontal grafting was the easiest and most successful method for cactus pear micrografting and that vascular connections occurred within the callus bridge between rootstocks and scions within 28 days. In addition, homografts grew significantly faster than the heterografts 90 days after ex vitro transfer. It has been concluded that the in vitro horizontal graft of cactus pear is a successful, easy and reliable method to graft micropropagated plants and that this grafting technique has a potential application in large-scale and commercial production of cactus pear plants.

8.3 Germplasm Biodiversity and Conservation

8.3.1 Germplasm Diversity

The genetic diversity within and among cactus pear species has been investigated by researchers from different countries using different types of markers in order to characterize and determine relationships between genotypes, and for breeding and genetic resource conservation programs (Table 8.2). According to Labra et al. (2003), phenotypical characterization of cactus pear species cannot serve to produce a stable classification due to the large morphological variation among species. Molecular markers represent a strong tool to assess genetic diversity since they are not influenced by the environment (Valadez-Moctezuma et al. 2015). Generally speaking, various reports on the genetic diversity within and among cactus pear species using molecular markers have been published. In this section, a review of some of the most recent studies is presented.

In Greece, Ganopoulos et al. (2015) evaluated the genetic diversity among 22 accessions of *Opuntia ficus indica* using 6 ISSR markers. A total of 57 bands were generated with approximately 50% polymorphism, and a high genetic variability was revealed in the Greek germplasm.

In Morocco, the genetic diversity within and among 7 cactus pear species (124 accessions) from different regions was evaluated using morphological traits and molecular markers (El Kharrassi et al. 2017). ISSR markers showed 64 polymorphic bands (96.9% polymorphism) while RAPD markers exhibited 6 polymorphic bands (100% polymorphism). The main finding of this study was the high genetic similarity between the accessions of *O. ficus indica* and *O. megacantha* in Morocco. In a different study, El Finti et al. (2016) evaluated the genetic variability of 13 genotypes of *O. ficus indica* collected from different regions of Morocco, using 16 SSR markers. These authors reported a high level of genetic diversity among genotypes, and that some markers can be used for genetic identification.

In Italy, Labra et al. (2003) used chloroplast simple sequence repeat (cpSSR) and amplified fragment length polymorphism (AFLP) markers to evaluate the genetic relationship among 11 cactus pear species and 6 populations of *Opuntia ficus indica* and *O. megacantha*. These authors revealed the high genetic similarity between *O. ficus indica* and *O. megacantha* and their difference from the other cactus pear species, and suggested that they should be considered as a unique and distinct taxon. In addition, based on molecular data, morphological traits and biogeographical distribution, they concluded that *O. ficus indica* should be considered as a domesticated form of *O. megacantha*, and that a revision of *Opuntia* genus classification using molecular, morphological and biogeographical analysis should be carried out. Zarroug et al. (2015a) evaluated the genetic variability among 62 cactus pear accessions grown in Italy, but some of them originated from other countries. Ten SSR markers were used and a medium to high levels of polymorphism were reported.

In addition to the above results obtained on Moroccan and Italian accessions, the close genetic relationship between *Opuntia ficus indica* and *O. megacantha* has been

Table 8.2 Examples of molecular markers used to assess the genetic diversity within and among cactus pear species in different countries

Marker type	Genus/species	Number of genotypes	Country	References
RAPD	*O. ficus indica*	28	Tunisia	Bendhifi et al. (2013)
RAPD ISSR	*O. ficus indica* *O. megacantha* *O. robusta* *O. aequatorialis* *O. dillenii* *O. leucotricha* *O. inermis*	22	Morocco	El Kharrassi et al. (2017)
SSR	*O. ficus indica*	13	Morocco	El Finti et al. (2016)
RFLP	*Opuntia* spp.	103	Mexico	Samah et al. (2016a)
RAMPO	*O. ficus indica* *O. en-gelmannii* *O. tomentosa* *O. undulata* *O. ellisiana* *O. streptacantha* *O. robusta*	25	Tunisia	Zarroug et al. (2015b)
RAPD ISSR	O. *megacantha* *O. streptacantha* *O. hyptiacantha* *O. affinis* *O. lasiacantha* *O. undulata* *O. joconostle* *O. matudae* *O. robusta* *O. albicarpa* *O. ficus indica* *O. chaveña*	52	Mexico	Valadez-Moctezuma et al. (2015)
SSR	*O. chaveña* *O. cochenillifera* *O. cochinera* *O. ficus indica* *O. hyptiacantha* *O. joconostle* *O. lasiacantha* *O. leucotricha* *O. megacantha* *O. robusta* *O. rzedowskii* *O. streptacantha* *O. undulata* *O. matudae*	88	Mexico	Samah et al. (2016b)

(continued)

Table 8.2 (continued)

Marker type	Genus/species	Number of genotypes	Country	References
cpSSR AFLP	*O. tuna* *O. humifusa* *O. monacantha* *O. stricta* *O. dillenii* *O. ficus indica* *O. undulata* *O. spinulifera* *O. megacantha* *O. amyclaea* *O. robusta*	17	Italy	Labra et al. (2003)
SSR	*Opuntia* spp. *O. ficus indica* *O. leucosarca* *O. litoralis* *O. pelosa* Hybrids	62	Italy	Zarroug et al. (2015a)

revealed in Mexico as well. Valadez-Moctezuma et al. (2015) assessed the genetic diversity of 52 Mexican cactus pear genotypes belonging to 12 different species using RAPD and ISSR markers. A total of 179 RAPD bands, of which 86 were polymorphic, and a total of 158 ISSR bands, of which 70 were polymorphic, were generated, and a wide genetic variation was revealed. These authors suggested that *O. ficus indica*, *O. albicarpa* and *O. megacantha* possibly have common ancestry, and that there is a smaller number of *Opuntia* species compared to those currently known, but with high intraspecific genetic variation. Samah et al. (2016a) evaluated the genetic diversity of 103 cactus pear accessions from Mexico using 9 restriction fragment length polymorphism (RFLP) markers. The results of this study showed that 37 fragments out of 136 were polymorphic (27.2%), and a close relationship between the studied accessions was revealed. Here again, it has been suggested that the number of *Opuntia* species in Mexico should be reviewed and that genomic sequencing should be performed. In a different study, Samah et al. (2016b) assessed the genic diversity of 88 Mexican cactus pear accessions belonging to different species, using 18 SSR markers. They reported that the delimitation of species in this genus is incorrect, and that *O. ficus indica*, *O. albicarpa*, *O. megacantha*, *O. streptacantha*, *O. lasiacantha*, and *O. hyptiacantha* have no clear boundaries.

In Tunisia, the genetic diversity of 28 *Opuntia ficus indica* ecotypes using morphological parameters and RAPD markers was evaluated. The RAPD analysis showed 41 polymorphic bands (73.21%), which reveals a strong difference among accessions, and a positive significant correlation was observed between morphological traits and RAPD markers (Bendhifi et al. 2013). Zarroug et al. (2015b) studied the genetic diversity among 25 cactus pear genotypes belonging to 7 species, grown in Tunisia but originated from different countries. A total of 16 RAMPO markers (a

combination of RAPD and ISSR markers) were used, resulting in 115 bands out of which 72 were polymorphic. These authors reported a relatively high degree of genetic diversity in the collection studied and suggested the utilization of RAMPO markers as a useful approach to characterize cactus pear germplasm.

In Turkey, Tütüncü et al. (2016) analyzed the genetic diversity of cactus pear plants collected from different regions using 50 RAPD markers. The markers generated 250 bands out of which 180 were polymorphic and a high level of genetic diversity among the Turkish cactus pear genotypes was reported.

8.3.2 Cultivars Characterization

In addition to molecular markers that have been widely used to assess the genetic relationships and diversity within and among cactus pear species, other types of descriptors, mainly the morphological and phytochemical ones, have been used to characterize cactus pear genotypes. The characterization was essentially based on fruits, seeds and cladodes. For example, Bendhifi et al. (2013) used quantitative and qualitative morphological traits to analyze the genetic diversity of Tunisian *Opuntia ficus indica* accessions. The 20 morphological descriptors used, which corresponded to growth of the tree (e.g. vigor, size and shape), cladode (e.g. size and color) and fruit (e.g. shape, weight and color), revealed a strong variability among accessions. El Kharrassi et al. (2017) attempted to characterize 124 2-year-old accessions of 7 Moroccan cactus pear species based on 10 morphological descriptors. Some of the studied parameters showed the following results: the plant height ranged from 19.85 cm in *O. dillenii* to 48.25 cm in *O. ficus indica*; the average number of cladodes per plant reached 7.75 in *O. dillenii*; and the cladode lengths ranged from 13.7 cm in *O. ficus indica* to 37 cm in *O. megacantha*. It was also reported that the species *O. ficus indica, O. dillenii* and *O. inermis* are spineless. In a different work, El Kharrassi et al. (2016) studied the fruit and juice characteristics of 30 Moroccan accessions of *O. ficus indica* and *O. megacantha*, and reported that parameters such as fruit length and weight, fresh and dry skin weight, fresh skin to fruit ratio, and water content in the skin varied significantly within and between the two species. They also reported the following fruit juice characteristic ranges: pH, 3.3–4.8; Brix value, 6.2°–12.6°; titratable acidity, 0.14–0.88 mmol H^+/L; total carotenoids content, 4.8–20.8 mg/L; and vitamin C content, 11.1–29.2 mg/L. A physicochemical characterization has also been carried out on cladodes of 6 Moroccan *Opuntia* species, and significant differences were reported in terms of cladode length (13.75–30.63 cm), width (6.25–17.33 cm), thickness (0.65–1.38 cm), fresh weight (67.50–766.00 g), dry weight (8.75–67.15 g), water content (86.67–92.04%), ash content (12.97–22.08 g/100 g DW), total protein concentration (4.64–11.56 g/100 g DW) and total sugar concentration (3.22–12.51 g/100 g DW; El Kharrassi et al. 2015). Abdel-Hameed et al. (2014) compared the phytochemical, nutritional and antioxidant properties of peels and pulps of two *O. ficus indica* cultivars grown in Saudi Arabia, which produce fruits of different colors (red and yellow). It has been found

that the antioxidant activity and phenolic compounds of the red cultivar were higher than those of the yellow cultivar, while they have almost the same content of copper, sodium and potassium. Accordingly, they suggested the use of these two cultivars in the food industry as powerful natural antioxidants. The seeds of cactus pear have also been used to characterize species and accessions. Samah and Valadez-Moctezuma (2014) used 19 morphological descriptors of fruit seeds (e.g. 100 seeds weight, seed area and seed perimeter) as a tool to characterize 110 Mexican accessions belonging to different cactus pear species. These authors reported that seed variables have a high discriminatory power and can be used to characterize and differentiate cactus pear genotypes. Seed protein content has been used to characterize 102 accessions of various Mexican cactus pear species (Samah et al. 2015). The quantity of total protein varied considerably among the seeds of different accessions, from 1.1 (in *O. ficus indica*) to 7.1 mg/mL (in *O. albicarpa*). Albumins (2.6–11.9 mg/mL) and globulins (2.6–9.5 mg/mL) were the most abundant protein fractions, followed by glutelins (2.3–8.5 mg/mL) and prolamins (1.1–7.9 mg/mL). More recently, a phytochemical characterization of *O. megacantha* seed oil and cladode essential oil from Morocco was reported (El Kharrassi et al. 2018; Table 8.3).

8.3.3 Phylogeny

Many researchers have studied the phylogenetic relationships among cactus pear species. Griffith (2004) investigated the origin of *Opuntia ficus indica* through the use of Bayesian phylogenetic analyses of nrITS DNA sequences, and reported that all 7 specimens of *O. ficus indica* evaluated are included in the same clade with *O. hypiacantha*, *O. leucotricha*, *O. megacantha*, *O. streptacantha* and *O. tomentosa*, and that this clade excluded *O. durangensis* and *O. robusta*. It was suggested that *O. ficus indica* is a crop domesticated from ancestral stock of arborescent, fleshy-fruited plants growing in Central Mexico. Realini et al. (2015) evaluated the genetic and phylogenetic relationships between various cactus pear species from Argentina, Bolivia, Brazil, Paraguay and Uruguay by ISSR genotyping and the sequencing of plastid intergenic spacers *trn*L-*trn*F and *psb*J-*pet*A. Considerable intraspecific variations in *O. aurantiaca*, *O. elata*, *O. discolor* and *O. salmiana* were revealed, and close relationships between *O. salmiana* and *O. schickendantzii*, as well as between *O. anacantha*, *O. aurantiaca*, *O. colubrina* and *O. discolor* were highlighted. Srikanth and Whang (2015) examined the taxonomic position of Korean cactus pear species based on DNA sequence analysis of *matK*, *trnL-F*, *atpB-rbcl*, and ITS regions. They found that *O. humifusa* f. *jeollaensis* belongs to the Macrocentra clade rather than the Humifusa clade, and that its genetic distance with *O. camanchica* was the lowest among all *Opuntia* species evaluated. In addition, *O. ficus indica* from Korea was genetically close to *O. engelmannii*. This suggests that the Korean *O. ficus indica* is a relative of *O. engelmannii*. Las Casas et al. (2017) evaluated the phylogenetic relationships among 72 cultivated and wild genotypes belonging to approximately 15 different cactus pear species using cpSSR and SNV markers. 11 chlorotypes

Table 8.3 Phytochemical composition of cactus pear (*O. megacantha* from Morocco) seed oil and cladode essential oil

	Cactus seed oil	Cactus cladode essential oil
Fatty acids C16:0 (palmitic acid) (%)	12.10 ± 0.13	17.09 ± 0.51
C16:1, ω-7 (palmitoleic acid) (%)	0.73 ± 0.05	0.43 ± 0.03
C18:0 (stearic acid) (%)	3.41 ± 0.10	2.15 ± 0.13
C18:1, ω-9 (oleic acid) (%)	18.48 ± 0.09	12.84 ± 0.31
C18:2, ω-6 (linoleic acid) (%)	62.99 ± 1.29	34.81 ± 1.11
C18:3, ω-3 (linolenic acid) (%)	0.25 ± 0.02	26.65 ± 0.50
SFA (%)	15.51 ± 0.10	19.24 ± 0.48
MUFA (%)	19.21 ± 0.12	13.28 ± 0.31
PUFA (%)	63.24 ± 1.27	61.47 ± 1.11
Total phytosterols (mg/100 g)	916.20 ± 4.08	2131.4 ± 15.25
Cholesterol (%)	1.23 ± 0.01	5.88 ± 0.06
α-cholestanol (%)	2.59 ± 0.08	1.13 ± 0.02
24-Methylene-cholesterol (%)	10.42 ± 0.05	0.04 ± 0.00
Campesterol (%)	0.56 ± 0.05	9.52 ± 0.10
Campestanol (%)	1.89 ± 0.08	1.04 ± 0.05
Clerosterol (%)	0.51 ± 0.01	0.28 ± 0.02
β-Sitosterol (%)	70.43 ± 0.11	0.82 ± 0.04
Sitostanol (%)	2.99 ± 0.02	64.97 ± 0.10
Δ^5-Avenasterol (%)	3.77 ± 0.04	7.90 ± 0.07
Δ^7-Stigmastenol (%)	1.19 ± 0.01	6.03 ± 0.09
Δ^7-Avenasterol (%)	1.50 ± 0.06	0.34 ± 0.05
Stigmasterol (%)	1.80 ± 0.03	1.44 ± 0.03
$\Delta^{5,23}$-Stigmastadienol (%)	–	0.34 ± 0.02
$\Delta^{5,24}$-Stigmastadienol (%)	–	0.21 ± 0.02
Spinasterol (%)	0.84 ± 0.02	–
Schottenol (%)	0.23 ± 0.01	–
Total tocopherols (mg/kg)	117.60 ± 2.96	488.80 ± 9.20
α-Tocopherol (%)	1.27 ± 0.02	100 ± 0.00
β-Tocopherol (%)	36.89 ± 0.16	–
γ-Tocopherol (%)	58.07 ± 0.10	–
δ-Tocopherol (%)	3.77 ± 0.07	–
Saturated hydrocarbons (mg/kg)	0.16	17.82

Source El Kharrassi et al. (2018)

were observed, and 50 genotypes, belonging to 9 species, were included in one wide chlorotype referring to *O. ficus indica*. These species are: *O. albicarpa*, *O. fusicaulis*, *O. amyclaea*, *O. elizondoana*, *O. megacantha*, *O. joconostle*, *O. undulata* and *O. vulgaris*, which shows their close relationship to *O. ficus indica*. The relationships among the 11 chlorotypes were further assessed through a median-joining network and 4 chlorotypes (Hcp1: including many of the *O. ficus indica* accessions; Hcp2: *O. leucotricha*; Hcp3: including *O. oligacantha* and Hcp4: *O. spinulifera*) showed a close genetic similarity. The analysis revealed the multiple maternal phylogeny for the fleshy fruit varieties classified as *O. ficus indica*.

8.3.4 Genetic Resources Conservation Approaches

Due to the high economic, agronomic and ecological importance of cactus pear, conservation of its genetic resources has become important to maintain biodiversity and for genetic characterization and breeding programs. Ex situ conservation is the main approach used for cactus pear. Accordingly, many collection sites have been established in different countries and used for various research activities.

Romano (2013) reported that, in the fields of the National Arid Land Plant Genetic Resources Unit (NALPGRU) of the National Plant Germplasm System (NPGS, USA), there are more than 20 species and 240 accessions of cactus pear, and that the majority of accessions belongs to *Opuntia ficus indica*. In South Africa, the Department of Agriculture in Limpopo Province has been conserving more than 70 accessions (cultivated as well as hybrids from spontaneous crossings) at Mara Experimental Farm, Makhado (Potgieter and Mashope 2009). In Italy, there is an *Opuntia* spp. collection in Sardinia hosted by the University of Sassari. This collection contains more than 2200 accessions of different cactus pear species including wild genotypes and ecotypes, international and local varieties, selected lines, and seedlings obtained from free pollination, and by inter- and intraspecific crosses and embryo culture. The accessions originate from different countries such as Argentina, Chile, France, Mexico, Morocco, South Africa and the USA, but the majority are from Italy (Chessa 2010). In Morocco, the National Institute of Agronomic Research has established many collection orchards of cactus pear species in their regional experimental stations (e.g. in Marrakech, Settat and Agadir; Fig. 8.5) for genetic characterization and ex situ conservation. The accessions that were collected from different regions of Morocco belong to different species. Studies on their morphological, molecular and physicochemical characteristics have been reported. Boujghagh (2011) published a full agro-morphological characterization of 127 accessions that belong to *O. ficus indica*, *O. megacantha*, *O. robusta*, *O. aequatorialis*, *O. dillenii*, and *O. schumannii*. These accessions are grown in the experimental station Melk Zhar (Agadir). El Kharrassi et al. (2015) investigated and reported the phenological behavior and the physicochemical characteristics of 14 accessions of 6 cactus pear species (*O. ficus indica*, *O. robusta*, *O. aequatorialis*, *O. dillenii*, *O. leucotricha* and *O. stricta*) grown in the experimental station Ain Zagh (Settat). A Morphological

characterization of 124 accessions and a genetic diversity assessment of 22 accessions using molecular markers were also performed on 7 species (*O. ficus indica, O. megacantha, O. robusta, O. aequatorialis, O. dillenii, O. leucotricha* and *O. inermis*) grown in this same experimental station (El Kharrassi et al. 2017). In addition, fruit and juice characteristics of 30 accessions of *O. ficus indica* and *O. megacantha* growing at the experimental station Jamaat Riah (Settat) has been reported (El Kharrassi et al. 2016).

In addition to these examples, other ex situ conservation collections of cactus pear have been used by researchers to sample and use plant material for various research purposes. For example, the collection established at the National Institute of Agronomic Research of Tunisia (Zarroug et al. 2015b); the CRUCEN germplasm bank in Zacatecas, Mexico (Samah et al. 2015; Valadez-Moctezuma et al. 2015); the Nopalera-UACh germplasm bank in Texcoco, Mexico (Samah et al. 2015); the experimental field of the Banco de Germoplasma del Programa de Nopal y Frutales, Instituto Nacional de Investigaciones Forestales y Agrícolas y Pecuarias (INIFAP) in North Guanajuato, Mexico (de Lyra et al. 2013); the *Opuntia* collection of the Texas A&M University-Kingsville in USA (Wang et al. 1997); and the ex situ collection hosted by the Universidad Nacional de Santiago del Estero in Argentina (Chessa et al. 2013). On the other hand, in vitro conservation of *O. ficus indica* was reported in the in vitro collection of the National Genebank of China (Zhang et al. 2014).

8.3.5 Cytogenetics

Cactus pear species have different ploidy levels. According to Bravo-Hollis and Scheinvar (1995), the chromosome base number of *Opuntia* species is x = 11. Mondragón-Jacobo and Bordelon (1996) reported that the highest numbers of chromosomes are found in the cultivated populations. Information on the ploidy level of cactus pear species can help in breeding and conservation programs (Segura et al. 2007). Accordingly, the ploidy level of the most important species was determined. For example, Segura et al. (2007) used flow cytometry to determine the ploidy level among 23 fruit-edible cactus pear species from Mexico. Four different ploidy levels were reported (2n = 2x, 2n = 4x, 2n = 6x, 2n = 8x). It has been found that *O. heliabravoana* is diploid; *O. leucotricha*, *O. spinulifera*, *O. elizondoana* and *O. robusta* var. Larreyi are tetraploids; *O. oligacantha*, *O. incarnadilla* and *O. matudae* are hexaploids; and that *O. zamundioi*, *O. lasiacantha*, *O. hyptiacantha*, *O. streptacantha* ssp. Streptacantha, *O. streptacantha* ssp. Aguirrana, *O. megacantha*, *O. joconostle*, *O. ficus indica*, *O. albicarpa*, *O. amarilla*, *O. chavena*, *O. cochinera*, *O. fuliginosa*, *O. pachona*, *O. cretochaeta*, *O. rzedowskii*, *O. robusta* var. Robusta and *O. robusta* var. Guerrana are octoploids. Similarly, Majure et al. (2012) reported different ploidy levels among other *Opuntia* species. For example, *O. ammophila* was reported to be diploid, *O. nemoralis* is tetraploid while *O. tortispina* has been recorded as either tetra- or hexaploid. On the other hand, some authors examined the ploidy level of interspecific hybrids. For example, McLeod (1975) reported that the

Fig. 8.5 Ex situ conservation of cactus pear in an experimental station of the National Institute of Agronomic Research of Morocco (INRA). Photos show accessions with different fruit colors as well as spiny and spineless plants

interspecific natural hybrid of *O. ficus indica* and *O. phaeacantha* var. Major is oddploid (2n = 77). Grant and Grant (1982) indicated that some pentaploid individuals (2n = 55) are natural hybrids of *O. lindheimeri* (6x) and *O. edwardsii* (4x); and of *O. phaeacantlha* var. Major (6x) and *O. edwardsii* (4x).

8.4 Functional Genomics

Functional genomics have not been investigated extensively in cactus pear. In fact, few studies have been performed in this field, and have mainly concerned ESTs related to abiotic stress tolerance. Expressed sequence tags (ESTs) are short sequences derived from both ends of a cDNA clone; they are randomly sequenced from various tissues and species and are very useful to identify and characterize new genes (Gill and Sanseau 2000). The tolerance of cactus pear species to extreme environments make them valuable to study molecular mechanisms underlying abiotic stress tolerance (Ochoa-Alfaro et al. 2012). Salas-Muñoz et al. (2012) reported that the *OpsHSP18* gene from *Opuntia streptacantha* has an important role in abiotic stress tolerance, in particular salt and osmotic stress. In fact, the overexpression of the *OpsHSP18* gene in *Arabidopsis thaliana* increased the seed germination rate under salt (NaCl) and osmotic (glucose and mannitol) stress. Ochoa-Alfaro et al. (2012) constructed a directional cDNA library from *O. streptacantha* cladodes, with 329 cactus pear unigenes classified into 11 functional categories, and a collection of ESTs accumulated under abiotic stress was reported. The most abundant EST (unigene 33) encodes a dehydrin protein and was named *OpsDHN1*. The *OpsDHN1* full length cDNA sequence of 905 bp includes 76 bp of 5′-UTR and 82 bp of 3′-UTR. This cDNA encodes a SK3-type acidic dehydrin of 248 amino acids. The *OpsDHN1* transcript is specifically accumulated in response to cold stress, and induced by abscisic acid. The overexpression of the *OpsDHN1* gene in *Arabidopsis thaliana* leads to enhanced tolerance to freezing treatment. Ochoa-Alfaro et al. (2012) indicated that all the nucleotide sequences of the *O. streptacantha* cDNA library were deposited at the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/). Silva-Ortega et al. (2008) evaluated the relationship between proline accumulation, P5CS activity and *Osp5cs* gene expression under salt stress in *O. streptacantha*. RT-PCR analysis revealed that the *Osp5cs* gene was induced by salt stress at 9 and 11 days of treatment, and that ABA-induced *Osp5cs* gene expression was observed in cladodes of young plants. The authors also reported correlation between the transcript up-regulation and the proline accumulation under salt stress, and suggested that this proline accumulation functions as an osmolyte for the intracellular osmotic adjustment and might play a critical role in protecting photosynthetic activity in *O. streptacantha* plants under salt stress. Rosas-Cárdenas et al. (2007) cloned and characterized a cDNA *OsPG* product of 282 bp with predicted sequence of 94 amino acids. Northern blot analysis did not detect the transcript of *OsPG* in flowers, roots and cladodes of prickly pear cv. Blanca Cristalina, but it was detected in fruit, which suggests that *OsPG* is expressed in a fruit ripening specific way. In addition, the *OsPG*

transcript changes substantially in abundance during the process of fruit ripening: the transcript was not detectable at the first green stages but the level increased markedly at middle and at ripe stages. However, *OsPG* expression at the green stage could be stimulated by treatment with exogenous ethylene, cold storage and wounding. Mallona et al. (2011) identified and studied genes involved in circadian regulation and crassulacean acid metabolism (CAM) in *O. ficus indica*. They found that *OfiSAND* and *OfiTUB* are appropriate standards for use in the quantification of gene expression, and reported 3 kinds of expression profiles: transcripts of *OfiPPCK* oscillated with a 24-h periodicity, transcripts of the light-active *OfiNADP-ME* and *OfiPPDK* genes adapted to 12-h cycles, while transcript accumulation patterns of *OfiPEPC* and *OfiMDH* were arrhythmic. They also reported that the expression of the circadian clock gene *OfiTOC1*, oscillated with a 24-h periodicity, peaking at night, and that the expression of *OfiCCA1* and *OfiPRR9*, adapted best to a 12-h rhythm, has a first peak during the morning and a second peak at dusk. The authors suggested that the evolution of CAM metabolism could be the result of modified circadian regulation at both the transcriptional and posttranscriptional levels.

8.5 Genetic Engineering

Genetic engineering is a powerful tool to transfer genes of agronomic interest into plants and then contributing significantly to their improvement. It also overcomes some limitations of conventional breeding such as time consumption and high labor intensity. The two genetic transformation techniques widely used and reputed to generate completely stable-transformed plants are *Agrobacterium tumefaciens*-mediated transformation and particle bombardment, or biolistics. *Agrobacterium tumefaciens*-mediated transformation has the advantages of a reduced abnormal transgene expression, a minimal equipment cost and a reliable insertion of a single copy or low copy of transgene, whereas biolistics allows transferring genes to any type of cell or tissue without considering the compatibility between the host and bacterium as a prerequisite for the *Agrobacterium* system (Krishna et al. 2015).

Thus far, very few studies have been reported on genetic transformation of cactus pear. Llamoca-Zárate et al. (1998) used friable calli initiated from cotyledons and hypocotyls of *Opuntia ficus indica*; and the vector pARGUSH that contains the *npt*II gene controlled by the *nos* promoter, the *uid*A gene controlled by the *par* promoter and the *tet* gene controlled by a bacterial promoter to achieve transformation by biolistics. Transgenic friable calli were obtained on kanamycin containing medium and the successful integration of foreign DNA into cells of *O. ficus indica* was confirmed by histochemical and photometric assays for GUS activity and by PCR amplification of DNA with *gus* gene primers, *npt*II gene primers and *tet* gene primers. In a different study, Llamoca-Zárate et al. (1999) bombarded shoot apical meristems of *O. ficus indica* with plasmids pFF19G, pNG and pARGUSH, containing the *uid*A gene (controlled by the CaMV35S promoter), the *uid*A and *npt*II genes (controlled by the CaMV35S promoter), and the *npt*II gene (controlled by the *nos*

promoter) and the *uid*A gene (controlled by the *par* promoter), respectively. The histochemical determination of GUS activity was performed and blue meristems indicating the expression of the *uid*A gene were observed. Thus, a successful delivery of foreign DNA into apical meristem cells of *O. ficus indica* was achieved. These authors also reported that there was no difference in the frequency of expression of the transgene when the meristems were bombarded with plasmids pFF19G, pNG and pARGUSH. More recently, Cruz et al. (2009) bombarded apical regions of *O. ficus indica* with the plasmid pAG1, containing the *gus* gene and the *Atahas* gene, which confers resistance to imazapyr. Genetic transformation was demonstrated by PCR and, out of 120 bombarded explants, 1 showed *gus* gene activity and 4 survived in 300 nM imazapyr. Silos-Espino et al. (2006) developed *O. ficus indica L. cv. Villa Nueva* genetic transformation system by *Agrobacterium tumefaciens*. A direct bacterial infection of areoles was performed using a hypodermic syringe and transgenic plants were selected on media supplemented with 100 mg/l kanamycin. Kanamycin-resistant shoots and regenerated plants exhibited transient and stable GUS activities, and the genetic transformation of regenerants was confirmed by PCR and Southern blot analysis; and the transformation rate was 3.2%.

8.6 Mutation Breeding

Induced mutations are carried out by using mutagens such as radiation or chemical compounds. It aims to create new crop varieties with improved yield and resistance to biotic and abiotic stresses (Gulfishan et al. 2015). To date, cactus pear mutagenesis is still in its infancy. Boujghagh et al. (2015) tried to determine an ideal radiation dose for cactus pear seeds. Accordingly, seeds were exposed to increasing doses (50–200 Grays) of gamma rays and compared to an untreated control group. The cumulative rate of germination was very high (96–98%) in the untreated group compared to the irradiated seeds (36–75%), and a decreasing cumulative rate of germination was observed when the dose of radiation increased. Moreover, irradiation has prolonged the latency period before germination. It has been concluded that the optimal radiation dose for cactus pear seeds is 50–125 Grays.

8.7 Hybridization

8.7.1 Conventional Hybridization

Sexual hybridization is a powerful breeding tool and an important generator of biodiversity that allows breeders to introduce new genes for improving plant traits (Aversano et al. 2012). The first hybridization experiments in cactus pear had very little success for many reasons such as the lack of registers and formal publications, and

the absence of clear advantages over the existing genotypes (Mondragón-Jacobo and Bordelon 1996). However, since the 1990s, more hybridization experiments have been carried out between and among *Opuntia* species. According to Wang et al. (1997), the ideal cactus pear genotype should have high yield, high fruit sugar content, good cold hardiness, disease resistance and attractiveness to the consumer. Conventional hybridization of cactus pear is mainly hampered by the seedlings need of approximately 6–8 years to reach full maturity (Mondragón-Jacobo and Bordelon 1996).

Wang et al. (1996) initiated a hybridization program between various cactus pear species and evaluated numerous parameters such as floral development, pollen germinability, breeding system and germination conditions. It was reported that *Opuntia ficus indica* could hybridize with *O. hyptiacantha*, and that *O. streptacantha* could hybridize *O. hyptiacantha*, *O. ficus indica* and *O. megacantha*, and that up to 50% fruit set was obtained with some hybridizations. Wang et al. (1997) attempted to combine traits such as high productivity, high-fruit-sugar content and cold hardiness. Accordingly, a series of hybridizations between different cactus pear accessions was carried out, and fruit set and germination frequency were evaluated. These authors expressed a particular interest to the cross between *O. lindheimerii* that has great cold hardiness and the highly productive accessions of *O. streptacantha* and *O. ficus indica*, and highlighted the close genetic affinity among the accessions of *O. lindheimerii*, *O. ficus indica* and *O. hyptiacantha*. Griffith (2001) evaluated fruit set and hybrid embryos after 11 reciprocal crosses between putative parental taxa of field-observed putative hybrids. Fruit set was observed in all reciprocal crosses except in those between *O. aureispina* and *O. Xspinosibacca*, and between *O. chisosensis* and *O. Xspinosibacca*. In addition, interfertility between *O. aureispina* and *O. macrocentra* "azurea type" was demonstrated which supports the hypothesis that *O. Xrooneyi* is the cultivar result of hybridization between these two species. This author also reported that in the cross between *O. Xspinosibacca* (2n = 44) and *O. engelmannii* var. engelmannii (2n = 66), and that between *O. macrocentra* var. minor (2n = 44) and *O. macrocentra* "azurea type" (2n = 22) or *O. macrocentra* var. macrocentra (2n = 22), hybrids survive only if the male parent is of the higher ploidy level. Felker et al. (2010) evaluated fruit characteristics of a 4-year-old sexual progeny of a cross between *O. lindheimerii* (male parent) and *O. ficus indica* (female parent). It has been found that the largest fruits of the progeny approached the size of commercial fruits, and that one of the spiny, non-apomictic progeny has produced the highest number of fruits. However, fruits of a considerable number of the non-apomictic seedlings matured much later than did the female parent, with 78 days as the longest delay observed.

8.7.2 Somatic Cell Hybridization

Somatic cell hybridization is highly effective to manipulate cellular genomes by protoplast fusion. It is an efficient means to overcome common crossing barriers

among plant species, which contribute significantly to plant breeding (Sink et al. 1992). To date, some protocols to isolate and culture protoplasts were reported in cactus pear (Llamoca-Zárate et al. 2006; Rosiles-Ortega et al. 2015). However, no regeneration from protoplast fusion has been reported.

8.8 Conclusion and Prospects

8.8.1 An Overview of the Current Status

To date, the majority of research on cactus pear focuses on the genetic diversity and relationships within and among species, and on the phytochemical characterization of fruits and cladodes to highlight their beneficial effects to the health and well-being of consumers, due to the antioxidant, antibacterial and antiviral properties of their compounds. On the other hand, the high importance of cactus pear and the high variability among its accessions led many research institutions in several countries such as Mexico, Italy and Morocco, to establish ex situ conservation collections in order to maintain biodiversity and to be used in various research activities as well as in breeding programs. Given the high agronomic, economic and ecological values of the genus *Opuntia*, it is henceforth necessary to use the most advanced breeding techniques to develop new commercial cultivars.

8.8.2 Cactus Pear and Global Climate Change

The frequent and extended drought periods, rainfall reduction, global warming and the increase of atmospheric concentrations of greenhouse gases such as CO_2 are some aspects of climate changes. These aspects have direct effects on plant growth and transpiration as well as on soil water availability (Polley et al. 2017). Cactus pear plants, which are well adapted to drought, high temperatures and low water availability, are of great utility in such situations. Cactus pear species are crassulacean acid metabolism plants. They are characterized by nocturnal stomatal opening, thus CO_2 uptake and water loss occur primarily at night when photosynthetically active radiation is zero (De Cortázar and Nobel 1990; Nobel 2001). This confers them a high water-use efficiency, and, as already reported, they are able to absorb and hold CO_2 excess from the atmosphere and to conserve water, which allows them to survive in extremely arid and hot environments, and to tolerate drought periods. Owen et al. (2016) reported that simulations on low-grade lands suggest that *Opuntia ficus indica* is highly resilient to climate change, with high productivity potential and the capacity to meet extreme bioenergy demand scenarios. All these characteristics make cactus pear plants an interesting choice for various climate change scenarios.

8.8.3 Recommendations for Future Research

With the ongoing development of biotechnological techniques, and due to the high importance of cactus pear and its capacity to adapt to hot and drought conditions, it is recommended to use the most advanced tools for its breeding. As shown in this chapter, methodologies such as genetic engineering, functional genomics, in vitro mutagenesis and somatic cell hybridization are still in their infancy. Furthermore, QTLs for agronomic traits should be identified. All these techniques should be used in future research to meet the objectives of cactus pear breeding.

Appendix I: A Non-exhaustive List of Organizations, Institutes and Universities Involved in Research Related to Cactus Pear

Institute	Research specialization/activities	Country	Website
El Instituto Argentino de Investigaciones de Zonas Áridas (IADIZA)	Utilization as forage Cold tolerance Salt stress tolerance Biomass production Micropropagation	Argentina	http://www.mendoza-conicet.gob.ar
Università degli Studi di Sassari	Germplasm conservation Genetic diversity assessment Post-harvest technology	Italy	https://www.uniss.it
Food and Agriculture Organization of the United Nations (FAO)	Agro-industrial utilization	Italy (Headquarters)	http://www.fao.org
International Center for Agricultural Research in the Dry Areas (ICARDA)	Utilization as forage Abiotic stress tolerance	Lebanon (Headquarters)	http://www.icarda.org
El Centro Regional Universitario Centro Norte (CRUCEN)	Germplasm conservation Morphological characterization Phytochemical characterization Genetic diversity assessment	Mexico	http://chapingo.mx/scru/crucen.html

Institute	Research specialization/activities	Country	Website
Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP)	Germplasm conservation Molecular characterization	Mexico	http://www.inifap.gob.mx
Instituto Potosino de Investigación Científica y Tecnológica (IPICYT)	Functional Genomics Metabolite production	Mexico	http://www.ipicyt.edu.mx
Universidad Autónoma Chapingo (UACH)	Germplasm conservation Morphological characterization Phytochemical characterization Genetic diversity assessment	Mexico	http://www.chapingo.mx
Institut National de la Recherche Agronomique (INRA)	Germplasm conservation Genetic diversity assessment Morphological characterization Phytochemical characterization	Morocco	http://www.inra.org.ma
Institut National de la Recherche Agronomique de Tunisie (INRAT)	Germplasm conservation Genetic diversity assessment Micropropagation	Tunisia	http://www.inrat.agrinet.tn
Texas A&M University-Kingsville	Germplasm conservation Antioxidant potential Morphological characterization Genetic diversity assessment Cross-hybridization Water use efficiency	USA	http://www.tamuk.edu

Appendix II: A Non-exhaustive List of Cactus Pear Species Cultivated for Edible Fruit Production

Species	Cultivar	Cultivation location
Opuntia ficus indica	Amarilla sin Espinas	Santiago del Estero, Argentina
	Gigante	Ceará, Brazil
	Bianca	Sicily, Italy
	Gialla	Sicily, Italy
	Muscaredda	Sicily, Italy
	Rossa	Sicily, Italy
	Sanguigna	Sicily, Italy
	Surfarina	Sicily, Italy
	Alfajayucan	Actopan, Mexico
	Blanco sin Espinas	Guanajuato, Mexico
	Redonda	Guanajuato, Mexico
	Rojo Pelón	Zacatecas, Mexico
	Rojo Vigor	Zacatecas, Mexico
	Aïssa	Tiznit, Morocco
	Moussa	Tiznit, Morocco
	Gialla	Grombalia, Tunisia
	Algerian	Bloemfontein, South Africa
Opuntia albicarpa	Cristalina	Zacatecas, Mexico
	Reyna	San Martín de las Pirámides, Mexico
Opuntia megacantha	Achefri	Tiznit, Morocco
	Rubi Reina	Zacatecas, Mexico
Opuntia streptacantha	Tuna Loca	Zacatecas, Mexico

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Chapter 9
Improvement of Fig (*Ficus carica* L.) by Conventional Breeding and Biotechnology

Fateh Aljane, Awatef Essid and Sabrine Nahdi

Abstract The common fig (*Ficus carica* L.), family Moraceae, is the only member in the genus cultivated for its fruits. For the past several decades, severe genetic erosion has threatened fig germplasm. Therefore, several fig collections were established in Asia, Europe, North America and North Africa. Fig cultivars are usually characterized using traditional methods based on phenotypic characters. However, molecular identification of fig cultivars has been carried out using random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) and single sequence repeats (SSR) molecular markers. Marker-assisted selection is used to identify a character of interest such as yield, fruit quality, biotic and abiotic stress resistance. Induced mutations are desirable in fig improvement for important fruit characters such as small ostiole size, large fruit size and fruit flesh quality. Fig mosaic disease (FMD) is one of the major diseases of fig found throughout the world. Different fig improvement breeding methods are described in this chapter including conventional breeding and biotechnology approaches.

Keywords *Ficus carica* · Germplasm · Hybridization · Molecular marker Pollination · Selection · Variety characterization

F. Aljane (✉) · A. Essid
Arid Land and Oasis Cropping Laboratory, Institute of Arid Regions of Medenine, 4119 Medenine, Tunisia
e-mail: fateh_aljane@yahoo.fr

A. Essid
e-mail: awatef.essid@yahoo.fr

S. Nahdi
Department of Plant Breeding and Crop Protection, Higher School of Agriculture, 7119 El Kef, Tunisia
e-mail: nehdimah@yahoo.fr

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9.1 Introduction

Fig (*Ficus carica* L.) is one of the oldest domesticated fruit species in the world. The English word fig is of ancient origin, derived from the Latin *ficus*. The species name c*arica* refers to Caria, an ancient region of Asia Minor noted for figs (Ferguson et al. 1990). The Phoenicians, Carthaginians and Greeks expanded the growing of figs. The Romans propagated it throughout the Mediterranean basin (IBPGR 1986; Roger 2003). The fig tree gradually spread and reached the most distant regions of the world such as China, India, Ethiopia, Arabia, Australia and the Americas, where it was introduced and expanded widely (Esclapon 1976). The ancestral fig tree (*Ficus carica* L.) was monoecious and later evolved into a functionally dioecious species (Nabli 1989).

The fig tree is known under several names in various civilizations: *mgyz* (Persians), *erineos* (Greeks), *teb* (Egyptians), *caprificus* (Romans) and *ettine* or *dhokkar* (Arabs). The Arabs promoted fig cultivation in Mediterranean countries. The Portuguese called them *figo de toco* and the Italians *profico* or *caprificus* (Lahbib 1984).

The genus *Ficus* is found mainly in the tropics but *Ficus carica* L. is a subtropical fruit tree (Ferguson et al. 1990). Figs are currently cultivated in most warm and temperate climates, and has been grown as a Mediterranean fruit tree for many years (Sahin 1998). Figs are often cultivated in association with others fruit trees such as olives, almonds, pomegranates and grapevines (Aljane 2011).

The common fig (*Ficus carica* L.) belongs to the large family Moraceae, with over 1400 species classified into about 60 genera. The somatic chromosome number is $2n = 2x = 26$ (Ferguson et al. 1990). The genus *Ficus* includes more than 700 species (Berg 2003). In these species, the fruits are drupelets developing from the ovaries in a closed syconium. The tiny fig wasp pollinator (*Blastophaga psenes* L.) gains access via a small opening (ostiole) in the fruit. *Ficus carica* is the only member in the genus cultivated for its fruit (Ferguson et al. 1990). Four types of figs are cultivated in the world, based on Storey (1976):

(a) The common type which produces figs without pollination where the fruit contains infertile seeds. Some cultivars bear a first crop (breba crop) and then produce a good second crop (main crop);
(b) San Pedro type produces a first crop (breba crop) that persist to maturity and is parthenocarpic. The second crop (main crop) requires pollination to develop to maturity. The production of the breba crop is a specialty of this type;
(c) Smyrna type that requires pollination with pollen from caprifigs (caprification) and bears mature fruit with viable seeds. However, without pollination the fruits will fall from the tree before maturation;
(d) Caprifig type produces syconia flowers, which contain pollen that is destined to pollinate the Smyrna and San Pedro types.

The aim of this chapter is to present an overview of fig cultivation, germplasm diversity, conservation and utilization, and the improvement of fig breeding through conventional and biotechnology methods.

9.2 Fig Production and Economic Importance

The total world fig cultivated area is over 390,000 ha, with recent annual production of 1,117,452 mt (FAO 2015). Turkey produces 70% of the total world production, followed by Egypt, Algeria, Tunisia, Iran, Morocco and Syria. Annual fig production in Tunisia is 29,000 mt, representing 3% of world production (FAO 2015). In Europe, Spain produces 23,285 mt, accounting for 25% of European production and about 3% of world production, followed by Albania, Germany, Portugal, Italy, Greece, France and Croatia.

The economic importance of fig production will continue in future to meet the increasing demand for fresh and dried figs in the world market. Fresh figs are characterized by a short shelf-life, an important challenge for commercial production and international marketing. Recently, Turkey and Mexico have expanded their fig production. Moreover, the development of the Calimyrna cv. in the USA and Europe, combined with improved cultural practices and better fresh-fruit postharvest practices, are opening new prospects for this crop worldwide (Flaishman et al. 2007).

9.3 Origin and Centers of Diversity

The fig found naturally in the Middle East and the Mediterranean regions, is of Smyrna type (Kjellberg and Valdeyron 1984). However, outside the Mediterranean regions, it is rarely found in the natural habitat (Nabli 1989). Spontaneous fig trees can potentially exchange genetic material each year (Kjellberg et al. 1987). A study of enzymatic polymorphism revealed that the spontaneous fig trees of southern France show that alleles are not randomly distributed; their frequencies vary regularly with the climate and reflect the role of adaptation (Kjellberg and Valdeyron 1984).

Human interest in the fig tree has led to its dispersal and cultivation in many parts of the world, proving its great adaptability and affinity with warm climates. Apparently all the varieties cultivated in the world over the centuries have been selected and propagated by vegetative multiplication (IBPGR 1986; Storey 1975). Some Mediterranean countries (Spain, Turkey, Algeria, Tunisia and Morocco) are considered to be centers of diversity of the fig. Plant material, traditionally grown in different areas called *microgenecenters*, is considered the primary genepool (Mars 2003).

9.4 Cultivation and Agricultural Practices

Fig is traditionally cultivated in only a few countries in the world and adapts easily to marginal conditions (Aksoy 1998; Golombek and Ludders 1990). Fig trees grow under different climates and variable agricultural practices (Fig. 9.1) and are culti-

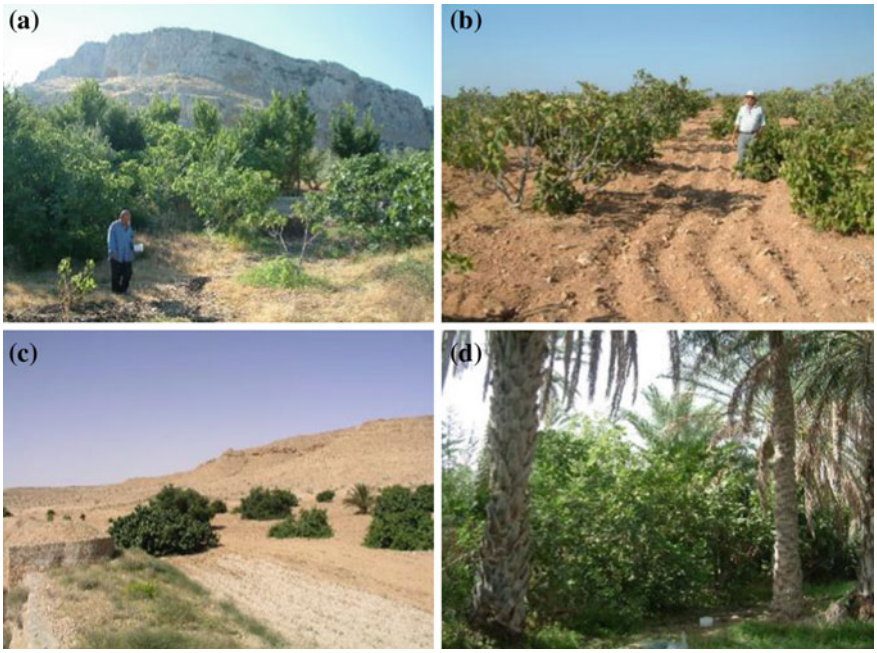

Fig. 9.1 Different fig culture systems depending on the climate, irrigation techniques and cultural practices. **a** Figs in sub-humid climate under irrigation, **b** Figs in semiarid climate under rainfed and supplementary irrigation, **c** Figs in an arid climate under rainfed conditions, **d** Figs in an oases ecosystem. Source: Photos by Fateh Aljane

vated in a wide range of soils (heavy clays, loams, and light sands) and well-drained soils. Fig trees are resistant of high salinity (Golombek and Ludders 1990).

Fig trees can be propagated by seed, cutting, layering and grafting. Figs obtained from seeds are used only in breeding programs (Aljane and Nahdi 2014). Fig nursery plant production is mainly done by planting cuttings in soil to establish new trees (Aljane 2006; Aljane and Nahdi 2014; Antunes et al. 2008). In common practice, cuttings should be 20–70 cm long and contain several nodes. Furthermore, farmers have used layering methods to propagate fig orchards (Dolgun and Tekintas 2008). In addition, in vitro methods have been developed and may be very useful in obtained new varieties and have been used in sanitation of fig cultivars from viruses (Aljane and Nahdi 2014; Elair et al. 2014; Mars 2003). Young trees planted in new orchards are spaced from 3–8 m to 9–14 m apart, depending on the fig type and the cultivar (Flaishman et al. 2007).

Fig trees tolerate droughty conditions and represent a suitable fruit crop for arid and semiarid regions. Moreover, figs require less water than other fruit species. The frequency of irrigation depends on tree size, vigor, soil type and rainfall. Fig trees may become stressed during dry periods because of their shallow root systems (Flaishman et al. 2007).

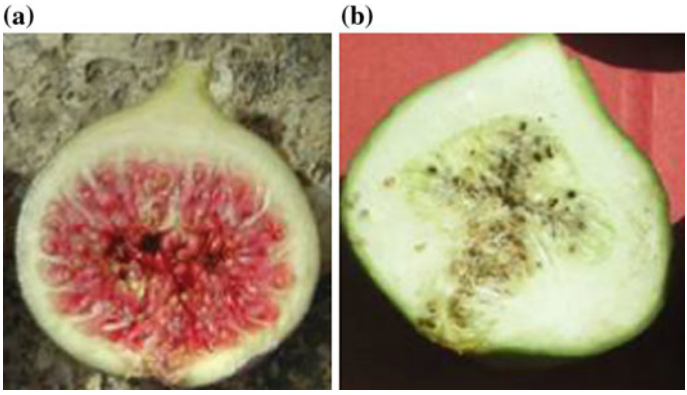

Fig. 9.2 *Ficus carica* fruit types. **a** Female fig fruit with female flowers, **b** Caprifig fruit with male and female flowers and the pollinator fig wasp *Blastophaga psenes* L. *Source* Photos by Fateh Aljane

9.5 Mutualism and Pollination

The common fig is dioecious, but functionally gynodioecious. Male trees (caprifigs) produce two types of flowers, male and female. Nevertheless, female trees produce only female flowers which develop into fertile seeds after pollination (Fig. 9.2). The pollination of *Ficus carica* L. is dependent on mutualism with the pollinating fig wasp (*Blastophaga psenes* L., Agaonidae) (Herre et al. 2008). Caprifig or male figs produce three crops per growing season; *profichi* in summer, *mammoni* ripening in autumn and *mamme* in spring (Aljane and Ferchichi 2007; Vidaud et al. 1997). Caprification is realized naturally or performed by farmers. Caprification problems are related to the disruption of the cycle of *Blastophaga psenes* in cold areas and the unavailability of mature male syconia at the time female fruits are receptive (Aljane and Ferchichi 2007; Essid et al. 2015, 2017).

Smyrna cultivars are pollinated in early summer with pollen from the caprifig (Essid et al. 2015, 2017). Usually, one male tree can pollinate 15–20 female fig trees. The *profichi* caprifigs are picked and placed in the orchard when the first wasps start to emerge. Caprifigs are replaced during the receptive time of female fig. (Aljane and Nahdi 2014; Essid et al. 2017). The pollen origin has a significant effect on fruit characteristics (Aksoy et al. 2003).

9.6 Germplasm Diversity and Conservation

For several decades, severe genetic erosion has been threatening fig germplasm. Therefore, it is imperative to establish programs to preserve and characterize fig genetic resources in the world and programs have been developed in several coun-

Fig. 9.3 Fig germplasm collection of the Institute of Arid Regions (IRA) of Medenine, Tunisia. *Source* Photo by Fateh Aljane

tries. Fig germplasm accessions are reported in Asia, Europe, North Africa and North America. The reported fig germplasm collections are listed in Tables 9.1, 9.2 and 9.3. The largest collections are in Italy, Turkey (Elbreyli Fig Research Institute), California (National Clonal Germplasm Repository (NCGR) Davis), Turkmenistan (TES-PGR) and France (CBNM Porquerolles) containing 442, 314, 190, 180 and 154 accessions, respectively. The CBNM Porquerolles collection has 154 different fig cultivars collected from Italy, North Africa, Greece, Turkey, France, Tunisia and Spain. The NCGR fig collection contains 190 accessions. The Arid Land and Oasis Cropping Laboratory germplasm collection of the Institute of Arid Regions of Medenine, Tunisia, holds 119 local fig accessions and 20 caprifigs collected from Tunisia, Algeria and Egypt (Fig. 9.3).

In situ conservation or on-farm conservation of fig cultivars in Tunisia is a challenge and is being carried out by research institutes, local nongovernmental organizations (NGOs), development agencies and farmers. Certain geographic sites were selected to conserve and to replant local fig cultivars. The regions of Zammour and Douiret in southeastern Tunisia were considered as traditional areas for the conservation fig cultivars (Mars et al. 2008).

In vitro conservation of fig germplasm has become extremely important because of changing environmental conditions associated with climate change. Fig genetic resource collections are conserved in vitro using tissue culture (IPGRI and CIHEAM

Table 9.1 Fig germplasm collections in Europe

Country	Location and number of accessions	Examples of accessions	Origin	References
France	CBNM Porquerolles: 154	Abicou Bourjassotte noire Col de dame noir Dauphine Longue d'août Madeleine des deux saisons Marseillaise Précoce ronde de Bordeaux Sultane	Italy North Africa Spain France Turkey France Greece France Tunisia	Khadari et al. (1995) Vidaud et al. (1997) CBNMP (1979) http://www.cbnmed.fr/pres/index.php
Italy	442	San Pietro Mattalona Dottato	Italy Italy Italy	Grassi and Santonastaso (1998) Piga et al. (2008)
Portugal	Faro (Algarve): 60 Alcobaça: 50	San Joao Branco Lampa Preta	Portugal Portugal	Antunes et al. (2008)
Spain	Badajoz: 85 Estremadure (SIDT): 35	Alacantina Albatera Goen	Spain Spain Spain	Giraldo (2005) Giraldo et al. (2008)
Greece	Athens: 16 Kalamata (N.A. G.G.RE. F.): 40 Haoussa: 25	Tsapela Basanata Livano	Greece Greece Greece	Tsantili (1990) Lionakis (1996) Papadopoulou et al. (2002)
Turkey	Mersin: 40 Adana (U. Cukurova): 35 Elbreyli (Fig Research Institute): 314	Degirmen Ipek Istanbul Pamuk Patlican Kabak Sarilop (Calimyrna)	Turkey Turkey Turkey Turkey Turkey Turkey Exotic	Aksoy (1995) Bostan et al. (1998) Karadeniz (2008)

2003). Two in vitro conservation protocols have been developed: cryopreservation and the slow-growth method (Engelmann 2000).

In cryopreservation, germplasm is preserved in liquid nitrogen at −196 °C for long-term storage; cellular divisions and metabolic processes are stopped at that temperature. However, stored plant materials are threatened by genetic instability (Panis et al. 2001). Two methods are employed for cryopreservation of fig materials: the classical technique and the vitrification technique (Engelmann 2000). The classic protocol is based on two steps: freezing by slow controlled cooling and rapid

Table 9.2 Fig germplasm collections in Asia

Country	Location and number of accessions	Examples of accessions	Origin	References
Georgia	Abkhazia: 120			Petrova and Voronova (1984)
Albania	Tirana: 21	Cingell Tivaras Stambolli	Albania Albania Albania	Koka (2008)
Serbia	Trebinje: 40	Tenica Zimmica Sargulja	Serbia	Kulina et al. (2002)
Slovenia	4	Dobrica Bilicia Zimica	Slovenia	Zigo and Stampar (2002)
China	Xuchang (Institute Forestry Henan): 21	Masui Dauphine Branswisck He-18	China China China	Jianye et al. (1997)
Turkmenistan	TES-PGR: 180			Levina (1984)
Syria	Idleb: 74	Abidi Ghazleni Sahli	Syria	Al Ibrahim (2000) Al Ibrahim and Bari (2006)

immersion of plants materials in liquid nitrogen. In this method, samples are treated with a penetrating cryoprotectant such as dimethyl sulfoxide, ethylene glycol or polyethylene glycol. This treatment prevents intracellular crystallization of water and subsequent cell damage (Panis et al. 2001). In addition, it should be noted that classical cryopreservation is unsuitable for fig tissue like callus and cell-suspension cultures. The vitrification method was applied for plant tissue cultures and qualifies as the most widely applicable of plant cryopreservation techniques (Panis et al. 2001).

Concerning the slow growth method, plant materials such as shoot tips, buds and meristem are cultured under cold conditions which keep the plant material alive but retard the growth significantly (Engelmann 2000). The slow-growth technique is considered the best for in vitro conservation of shoot tips. A survival rate of 50% plant regeneration was achieved by treating shoot tips with cryoprotectant for 20 min and then cooling them to −40 °C. Slow growth is the most commonly used technique for the cryopreservation of shoot tips, meristems, cell cultures and somatic embryos. As for root tip explants no difference was found between the cryopreservation and the slow-growth methods; nevertheless, plant regeneration from cryopreserved root tips was obtained from adventitious bud formation (Towill and Bajaj 2002).

Table 9.3 Fig germplasm collections in the North Africa and North America

Country	Location and number of accessions	Examples of accessions	Origin	References
United States of America	NCGR Davis California: 190	Panachée jaune verte Brunswick Violette de Bordeaux	California France	Vidaud et al. (1997) Simon (2002) Stover and Aradhya (2008) GRIN (1990) https://www.ars-grin.gov
Algeria	58	Alekake Tabouharchaout Taanimt Tabarant Tamaghroust Azendjar Bakkor (parthenocarpic)	Algeria Algeria Algeria Algeria Algeria Algeria Algeria	IBPGR (1986) Mazri-Kartout and Aid-Houchi (2001)
Morocco	Ain Taoujdate (INRA Meknès): 72	Nabout, Noukali Hafer El-Bghal Ghoddane Bousbati Filalia Fassi Embar El-Khel El Quolti Lebied Abacor Blanca Cuello Damo Blanca Kadota Trojano	Morocco Morocco Morocco Morocco Morocco Morocco Morocco Morocco Exotic Exotic Exotic Exotic	Oukabli et al. (2003) Khadari et al. (2004) Messaoudi (2003)
Tunisia	Tataouine (Institute of Arid Regions): 139 (119 female figs and 20 male figs)	Zidi Soltani Temri Wedlani Magouli Sawoudi Makhbech Bouholi Wahchi Bouharrag (caprifig) Magouli (caprifig) Jrani (caprifig) Asafri (caprifig) Limi (caprifig) Tebassi (caprifig) Masria Zazairia	Tunisia Tunisia Tunisia Tunisia Tunisia Tunisia Tunisia Tunisia Tunisia Tunisia Tunisia Tunisia Tunisia Tunisia Tunisia Tunisia Egypt Algeria	Aljane and Ferchichi (2009a) Aljane and Ferchichi (2010) Aljane (2011) Aljane et al. (2012) Aljane (2016) Essid et al. (2017)

9.7 Breeding Methods

The fig is a dioecious tree (separate male and female trees), characterized by three floral types; long-styled female, short-styled female and male flowers. Fig breeding is difficult because of the occurrence of parthenocarpy and seedlessness, a complex pollination mechanism, lack of reference collections, absence of a universal descriptor list to differentiate different cultivars, presence of large intra-varietal diversity, incidence of synonymies and homonymies and the closeness of wild types to cultivated plants in some regions (Aljane 2016; Aljane and Ferchichi 2010; Beck and Lord 1988). The approach to fig breeding is based on many important traits, (e.g. yield, fruit quality, and elimination of pollination, biotic and abiotic stress resistance, fruit ripeness and persistence, hardiness to environment constraints (Jona and Grihaudo 1991; Storey 1975). A total of 600 fig cultivars have been characterized and described worldwide (Wang et al. 2017).

Recent developments in conventional breeding, molecular approaches, phytochemical characterization, mutation, hybridization and fig sanitation are reviewed below.

9.7.1 Conventional Breeding

Figs have been conserved clonally by rooted cuttings and have acquired local denominations (Flaishman et al. 2007). Fig genetic resources indicate considerable potential for improving this species. A large number of fig cultivars have been developed and planted in several countries (Singh et al. 2015). The traditional selection approach in fig is based on yield, quality and biotic and abiotic stress resistance (Aljane and Ferchichi 2010; Aljane et al. 2012; Ferguson et al. 1990; Mars 2003; Storey 1975, 1976, 1977).

Some fruit quality characters are very interesting, such as fruit shape, color, weight, dimensions (Fig. 9.4), acidity and sugar content, and ostiole diameter. (Aljane and Ferchichi 2010). Shape is very important for packing and transportation. The most suitable fruit shape is globular (Caliskan and Polat 2008; Condit 1941). Ease of peeling is critical to satisfy local and global customer preferences (Caliskan and Polat 2008; Can 1993). Fruit weight is very important for fresh fig consumption (Aksoy et al. 1992). Aljane and Ferchichi (2010) cited data that fig fruit weights varied from 63–172 g. In other studies, Ozeker and Isfandiyaroglu (1988) recorded a fig fruit weight of 30–90 g, (Caliskan and Polat 2008) 24–92 g and (Polat and Ozkaya 2005) 28–107 g. Higher fruit weights are explained by the difference in the genetic potential of cultivars, cultural practices, water quality and climatic conditions. Mean fruit lengths varied from 51 mm in cv. Wahchi to 80 mm in cv. Faouari and mean fruit diameter between 47 mm in cv. Soltani Abiadh and 76 mm in cv. Faouari (Aljane and Ferchichi 2010). The ostiole opening size range is 0.6–9 mm (Polat and Ozkaya 2005). A large ostiole is an undesirable characteristic because pests and pathogens

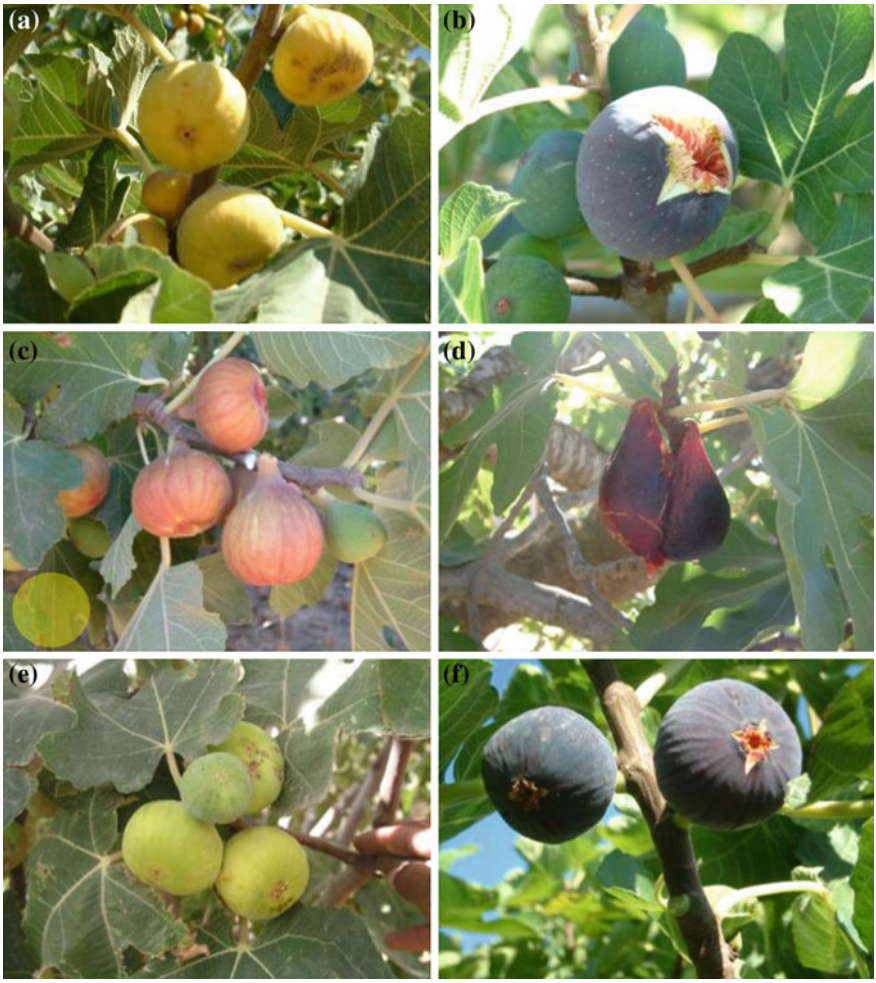

Fig. 9.4 Examples of the variability of fig varieties in the size, shape and color fruits cultivated in Tunisia. **a** Safouri, **b** Bouholi, **c** Jemâaoui, **d** Baghali, **e** Bither Akhdhar, **f** Zidi. *Source* Photos by Fateh Aljane

can easily enter the fruit (Caliskan and Polat 2008; Can 1993). The mean fruit skin thickness is between 0.3 mm in cv. Bouholi and 1.4 mm in cv. Boukhobza; this character is of great importance in packing and transportation of figs (Condit 1941).

Total acidity, pH, total soluble solids contents of fig cultivars are 0.36–1.58 g/l, 4.76°–5.31° and 11.15°–19.55° Brix, respectively (Aljane and Ferchichi 2010). These results are similar to reports by Aksoy et al. (1992), Khadari et al. (1995) and Mars et al. (1998). Reducing sugars (fructose and glucose) range from 1.50–13.50 g/100 g of fresh matter (Aljane and Ferchichi 2010).

Cultivar Gobernador shows the lowest glucose content, while cvs. Tioantonio and Calar were the sweetest, with 15.89 and 13.41 g/100 g of fresh matter, respectively (Melgarejo et al. 2003). Sugar is one of the most important factors in fruit taste; the cultivars with sweet fruits produce high-quality dried figs. Differences in results can be explained by various factors: different genetic potential of the cultivar, plant nutrients and differences in ripening.

In Turkey, 3 fig cvs. (C1, C2 and C4) were selected by Ozeker and Isfandiyaroglu (1988) from 12 early-fruiting cultivars. In Italy, Grassi and Santonastaso (1998) identified 442 clones from different regions. Furthermore, some morphological and phenological characteristics resolved many synonymies.

9.7.2 Phytochemical Compounds Characterization and Genotype Effect

Fig fruits are an important source of phytochemical compounds and exhibit antioxidant activity (Aljane and Ferchichi 2009b; Duenas et al. 2008; Ercisli et al. 2012; Vinson 1999). Soloman et al. (2006) determined the polyphenol, anthocyanin and flavonoid contents of some fig varieties. The contents of the phenolics compounds are depended on the fig cultivars; some studies have revealed that the cultivars and the skin color influenced the flavonoid and anthocyanin contents as well as the level of antioxidant activity (Caliskan and Polat 2011; Ercisli et al. 2012; Soloman et al. 2006; Veberic et al. 2008). Vallejo et al. (2012) studied the influence of fruit cultivars and harvest season on the phenolic compound content. Duenas et al. (2008) and Veberic et al. (2008) determined that fig fruit skin had the highest phytochemical composition.

Phytochemical characteristics of some Tunisian fig cultivars were studied; these were affected to different degrees by genotype and fruit skin color (Table 9.4). The total polyphenols (TP) contents of fig cultivars varied from 51.50 in cv. Bouholi to 100.23 in cv. Nasri mg GAE 100 g^{-1} FW. The purple blackish and red greenish fruit skin cultivars presented the highest values 72.19 and 77.60 mg GAE 100 g^{-1} FW, respectively (Bachir Bey and Louaileche 2015). The total anthocyanins amounts of the fig cultivars studied varied from 2.58 in cv. Baghali 3–11.67 in cv. Zidi 2, mg CGE 100 g^{-1} FW; cv. Zidi 2 cultivars had the highest content of 11.67 followed by cv. Sawoudi 3 at 9.7 and cv. Bouholi with 8.18, mg CGE 100 g^{-1} FW. It is clear that purple-blackish cultivars contain more anthocyanins. Fig skin color is influenced by the concentration and distribution of various anthocyanins (Gao and Mazza 1995). Statistical data analysis revealed that fruit skin color of these cultivars has no effect on total flavonoid (TF) content; the purple greenish cultivars such as cv. Soltani Ahmar had the highest contents of 17.59 mg catechin 100 g^{-1} FW, followed by the green yellowish cv. Mahdoui with 15.27 mg catechin 100 g^{-1} FW. Vallejo et al. (2012) revealed that the total flavonoid contents of dark-purple fig varieties were greater than those of lighter-colored ones.

Table 9.4 Effect of genotype on total phenolics (TP), total anthocyanins (TA) and total flavonoids (TF) characteristics of some Tunisian fig cultivars

Cultivar name	Total polyphenols (TP) mg GAE 100 g^{-1} FW	Total anthocyanins (TA) mg CGE 100 g^{-1}	Total flavonoids (TF) mg catechin 100 g^{-1} FW	Fruit skin color
Bither 1	60.51 ± 0.75 ef	3.76 ± 0.30 c	5.68 ± 0.20 de	Green yellowish
Jebali 1	76.48 ± 0.11 no	3.44 ± 0.91 c	11.50 ± 0.40 h	
Mahdoui	63.21 ± 0.32 h	3.74 ± 0.91 c	15.27 ± 0.13 j	
Bayouthi 1	76.63 ± 0.05 no	3.01 ± 0.11 c	0.33 ± 0.10 a	Green
Bayouthi 2	88.46 ± 0.47 r	5.62 ± 0.30 f	5.68 ± 0.10 de	
Besbessi	56.30 ± 0.77 c	3.33 ± 0.30 c	12.16 ± 0.27 hi	
Bither 2	65.54 ± 1.45 i	6.80 ± 0.30 g	8.59 ± 0.82 g	
Jemâaoui	76.16 ± 0.24 no	6.20 ± 0.69 g	2.78 ± 0.10 b	Red greenish
Rogabi	79.03 ± 0.15 p	3.96 ± 0.20 cd	3.77 ± 0.24 bc	
Gaa Zir	71.76 ± 0.08 k	4.54 ± 0.11 de	5.42 ± 0.38 de	Brown purplish
Temri	60.62 ± 0.04 ef	3.67 ± 0.30 c	5.68 ± 0.47 de	
Zergui	54.60 ± 1.36 b	5.58 ± 0.15 f	16.57 ± 0.09 k	
Baghali 2	69.93 ± 0.11 j	3.75 ± 0.91 c	6.15 ± 0.09 e	Purple greenish
Baghali 3	79.42 ± 0.61 p	2.58 ± 0.91 b	4.36 ± 0.04 cd	
Chetoui Akhal	73.36 ± 0.59 l	7.04 ± 0.20 h	12.30 ± 0.72 hi	
Croussi	74.58 ± 0.52 lm	4.29 ± 0.41 de	5.68 ± 0.24 e	
Kahli 2	62.34 ± 0.10 gh	4.22 ± 0.30 de	12.76 ± 0.09 i	
Nemri	59.34 ± 0.57 de	3.79 ± 0.82 cd	5.77± 0.35 e	
Soltani Ahmer	61.59 ± 0.66 fg	4.68 ± 0.15 de	17.59 ± 0.12 l	
Wedlani	63.57 ± 0.31 h	3.56 ± 0.20 c	1.78 ± 0.11 a	
Bouharrag	58.21 ± 1.59 d	5.13 ± 1.02 ef	7.47 ± 0.24 f	Purple blackish
Bouholi	51.50 ± 1.49 a	8.18 ± 0.20 i	1.78 ± 0.90 a	
Kahli 1	61.30 ± 0.63 fg	6.21 ± 0.91 g	11.70 ± 0.07 h	
Nasri	100.23 ± 0.38 s	3.95 ± 0.11 cd	1.85 ±0.94 a	
Sawoudi 3	75.45 ± 0.41 mn	9.70 ± 0.30 j	8.99 ± 0.48 g	
Sawoudi 5	77.37 ± 0.44 o	4.91 ± 0.91 e	11.70 ± 0.26 h	
Zidi 2	81.26 ± 0.99 q	11.67 ± 0.30 k	5.62 ± 0.16 de	
Total mean	68.93 ± 11.27	4.80 ± 2.23	7.60 ± 4.59	
F value	461.74	113.95	64.50	
P value	0.000	0.000	0.000	

0.001; 0.000: significant. High significant at $p < 0.05$ respectively, values in the same line with different lower-case letters are significantly different at $p < 0.05$ according to Duncan's test, *GAE* Gallic acid equivalent, *CGE* cyanidin-3-glucoside equivalents, *FW* Fresh weight

A study surveyed combined metabolomic and transcriptomic analyses between cv. Green Peel and its color mutant cv. Purple Peel. In total, 5 and 22 metabolite contents were analyzed and revealed a significant different within fruit peels. In this study, fruit color at the ripening stage of the two cultivars presented a considerable variation (Wang et al. 2017).

9.7.3 Molecular Breeding

Molecular marker techniques are very helpful to characterize and identify fig germplasm. Molecular markers such as RFLPs (restriction fragment length polymorphism) of the total genome, RAPD (random amplified polymorphic DNA), AFLPs (amplified fragment length polymorphism), ISSR (inter simple sequence repeat) and SSR (single sequence repeats) or microsatellites have been successfully used to characterize fig genetic resources. Therefore, a knowledge of the genetic diversity of fig germplasm is necessary to broaden the genetic base and utilization in breeding programs (Singh 2014).

Fig cultivars are numerous and genotypes are either misidentified or called by different denominations in different regions of the world (Aljane 2016; Aljane et al. 2012; Essid et al. 2017). Fig names were mainly given based on phenotypic and morphological characters such as fruit color, fruit shape, local geography and maturity period. (Aljane and Ferchichi 2009a; Caliskan and Polat 2008; IPGRI and CIHEAM 2003). This approach is influenced by environmental conditions in some traits. In addition, some isozyme markers are used (Cabrita et al. 2001). Molecular identification of fig cultivars was based on RAPD and AFLP markers (Aljane 2016; Cabrita et al. 2001; Galderisi et al. 1999; Khadari et al. 1995; Papadopoulou et al. 2002). Some other markers and microsatellites or SSRs are used to characterize and identify fig cultivars (Essid et al. 2015; McCouch et al. 1997). SSRs have become the markers of choice for fingerprinting purposes in most plant species due to their high polymorphism, codominance and reproducibility (Gupta and Varshney 2000). It is crucial to detect and analyze fig germplasm by molecular markers, both for conservation of fig germplasm and for purposes of crop improvement (Salhi-Hannachi et al. 2003). In fact, molecular markers (RAPD, ISSR and SSR) offer numerous advantages, since they are stable and detectable in all tissues and are not confounded by the environment (Agarwal et al. 2008). In fig, combinations of morphological traits as well as molecular markers are highly recommended for identification, characterization and selection of genetic resources (Almajali et al. 2012; Papadopoulou et al. 2002; Saddoud et al. 2008; Salhi-Hannachi et al. 2003).

Several Mediterranean Region countries have investigated the genetic diversity of figs. In this way, Aljane (2016) characterized 30 Tunisian fig accessions using RAPD markers (Fig. 9.5). At the molecular level, these results reflect one of the highest polymorphisms ratios (79.83%) among cultivars grown in the Mediterranean regions; 39% in 12 RAPD primers (Khadari et al. 1995), 67% in 7 RAPD markers (Papadopoulou et al. 2002), 70% in 13 RAPD markers (Akbulut et al. 2009) and

81% in 7 RAPD primers (De Masi et al. 2003). This high degree of polymorphism revealed the considerable genetic diversity among Tunisian fig accessions and indicates promising potential for selection and availability as a genetic resource. Among the studied accessions, cvs. Zidi 2 and Khdhouri 3 tended to show the lowest genetic distance of (0.269%), suggesting they are very closely related, and possibly might be the same genotype, but with different denominations. For the remaining accessions, the genetic distance matrix showed a high level of divergence at the DNA level (Aljane 2016).

Essid et al. (2015) used 13 SSR markers to characterize 20 Tunisian local caprifigs. Results showed successful and repeatable amplification fragments in all the accession studies, 37 alleles with varied from 2 (LMFC32, LMFC15, LMFC21, LMFC31, LMFC18, LMFC27 and LMFC23) to 6 (LMFC30) alleles per locus. These 13 loci presented an average of 2.85 alleles per locus and amplification fragment sizes within 120 and 278 bp. Some individuals revealed the amplification of 3 alleles in LMFC30, MFC1, MFC4 markers and 4 alleles in LMFC28) suggesting the amplification of more than one locus. Similarity, identification of 209 fig accessions from an ex situ germplasm collection in Spain were amplified four loci (Giraldo 2005). The study of 9 SSR markers revealed one or two bands per genotype and proved the amplification of a single locus (Callen et al. 1993). In addition, when one or two fragments were showed per locus, genotypes were considered homozygous or heterozygous, respectively (Callen et al. 1993).

The SSR markers applied to 20 Tunisian local caprifigs showed that the allelic frequencies varied from 0.03 to 0.98 with a mean of 0.45. In addition, 6 alleles were amplified in one accession; for example the allele 205 of LMFC32 is showed in cv. Jrani and the allele 193 of LMFC 28 in cv. Bouharrag 2 (Essid et al. 2015).

This work is the first molecular characterization of some male fig accessions using SSR markers. Results indicated that there is no clear genetic relatedness among the 20 genotypes conserved in an ex situ germplasm collection and collected from different geographic areas (Fig. 9.6). This suggests an exchange of plant materials within regions (Essid et al. 2015).

Marker-assisted selection (MAS) is used for indirect selection of a genetic determinant or determinants of characters of interest (yield, quality, disease resistance, abiotic stress tolerance). These are important in quantitative inheritance (Jona and Grihaudo 1991; Storey 1975).

Various DNA profiling methods are currently available and consist of two main categories, according to the information provided: specific locus and dominant markers and arbitrary and dominant. The combined action of multiple segregating genes and environmental factors are an excellent example of quantitative variation results. The identity of genotype marker segregation and phenotypic characters of cultivars revealed the detection and location of loci having an effect on the quantitative trait loci (QTL). In fact, QTL was used in MAS (Cabrita et al. 2001; Galderisi et al. 1999).

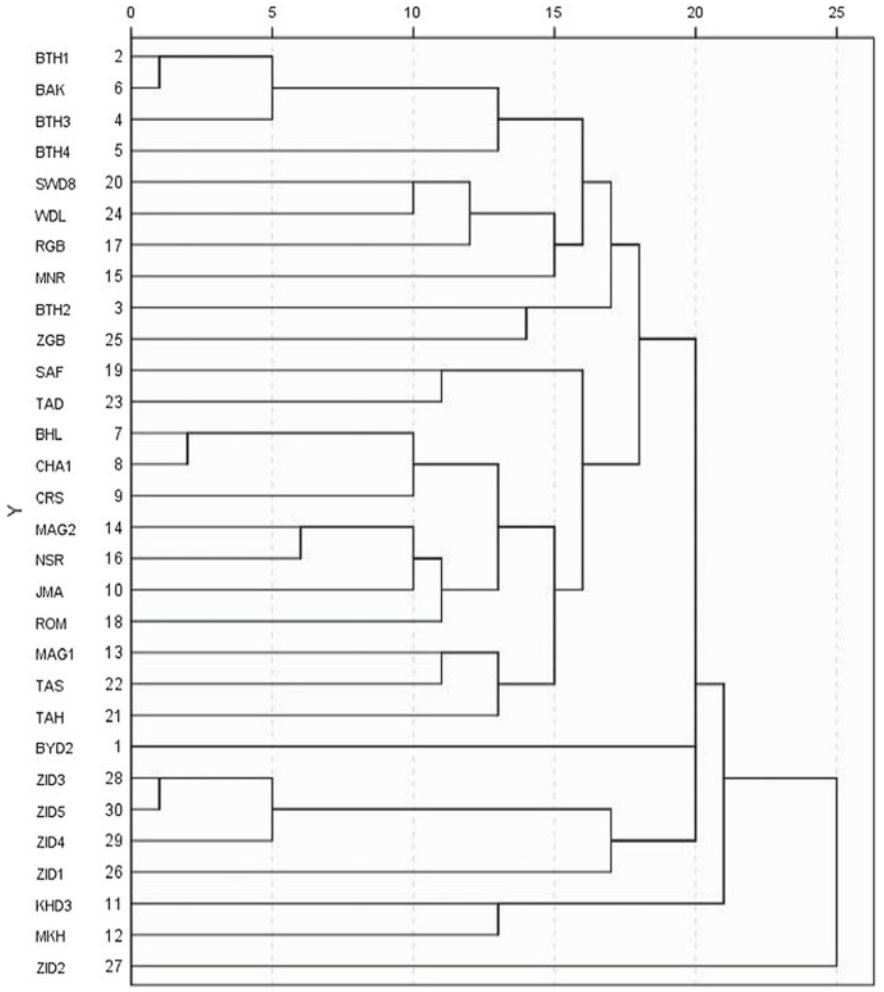

Fig. 9.5 Genetic relatedness among 30 Tunisian fig accessions generated by UPGMA cluster analysis based on RAPD markers. Abbreviations: *BYD2* Bayoudhi2, *HD3* Khadhouri3, *TAD* Tayouri Akhdhar, *BTH1* Bither1, *MKH* Makhbech, *SAF* Safouri, *BTH2* Bither2, *MAG1* Magouli1, *SWD8* Sawoudi8, *BTH3* Bither3, *MAG2* Magouli2, *WDL* Wedlani, *BTH4* Bither4, *MNR* Minouri, *ZGB* Zaghoubi, *BAK* Bither Akhal, *NSR* Nasri, *ZID1* Zidi1, *BHL* Bouholi, *RGB* Ragoubi, *ZID2* Zidi2, *CHA* Châari, *ROM* Romani, *ZID3* Zidi3, *CRS* Croussi, *TAH* Tayouri Ahmar, *ZID4* Zidi4, *JMA* Jemâaoui, *TAS* Tayouri Asfar, *ZID5* Zidi5. *Source* Figure by Fateh Aljane

9.7.4 In Vitro Micropropagation and Applications

In vitro micropropagation has been used successfully for the propagation of the fig tree (Fig. 9.7). Different types of explants have been used; the apical bud is the most important (Nobre and Romano 1998). Regeneration of fig trees was also possible from

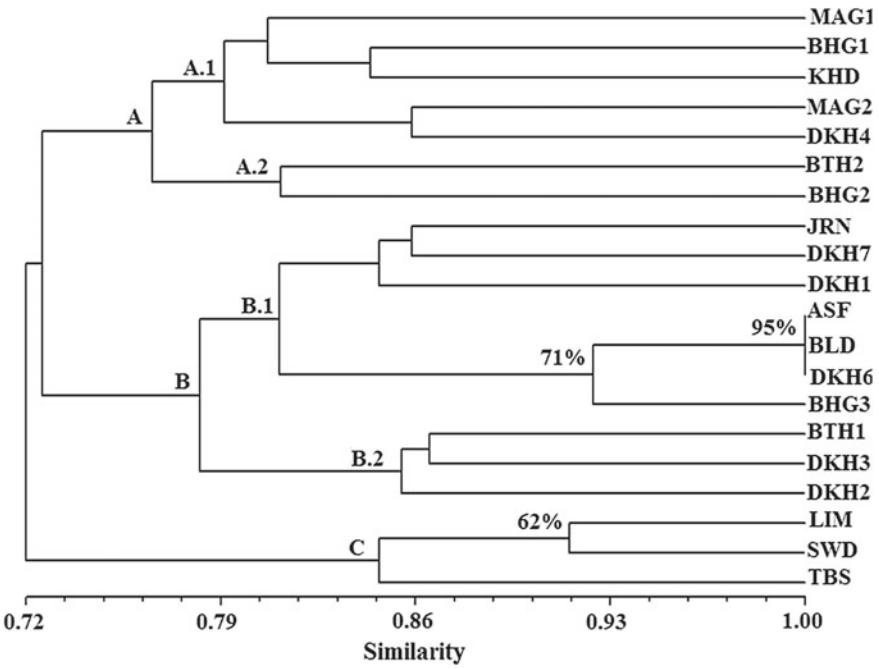

Fig. 9.6 Genetic relatedness among 20 Tunisian caprifig accessions generated by UPGMA cluster analysis based on SSR markers. Abbreviations: *MAG1* Magouli1, *MAG2* Magouli2, *BTH2* Bithri2, *BLD* Beldi, *JRN* Jrani, *DKH2* Dhokkar2, *DKH1* Dhokkar1, *DKH6* Dhokkar6, *BTH1* Bithri1, *DKH3* Dhokkar3, *LIM* Limi, *DKH7* Dhokkar7, *ASF* Assafri, *DKH4 Dhokkar4*, *TBS* Tebessi, *BHG3* Bouharrag3, *BHG1* Bouharrag1, *BHG2* Bouharrag2, *SWD* Sawoudi, *KHD* Khadhouri. *Source* Figure by Fateh Aljane

meristems in order to produce virus-free young plants. In vitro micropropagation will produce fruit after 2 years of planting in an orchard (Demiralay et al. 1998) and produce disease-free plants; the thermotherapy technique coupled with in vitro culture has effectively produced young plants (Lopez Corrales et al. 1998). Somaclonal variation is another source for selecting new variability to develop improved cultivars (Muriithi et al. 1982); however, no new cultivar has yet been released using this approach (Mars 2003).

A study was conducted to evaluate salt tolerance (NaCl) in 5 fig cultivars (Achtoy White, Masone Black, Zeiblly Red, Zeiblly Flair and Shami Stihy) using in vitro tissue culture. Some parameters such as regenerated shoots number, shoot length, leaves number per shoot, fresh weight and dry weight were followed and measured. The results showed that cvs. Masone Black and Shami Stihy were salt-tolerant as compared to the others (Soliman and Abd Alhady 2017).

To investigate the effect of different medium salt strengths and different sources of carbon (fructose and sucrose), shoots of fig cvs. Condaria and Black Mission

Fig. 9.7 Fig explants cultivated by in vitro propagation

were cultured in vitro on a modified medium supplemented (MS) with 0.5 mg/l 6-benzyllaminopurine.

Taha et al. (2013), showed that culture medium (MS) at full strength revealed the highest shoot and leaf number. But, MS with double strength displayed the greatest shoot length. Furthermore, fructose as a carbon source was better that sucrose for micropropagation of fig materials (Taha et al. 2013). Results revealed that fig cvs. Condaria and Black Mission presented a significant difference for all the parameters studied. However, cv. Black Mission produced higher shoot numbers in comparison to cv. Condaria. In addition, cv. Condaria recorded the highest values of both parameters; leaf numbers and shoot length (Taha et al. 2013).

9.7.5 Plant Material Sanitation

Fig mosaic disease (FMD) is one of the major diseases of this crop found throughout the world (Nahdi and Aljane 2014). Micropropagation of fig has been used in the sanitation of fig cultivars from viruses (Elair et al. 2014). FMD is characterized by mosaic, ring spot, discoloration and deformation of leaves; yellow spots on fruits, premature fruit drop and reduced fruit yield (Blodgett and Gomec 1967). Several viruses have been isolated from young leaves showing mosaic symptoms, but none has been identified as the causal agent of FMD (Castellano et al. 2007; Elbeaino et al. 2007; Gattoni et al. 2009).

A study was made of the ex situ fig germplasm collection of the Institute of Arid Regions of Medenine, Tunisia. In total, samples of leaves of 66 figs with mosaic virus-associated symptoms were collected to identify the virus. Fig mosaic virus (FMV) was the prevalent virus, detected in 12.28% of the 66 samples. However, 86.36% of the samples were positive for FBV-1. None of the samples were positive for the following viruses: FMMaV, FLMaV-2, AFCV-1 and FLV-1. Results suggested that a complex of viral infections was likely the probable cause of the mosaic disease symptoms (Nahdi and Aljane 2014).

In Tunisia, efficacy of tissue culture (meristem) in virus elimination from female fig cvs. (Zidi, Soltani and Bither Abiadh) and one caprifig (cv. Asafri) with multi-infections virus (FMV, fig mild-mottle-associated virus: FMMaV, fig fleck associated virus: FFkaV) were studied in vitro. Results showed sanitation rates of the 4 cvs. Zidi, Soltani, Bither Abiadh and Asafri were 79.46, 65.55, 68.75 and 70.83%, respectively. Generally, the elimination of FVC and FMMaV viruses established for a low meristem size (0.5 mm) given sanitation rates of 70–90% (Bayoudh et al. 2017) (Fig. 9.8).

9.7.6 Mutation

Spontaneous and induced mutations may play an important role in fig improvement for important characters such as small ostiole size, large fruit size, fruit flesh quality and tree productivity (Santoni et al. 2000). Mutations can be induced either by specific treatments with physical and chemical mutagens or by tissue culture. In addition, spontaneous mutants can occur in commercial orchards. Recently, a mutant cv. Red Kadota was selected from cv. Kadota, with a red skin that develops during fruit maturation (Flaishman et al. 2007).

Some fig cultivars were mutated by colchicine treatment of axillary buds and seeds (Storey 1975). The effect of gamma radiation on cuttings, seeds, and pollen in figs has been reported (Özen et al. 2017; Storey 1975). The most frequent finding was dwarfness and acceleration of fruiting (Mars 2003).

In Turkey, fig improvement was carried out by induced mutations; improved traits were fruit size, early ripening and small ostiole size. In this study, cvs. Sarılop and

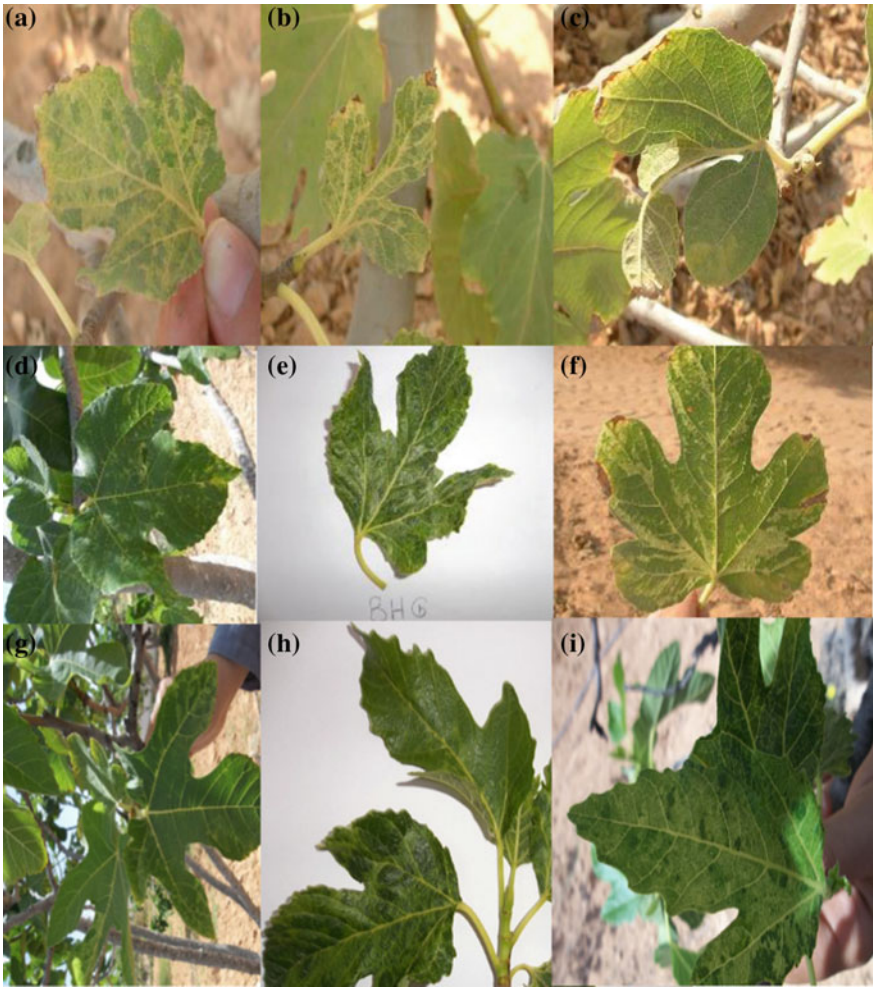

Fig. 9.8 Symptoms observed during the survey of fig germplasm collection of the Institute of Arid Regions (IRA) of Medenine, Tunisia. **a**, **f** Chlorotic spots and thinning of veins, **b**, **c** Deformation and necrosis, **d**, **g** Speckles, **e** Embossing, **h**, **i** Thinning of veins, deformation and yellow area. *Source* Photos by Fateh Aljane

Bursa Siyahı were used and 25 scions were treated with 6 different cobalt gamma radiation doses (0.0, 10.0, 16.9, 25.3, 50.7 and 67.0 unit of absorbed dose: gray). The M1V1 (mutation 1 vegetation 1), M1V2 and M1V3 were established by grafting in 3 years. Some parameters (shoot growing and survival rate) were investigated; the LD50 values were measured as 50.7 gray in cv. Sarılop, and 25.3 gray in cv. Bursa Siyahı (Özen et al. 2017). The results showed 992 cv. Sarılop M1V3 individuals, 836 cv. Bursa Siyahı M1V3 individuals and 1828 M1V3 individuals. Young plants were

planted in an experimental field and epistasis effect deaths in these individuals were examined (Özen et al. 2017).

Mutagenic methodologies have been used in fig breeding to improve genetic resources. In this way, 5 selected plants were treated with gamma ray. Genetic diversity among these 5 plants and the cv. Roxo-de-Valinhos, a commercial crop, was analyzed by using RAPD and AFLP molecular markers. In total, 140 primers (RAPD) and 12 primer combinations (AFLP) were tested and showed no genetic difference within the 5 selected plants and cv. Roxo-de-Valinhos. The 5 fig mutants did not differ genetically within themselves and within cv. Roxo-de-Valinhos; the application of DNA sequencing is necessary to detect polymorphism within treatments. The methylation-sensitive restriction enzymes method must be tested on fig plants to detect the epigenetic variation, which is expressed by the phenotypic variation (Rodrigues et al. 2012).

9.7.7 Hybridization

In California, fig hybridization has been developed, to produce figs with high quality fruit characters, large size and light external dried fig. In addition, this fig would not need to be pollinated and have a closed fruit ostile. A great number of fig hybrid seedlings (2400) were developed in University of California Kearney Agricultural Center (KAC), Parlier, California. A total of 45 seedlings were selected and evaluated based on certain fruit traits such as size, skin color, skin quality, ostiole size, flesh quality and density, fungal decay potential and tree productivity (Doyle et al. 2005). The most significant achievement of the California fig breeding was development of 5 hybrid cvs.: Conadria, Di Redo, Flanders, Tena and Excel. Furthermore, a common cv. Sierra was developed with late summer maturity and green color skin (Doyle and Ferguson 2005).

A research program on fig rootstocks was developed to provide resistance to the soil-borne disease *Ceratocystis canker.* Inter-cultivar differences have been reported in the resistance of fig trees to this disease, but all common fig cultivars contract it to some degree. Interspecific hybridization was performed between the common fig (seed parent) and the wild species *Ficus erecta* (pollen parent) through artificial pollination. Results suggest that interspecific hybridization within *F. carica* and *F. erecta* provides a new fig rootstock source with resistance to *C. canker* (Yakushiji et al. 2012).

The development of protoplast regeneration varieties is still very limited for *Ficus carica*. However, protoplast regeneration of plants has opened new horizons for practical breeding in a number of fruit species. Viable protoplasts from cell suspensions of *Ficus* were developed by incubating cells in medium and then transferring them to another medium with medium supplemented (MS) salts, vitamins and glucose. After cell division was detected 5×10^5 viable protoplasts/cm^3 were obtained (Liamoca Zarate et al. 2006).

9.7.8 Transgenic Varieties

Transgenic varieties are limited in fruit trees and their efficiency is very diverse according to the fruit species, some being recalcitrant (Chevreau 2009). Transgene technology offers many advances for fruit-tree breeding and the development of well-adapted genotypes. For example, the fire blight resistance character was induced into pear cv. Barlett, which is suitable for processing and without modification of fruit quality characters (Janick 2012). As well, transgenic *Ficus carica* trees were used to improve agricultural traits and for the production of foreign proteins and edible vaccines (Flaishman et al. 2012). Nevertheless, some fruit quality characters, biotic stress (virus, bacteria, fungal diseases) and reproductive cycle were modified by gene transfer in fruit trees (fig species). Two protocols have been developed to produce intragenic varieties which include only sequences from the species itself and from two related species (Chevreau 2009).

Transgenic varieties have been developed to improve agronomic traits, fruit storability and disease resistance. The transgenic approach for fig-tree breeding was described by Yancheva et al. (2005), wherein an *Agrobacterium*-mediated transformation of the cv. Brown Turley and Smyrna was realized. In fact, shoot regeneration was developed on medium supplemented with (MS) basal salt with indole butyric acid IBA and sucrose. Regeneration was maximal (100%) when the explants were oriented on the dorsovenetral and developed fig shoots/explants for the cv. Brown Turley (common type) and Smyrna. Technological progress enables gene transfer and permitted the production of desirable proteins in fig fruits, in order to enhance the nutritional and pharmaceutical constitution (Flaishman et al. 2008).

9.8 Conclusions and Prospects

The improvement of breeding approaches in fig has been based on yield, fruit quality, elimination of pollination, fruit ripeness persistence, hardiness to environmental constraints and biotic and abiotic stress resistance. The traditional selection approaches in fig were based on yield, quality and disease resistance. Among the different characters of quality, those of high discriminating level are fruit shape, color, weight, dimensions, acidity and sugar content, and ostiole diameter. Development of successful modern production depends on varietal selection and improvement combined with resolution of some of the problems currently plaguing the industry such as fruit cracking, handling and environmental impact.

Molecular-marker methods are very helpful in differentiation and selection of accessions and varieties according to DNA fragment lengths, Molecular markers such as RFLP, RAPD, ISSR and SSR have reportedly been successfully used for the characterization fig germplasm. Marker-assisted selection is an approach whereby a marker (morphological, biochemical, DNA variation) is used for indirect selection of a genetic determinant or determinants of a characters of interest such as yield, quality, disease resistance and abiotic stress tolerance.

Spontaneous and induced mutations may play an important role in fig improvement for important characters such as small ostiole size, large fruit size, and fruit-flesh quality. Micropropagation techniques of fig have been used in sanitation of fig cultivars from virus contamination and to produce new cultivars.

Morphological and molecular data of fig can be used to guide ex situ conservation measures and to improve conservation of this species. In many countries, the threat of genetic erosion is obvious, particularly for cultivars of the Smyrna type; however, an alternative approach for genetic resources management must be considered. The considerable genetic diversity available in the many regions of the world should be exploited for selection of new cultivars, which may be suitable for arid and semiarid areas. Data from analyzing quantitative variation and especially of uncovering its potential genetic basis are therefore of major importance for breeding programs.

Recently, research was carried out to study phytochemical composition in fig fruits. Fig fruits show high contents of phytochemical (polyphenols, anthocyanins, flavonoids) compositions and are an important source of natural antioxidants which can be valorized and used in the food industry, cosmetics and the medical sectors. Fig mosaic disease is one of the major diseases of fig found throughout the world. There should be an effective strategy for FMD, as it remains a serious pathological constraint to fig germplasm exchange in the world.

Development of successful modern production depends on varietal selection and improvement, combined with resolution of some of the current problems such as environmental impact, fruit cracking and handling problems.

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Appendix 1

Research Institutes

Institute/University/Faculty	Specialization and research activity	Contact information
Fig Research Institute—Erbeyli Incirliova, Aydin Turkey	Fig research	http://www.gfar.net/organizations/fig-research-institute-erbeyli
Ege University Faculty of Agriculture, Bornova Izmir Turkey	Breeding crop	Prof. Aksoy Uygun E-mail: uygun.aksoy@ege.edu.tr
National Clonal Germplasm Repository, University of California Davis California USA	Collection, preservation, evaluation, and distribution the genetic resources of the crops	https://www.ars-grin.gov/ https://www.ars.usda.gov/pacific-west-area/davis-ca/natl-clonal-germplasm-rep-tree-fruit-nut-crops-grapes/ Research leader E-mail: john.preece@ars.usda.gov
Estacion Experimental la Mayora, CSIC, 29750 Algarrobo-Costa, Malaga Spain	Genetic resources conservation and evaluation	Dr. Esther Giraldo E-mail: esthergiraldo@yahoo.es
Conservatoire Botanique National Méditerranéen de Porquerolles, Parc National de Port-Cros, Castel Ste Claire France	Collection, preservation, evaluation, genetic resources of the crops	http://www.cbnmed.fr/pres/index.php
Ecole Nationale d'Agriculture de Meknès Meknès Morocco	Agriculture development research	http://www.enameknes.ac.ma/
Arid Regions of Medenine (IRA) Médenine Tunisia	Agriculture research, conservation biodiversity and combating desertification	http://www.Ira.agrinet.tn Dr. Fateh Aljane E-mail: fateh_aljane@yahoo.fr
Pépinière Baud 910 Chemin du Parrot Le Palis Vaison La Romaine France	Conservation and propagation of pomegranate and fig trees	http://www.fig-baud.com/catalogue.html E-mail: pepinières@fig-baud.com

Appendix 2

Genetic Resources of Fig

Characteristics of some local fig (*Ficus carica* L.) cultivars maintained ex situ in the germplasm collection of the Institute of Arid Regions (IRA), Medenine, Tunisia Cultivars	Important traits				Cultivation location/department in Tunisia
	Fig type	Fruit shape	External color	Fruit weight	
Bayoudhi2	Common	Spheroid without neck	Green yellowish	Medium 20–39 g	Toujen (Médenine)
Bither1	San Pedro	Oblate without neck	Yellow/green yellowish	Medium 20–39 g	Ghadhabna (Mahdia)
Bither2	San Pedro	Globosely without neck	Light red	Medium 20–39 g	Kerkennah (Sfax)
Bither3	San Pedro	Oblate without neck	Yellow	Large 40–60 g	Kerkennah (Sfax)
Bither4	San Pedro	Oblate without neck	Green yellowish	Medium 20–39 g	Toujen (Médenine)
Bither Akhal	San Pedro	Oblate with neck/oblate without neck	Red yellowish/dark red	Large 40–60 g	Zarzis (Médenine)
Bouholi	San Pedro	Oblate with neck/spheroid without neck	Purple/light red/dark purple	Medium 20–39 g	Djébba (Béjà)
Châari	Smyrna	Spheroid with neck	Yellow	Medium 20–39 g	Zarzis (Médenine)
Croussi	Smyrna	Oblate without neck/oblate with neck	Dark red	Medium 20–39 g	Beni Kheddâche (Médenine)
Jemâaoui	Smyrna	Globosely without neck/pyriform with long and curved neck	Red yellowish	Medium 20–39 g	Zammour (Médenine)
Khadhouri3	Smyrna	Spheroid without neck	Green yellowish	Medium 20–39 g	Djerba (Médenine)
Makhbech	Common	Globosely with neck	Green	Medium 20–39 g	Bir Amir (Tataouine)
Magouli1	Smyrna	Oblate without neck	Green	Medium 20–39 g	Bir Amir (Tataouine)

Characteristics of some local fig (*Ficus carica* L.) cultivars maintained ex situ in the germplasm collection of the Institute of Arid Regions (IRA), Medenine, Tunisia Cultivars	Important traits				Cultivation location/department in Tunisia
	Fig type	Fruit shape	External color	Fruit weight	
Magouli2	Smyrna	Spheroid without neck	Yellow	Medium 20–39 g	Djerba (Médenine)
Minouri	Smyrna	Spheroid with neck	Red yellowish	Small < 20 g	Bir Amir (Tataouine)
Nasri	Smyrna	Globosely with neck/pyriform with massif neck	Red yellowish	Medium 20–39 g	Toujen (Médenine)
Ragoubi	Smyrna	pyriform with long and curved neck	Red yellowish	Medium 20–39 g	Zammour (Médenine)
Romani	Common	Oblate without neck	Red yellowish	Medium 20–39 g	Bir Amir (Tataouine)
Tayouri Ahmar	Smyrna	Spheroid with neck/globosely with neck	Yellow	Medium 20–39 g	Bir Amir (Tataouine)
Tayouri Asfar	Smyrna	Pyriform with long and curved neck	Red/dark red/purple blackish	Large 40–60 g	Bir Amir (Tataouine)
Tayouri Akhdhar	Smyrna	Oblate without neck/oblate with neck	Red yellowish	Large 40–60 g	Bir Amir (Tataouine)
Safouri	Smyrna	Spheroid without neck/pyriform with massive neck	Yellow	Medium 20–39 g	Zammour (Médenine)
Sawoudi8	Smyrna	Pyriform with massif neck	Green	Large 40–60 g	Toujen (Médenine)
Wedlani	Common	Spheroid without neck/oblate with neck	Purple greenish/dark red	Medium 20–39 g	Zammour (Médenine)
Zaghoubi	Smyrna	Pyriform with massive neck/pyriform with long and curved neck	Red yellowish	Medium 20–39 g	Beni Kheddâche (Médenine)
Zidi1	Smyrna	Pyriform with massive neck	Red purple blackish	Large 40–60 g	Bir Amir (Tataouine)

Characteristics of some local fig (*Ficus carica* L.) cultivars maintained ex situ in the germplasm collection of the Institute of Arid Regions (IRA), Medenine, Tunisia Cultivars	Important traits				Cultivation location/department in Tunisia
	Fig type	Fruit shape	External color	Fruit weight	
Zidi2	Smyrna	Pyriform without differentiated neck	Red	Medium 20–39 g	Djébba (Béjà)
Zidi3	Smyrna	Pyriform with massive neck	Light Red	Medium 20–39 g	Dégâche (Tozeur)
Zidi4	Smyrna	pyriform with long and curved neck	Red	Medium 20–39 g	Ghannouch (Gabès)
Zidi5	Smyrna	pyriform with long and curved neck	Purple	Medium 20–39 g	Massjed Aissa (Sousse)
Cultivars	Important traits				Cultivation location/department in Tunisia
	Fruit length	Fruit diameter	Skin thickness	Ostiole opening	
Bayoudhi2	Short 29–45 mm	Medium 39–49 mm	Semi-open 1–3 mm	Fine < 1 mm	Toujen (Médenine)
Bither1	Short 29–45 mm	Medium 39–49 mm	Semi-open 1–3 mm	Medium 1–105 mm	Ghadhabna (Mahdia)
Bither2	Short 29–45 mm	Small 28–38 mm	Semi-open 1–3 mm	Medium 1–105 mm	Kerkennah (Sfax)
Bither3	Short 29–45 mm	Large 50–60 mm	Semi-open 1–3 mm	Fine < 1 mm	Kerkennah (Sfax)
Bither4	Short 29–45 mm	Small 28–38 mm	Semi-open 1–3 mm	Fine < 1 mm	Toujen (Médenine)
Bither Akhal	Short 29–45 mm	Medium 39–49 mm	Open > 3 mm	Fine < 1 mm	Zarzis (Médenine)
Bouholi	Short 29–45 mm	Medium 39–49 mm	Open > 3 mm	Medium 1–105 mm	Djébba (Béjà)
Châari	Short 29–45 mm	Small 28–38 mm	Semi-open 1–3 mm	Medium 1–105 mm	Zarzis (Médenine)
Croussi	Short 29–45 mm	Medium 39–49 mm	Semi-open 1–3 mm	Medium 1–05 mm	Beni Kheddâche (Médenine)
Jemâaoui	Medium 46–54 mm	Small 28–38 mm	Semi-open 1–3 mm	Medium 1–105 mm	Zammour (Médenine)

Cultivars	Important traits				Cultivation location/department in Tunisia
	Fruit length	Fruit diameter	Skin thickness	Ostiole opening	
Khadhouri3	Short 29–45 mm	Small 28–38 mm	Semi-open 1–3 mm	Fine < 1 mm	Djerba (Médenine)
Makhbech	Short 29–45 mm	Medium 39–49 mm	Semi-open 1–3 mm	Medium 1–105 mm	Bir Amir (Tataouine)
Magouli1	Short 29–45 mm	Medium 39–49 mm	Semi-open 1–3 mm	Fine < 1 mm	Bir Amir (Tataouine)
Magouli2	Short 29–45 mm	Medium 39–49 mm	Semi-open 1–3 mm	Fine < 1 mm	Djerba (Médenine)
Minouri	Short 29–45 mm	Small 28–38 mm	Closed < 1 mm	Fine < 1 mm	Bir Amir (Tataouine)
Nasri	Short 29–45 mm	Small 28–38 mm	Semi-open 1–3 mm	Medium 1–105 mm	Toujen (Médenine)
Ragoubi	Long 55–75 mm	Small 28–38 mm	Semi-open 1–3 mm	Medium 1–105 mm	Zammour (Médenine)
Romani	Short 29–45 mm	Medium 39–49 mm	Open > 3 mm	Medium 1–105 mm	Bir Amir (Tataouine)
Tayouri Ahmar	Short 29–45 mm	Small 28–38 mm	Semi-open 1–3 mm	Medium 1–105 mm	Bir Amir (Tataouine)
Tayouri Asfar	Long 55–75 mm	Medium 39–49 mm	Open > 3 mm	Medium 1–105 mm	Bir Amir (Tataouine)
Tayouri Akhdhar	Short 29–45 mm	Medium 39–49 mm	Open > 3 mm	Medium 1–105 mm	Bir Amir (Tataouine)
Safouri	Medium 46–54 mm	Medium 39–49 mm	Semi-open 1–3 mm	Medium 1–105 mm	Zammour (Médenine)
Sawoudi8	Short 29–45 mm	Small 28–38 mm	Semi-open 1–3 mm	Medium 1–105 mm	Toujen (Médenine)
Wedlani	Short 29–45 mm	Medium 39–49 mm	Open > 3 mm	Fine < 1 mm	Zammour (Médenine)
Zaghoubi	Medium 46–54 mm	Small 28–38 mm	Semi-open 1–3 mm	Medium 1–105 mm	Beni Kheddâche (Médenine)
Zidi1	Long 55–75 mm	Medium 39–49 mm	Semi-open 1–3 mm	Medium 1–105 mm	Bir Amir (Tataouine)
Zidi2	Medium 46–54 mm	Small 28–38 mm	Semi-open 1–3 mm	Thick > 1.5 mm	Djébba (Béjà)
Zidi3	Medium 46–54 mm	Small 28–38 mm	Semi-open 1–3 mm	Medium 1–105 mm	Dégâche (Tozeur)
Zidi4	Medium 46–54 mm	Small 28–38 mm	Semi-open 1–3 mm	Thick > 1.5 mm	Ghannouch (Gabès)
Zidi5	Long 55–75 mm	Medium 39–49 mm	Semi-open 1–3 mm	Fine < 1 mm	Massjed Aissa (Sousse)

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Chapter 10
Kiwifruit (*Actinidia* spp.) Breeding

Zac Hanley

Abstract Kiwifruit are the first representatives of the genus *Actinidia* to be domesticated and the most recent fruit to be globally commercialized. They present challenges to breeders and orchardists due to long juvenility, dioecy, high heterozygosity, multiple ploidies and climbing growth habit. The first cultivars were selections from the wild or open-pollinated varieties but, since breeding began in New Zealand in the 1970s, progress has been made in several countries. Yellow-fleshed and red-fleshed kiwifruit are now found alongside the original green-fleshed varieties. Global production has increased significantly in recent years, as have the number of cultivars available in some countries. However, many commercially important and globally distributed *Actinidia* cultivars remain vulnerable to the bacterial canker of kiwifruit pandemic caused by *Pseudomonas syringae* pv. *actinidiae*. The molecular genetics revolution is poised to accelerate the development of new cultivars now that the *Actinidia* genome sequences and low-cost genotyping are becoming available. Conventional breeding is already augmented by marker-assisted selection and now genomic selection will be applied in kiwifruit and their smaller berry-like relatives. The results will soon provide consumers worldwide with a range of appealing flavors, colors, health benefits and fruit formats from the *Actinidia* genus.

Keywords Kiwifruit · *Actinidia* · Breeding · Domestication · Marker-assisted selection · Genomic selection

10.1 Introduction

The genus *Actinidia* includes the cultivars *A. chinensis* var. *chinensis* and *A. chinensis* var. *deliciosa*, vines whose fruit, commonly known as kiwifruit, are yellow- and green-fleshed. They are also known as *kiwis* in some countries where the flightless

Z. Hanley (✉)
The Institute of Plant and Food Research New Zealand Limited, Private Bag 92169, Auckland Mail Centre, Auckland 1142, New Zealand
e-mail: zac.hanley@plantandfood.co.nz

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birds known by that name for centuries are rarely thought of. Even the use of *kiwi* to denote the people of New Zealand predates the fruit use. Kiwifruit and their relatives have a distinctive taste, high in vitamin C and soluble fiber. They have been the subject of selective breeding for only a few decades. Kiwis are the most recently domesticated global fruit. *Actinidia* vines are temperate, long-lived, deciduous perennials which evolved in southwest China and are propagated clonally. This chapter discusses the breeding, both conventional and genomic, of new kiwifruit cultivars in the context of the special characteristics of the genus, being dioecy, ploidy races, long generation cycles, recent domestication and high heterozygosity. Conventional breeding has been successful; more advanced breeding is only just beginning to prove itself.

10.1.1 The Genus *Actinidia*

The genus *Actinidia* is part of the Actinidiaceae family within the Ericales order of dicotyledonous flowering plants. The geographic center of origin is in southwestern and central China and their current wild distribution ranges north from those near-tropical regions to Siberia and east to the Korean peninsula and Japan (Huang 2016b). The genus is thought to have experienced recurrent polyploidization, extensive intra- and interspecific hybridization, and diverse selective environments, resulting in a reticulate evolutionary pattern whose species-level phylogeny remains contentious (Liu et al. 2017). The most recent revision by Li et al. (2007) was updated by Huang (2016c), who denominated 54 species (52 endemic to China, 1 from Japan, 1 from Nepal) and 21 varieties. This may yet be revised as the genus is poorly sampled. However, commercial breeding and production has been based almost entirely on two species: *A. chinensis* and, to a much lesser extent, *A. arguta*.

The fruit of *Actinidiae* are defined botanically as berries, derived from a single ovary with many carpels. The fruit are zoned with a central core surrounded by seed-containing locules embedded in an inner pericarp, an outer pericarp and skin. All may be eaten, although commercially-available kiwifruit usually have dry, variably hairy skins that consumers typically remove. The pericarp zones are rich in vitamin C and colorful. Unripe fruit are typically green-fleshed. Ripe green flesh is a result of the persistence of chlorophyllous chloroplasts, while yellow is caused by carotenoids (predominantly lutein and beta-carotene) revealed when chlorophyll is lost, and red derives from the presence of anthocyanins such as cyanidin galactosides (Montefiori et al. 2005); pigments can also combine to create purples, oranges, olive greens and more. Fruit flesh textures range from juicy and melting to gelatinous, determined by the genetics of the cultivar and developmental maturity of the fruit. The flavor of the commonest kiwifruit cultivar, the widely grown green-fleshed *Actinidia chinensis* var. *deliciosa* cv. Hayward, is associated with a low ratio of sugars to organic acids and the presence of ethyl butanoate and (*E*)-2-hexenal; however, altered sugar-acid ratios and a range of volatiles modulate flavor, including terpenes in *A. arguta* varieties and esters in yellow-fleshed *A. chinensis* var. *chinensis*. The diversity and range of sizes and flesh colors is illustrated in Fig. 10.1.

Fig. 10.1 Top: a cross-section of fruit from different *Actinidia* species showing the diversity and range of sizes and flesh colors within the genus. Bottom: cultivars have divided the kiwifruit market according to their fruit flesh color. Images © The New Zealand Institute for Plant and Food Research Limited

All *Actinidia* species are functionally dioecious, using an active-Y sex determination mechanism (Ferguson and Huang 2007). Flowers are five-petalled and lure insect pollinators through visual and olfactory triggers. Pistillate flowers are morphologically perfect, having compound ovaries but also anthers bearing non-viable pollen. Staminate flowers are unisexual, lacking ovaries, so do not usually develop into fruit. Occasional adromonoecious (inconstant) males have been observed that produce small fruit and in *A. chinensis* var. *deliciosa* have been used to generate self-

fertile stable hermaphrodites (McNeilage and Steinhagen 1998). Hermaphroditism is rare and its broader distribution would radically affect the breeding of *Actinidia* cultivars.

The ploidy of an individual *Actinidia* plant cannot be taken for granted (Huang 2016c). Their basic chromosome number $x = 29$, a large number likely arising from rediploidization of a paleopolyploid progenitor (Ferguson and Huang 2007). Chromosome counts and flow cytometry have identified diploids (31 taxa), tetraploids (17 taxa), hexaploids (3 taxa), heptaploids (1 taxon), and octoploids (8 taxa); however, ploidy is not diagnostic of phylogeny and at least 13 species contain examples of more than one ploidy (Huang 2016c). Flow cytometry of a natural population of *A. arguta* has identified individuals with $4x$, $6x$, $8x$ and $10x$ ploidies. Ploidy races tend to be separated geographically in the wild, either regionally or by altitude. This complexity can stymie the exchange of genetic material in a breeding program but also offers opportunities where technologies can reunite diverse gene pools separated by time, geography and ploidy barriers.

10.1.2 Domestication and Commercial Exploitation

Kiwifruit have long been known to be delicious. Called *mihoutao* (macaque monkey peaches) in China, they were occasionally cultivated as far back as the Tang dynasty more than a thousand years ago. However, the successful development of kiwifruit as a global fruit is largely due to the excellent characteristics (and excellent marketing) of the green-fleshed cv. Hayward. This cultivar was selected by, and later named for, New Zealand nurseryman Hayward Wright around 1930. This was not through breeding: Wright did not carry out controlled crosses but instead grew a tangled patch of 14 plants from which he observed and isolated what he termed Wright's Large Oval, a superior fruit popular with local consumers. His starting material were plants a generation or two from a few seed brought from China to New Zealand by school headmistress Isabel Fraser after a visit to Yichang in 1903. Although other kiwifruit seeds and plants were taken to Europe and the USA, it was considered only an ornamental outside China and New Zealand, where its dioecious nature and cropping potential were recognized (Ferguson 2005).

10.1.2.1 Internationalization

In 1975, New Zealand exporters agreed that henceforth only cv. Hayward kiwifruit would be exported from the country. The Hayward fruit was large, handsome, high in vitamin C, and had a flavor preferred by consumers over that of fruit from other kiwifruit cultivars then available. Most importantly, it remained more palatable to consumer appeal after longer in cool storage and therefore allowed the development of an export industry based from New Zealand initially serving the USA and later the world. In that year, nearly three-quarters of a million tray-equivalents of kiwifruit

were exported; 40 years later, export volumes had grown 110-fold. Note that tray-equivalents (TE) are the standard unit of kiwifruit export from New Zealand. 1 TE has been set at 3.6 kg of fresh fruit since 2014.

While cultivars appeared slowly, agronomic techniques improved rapidly. Grafting onto established rootstocks allowed plants of known sex to be distributed, thus preventing growers from accidentally establishing monoecious gardens that required 4–5 years to show their inability to set fruit (Ferguson and Bollard 1990). In later high-productivity cropping, the benefit of grafting is to shorten the juvenility period. The kiwifruit vine was tamed by T-bars (later, pergolas) that exploit its climbing growth habit by providing alternative support structures to forest trees. Coupled with cane and bud management, this has created high-yielding growth environments that have proven increasingly profitable. Average per-hectare productivity in New Zealand has increased from 25 t ha^{-1} of Hayward cv. in the 1980s (Sale and Lyford 1990) to 38 t ha^{-1} for 2015/2016; mature yield of cv. Zesy002 (Zespri SunGold Kiwifruit) approach 50 t ha^{-1}. Postharvest studies have extended the harvest, storage and therefore marketing windows: kiwifruit can now be supplied for much of the year from a single geographic source.

10.1.2.2 Beyond New Zealand

Meanwhile, a systematic collection program in China began in 1978 (Huang et al. 2003). From this, most selections became widely grown cultivars (Ferguson and Huang 2007) e.g. the vigorous green-fleshed Chinese domestic variety Qinmei. Collected seed i.e. progeny of open-pollinated wild selections also led to important cultivars, including the red-fleshed *Actinidia. chinensis* var. *chinensis* cv. Hongyang (Wang et al. 2003). Cultivation technologies are being adopted, adapted, developed, and shared.

The success of the Hayward cultivar from New Zealand led to competition and a need for a new, differentiated kiwifruit offering to consumers. In 2000, the yellow-fleshed cv. Hort16A was launched as Zespri® Gold Kiwifruit and led to a second wave of global growth in kiwifruit consumption. By 2010, almost all of New Zealand kiwifruit production was based on two female cultivars, *Actinidia chinensis* var. *deliciosa* cv. Hayward and *A. chinensis* var. *chinensis* cv. Hort16A. Over the same period, orchard area in China for domestic consumption grew dramatically and now almost half of all kiwifruit are produced and consumed there.

The fruit of *Actinidia arguta* have also been collected from the wild in China, Japan and Siberia for many years. However, the commercialization of this species has not progressed to the same degree as the *A. chinensis* varieties. *A. arguta* fruit (sometimes known as hardy kiwi, baby kiwi, or kiwiberries) are sweet, grape-sized edible-skinned *Actinidia* but differing climatic and vine management requirements have inhibited commercial production. Kiwiberry vines have a longer chilling requirement, break bud earlier, and produce fruit that do not store as well as *A. chinensis*. For this reason, there are estimated to be only approximately 200 ha of commercial kiwiberry pro-

duction (NZ KiwiBerry Growers Inc. 2017), compared with 160,000 ha of kiwifruit worldwide (Ferguson 2016).

Current world production of kiwifruit is almost 3 mt, two-thirds of which are the green Hayward variety. Kiwifruit are a minor crop representing only 0.2–0.3% of the total annual global fresh fruit production. Just six countries (China, Italy, New Zealand, Iran, Chile and Greece) account for over 90% of production. Four-fifths of production and more than 90% of annual consumption are in the Northern hemisphere; Southern hemisphere producers New Zealand and Chile therefore enjoy an advantage of supplying the low-competition counter-season but endure the disadvantage of high shipping costs to northern markets. The larger main producer countries have breeding programs.

In the 2000s, the successful global industry was built on a narrow genetic base that could and did crumble. It was saved by changes to orchard management and by breeding.

10.1.3 Psa Pandemic

The single largest change in the breeding context of kiwifruit in recent years has been the arrival of the destructive pathogenic bacterium *Pseudomonas syringae* pv. *actinidiae* (Psa) in Italy and New Zealand in the last decade (Vanneste 2017). The bacterium causes kiwifruit bacterial canker disease, leading to necrotic leaf spots; bud rot; shoot die back; wilting of buds, shoots or canes; cankers on canes, leaders, or trunks; production of a red rusty exudate; (rarely, but diagnostically) a milky-white exudate from infected tissues, and extensive vine death. The initial economic losses were felt most severely in New Zealand, costing perhaps NZD 930 million in the first four years (Birnie and Livesey 2014) with the eventual impact perhaps as high as NZD 2 billion (Vanneste 2015).

Aggressive containment measures in infected countries had varying success. Upon first detection in New Zealand in 2010, the disease spread through kiwifruit production orchards more rapidly than anywhere else in the world. Within a year, orchards across an area over 4000 km^2 were infected. This was due to the climatic conditions, which favor infection for most of the year, and a concentrated production diculture, dominated by just two cultivars. The *Actinidia chinensis* var. *chinensis* cv. Hort16A was highly susceptible, represented approximately 20% of New Zealand's production area, and was often grown in contiguous orchards (Costa and Ferguson 2015), effectively forming a monoculture. Moreover, the climate in the primary growth region, the Bay of Plenty, North Island, favored the bacterium's development and dissemination, with average spring and summer temperatures in a band supporting optimal bacterial reproduction while winter temperatures were not low enough to inhibit growth. Precipitation rates and the frequency of mild frosts also favored the establishment of systemic infections and the transmission from vine to vine (Froud et al. 2015).

In 2011, the last pre-Psa harvest produced 29 million TE of Zespri® Gold kiwifruit for export from New Zealand. Within three years output shrank by three quarters and today it is virtually zero. The replacement, cv. Zesy002, sometimes known as cv. Gold3, was launched in 2012, marketed as Zespri® SunGold. It is a breeding success story, described below. Psa is now ubiquitous factor in kiwifruit breeding programs and no commercial cultivar is likely to succeed if susceptible to the disease.

10.1.4 Into the Future

Kiwifruit production and breeding are increasing for at least three reasons. First, kiwifruit remain under threat from disease incursions that may devastate global production—in recent years, the generalist pathogen *Ceratocystis fimbriata* has spread through Brazilian kiwifruit plantations and caused severe losses (Piveta et al. 2016). New cultivars will need greater tolerance to this and other pathogens and wild germplasm must be collected and preserved.

Second, the diversity of *Actinidia* species and its comparatively low market penetration to other fruit offer the opportunity to supply greater novelty to consumers.

Third, abiotic risks must also be mitigated. Varieties of kiwifruit that can withstand lower quality soil and water conditions found outside the current production areas are required. More importantly, climate change predictions show perturbations in seasonal temperature and precipitation cycles that will significantly affect flower, and therefore fruit, production and timing in New Zealand's largest production area by the second half of this century (Tait et al. 2017). Other production areas will be similarly affected. If current cultivars become non-viable in future climates, new cultivars will be required.

10.2 Breeding of *Actinidia* Cultivars

The first *Actinidia* cultivars were selections from open-pollinated seedlings ultimately derived from seed extracted from wild fruit e.g. green cv. Hayward in 1930, the male cv. Chieftain in 1989 and red cv. Hongyang in 1993. The intent was to provide fruit with appeal to consumers. Selections directly from the wild continue to be the main source of cultivars, such as the widely-planted cv. Jintao, commercialized as Jingold™, with its long, long-storing yellow-fleshed fruit. Some spontaneous clonal variations, including the Kramer strain of cv. Hayward common in New Zealand, have proven successful, as have occasional budsports (see below). However, systematic breeding is becoming more important in *Actinidia* cultivar development in order to meet increasingly high grower, marketer and consumer expectations. Breeding targets have consequently grown beyond flavor: breeders now seek high-yielding vines producing consistent flavorful fruit with visual appeal (unblemished skins, vivid flesh colors) and that are compatible with the producer country's growing environ-

ment, including soil quality, climatic profile, horticultural intensity, locally available pollinizers, rootstocks and of course the presence and severity of Psa.

10.2.1 Beginning Breeding

Controlled crosses and trait selection increase genetic gain and the consequent likelihood of providing a commercial cultivar. This contrasts with open pollination, where pedigree information is absent, the breeding value of at least one parent is unquantified, the predictability of the outcome is reduced, and accidental inbreeding is a risk. The most advanced kiwifruit breeding programs carry out within-family phenotypic selections in trait-specialized populations to maintain effective population size; potential cultivars are seedlings from interfamily terminal crosses.

Breeding pedigrees are short in the *Actinidia* because its members are still in the midst of domestication and so crosses tend to be between highly heterozygous individuals. Coupled with the permeability of the species barrier within the genus (ploidy considerations notwithstanding), this means that interfamily crosses can be between diverse individuals with high variance in specific combining ability. For highly-heritable traits, this can theoretically result in significant genetic gains and population improvement. However, in *Actinidia* genetic gain this must be balanced with considerations of gene pool size—it is generally accepted that *Actinidia* germplasm repositories even in China represent a small sample of the wild populations (Huang 2016c) and so inbreeding depression may counterbalance additive genetic gains from high heterozygosity. Repositories and vines in the wild remain under threat from diseases such as Psa or environmental disruption and there are efforts to conserve uncharacterized and underutilized germplasm e.g. Li and Lowe (2007).

The longest and most successful kiwifruit breeding program began with controlled pollinations in 1979 by Stuart Dawes and Ron Davison of New Zealand's Department of Scientific and Industrial Research (DSIR) (Alan Seal, pers comm). Forwards selection for fruit taste, fruit color and fruit size led to some cultivars e.g. early-maturing Tomua. However, the most significant breeding success from the *Actinidia* genus was cv. Hort16A, later marketed as Zespri® Gold Kiwifruit.

Yellow-fleshed *Actidinia chinensis* var. *chinensis* were introduced from China to New Zealand in 1977. Ten years later, Mark McNeilage, a kiwifruit breeder at DSIR, carried out crosses aimed at improving on a vine with small yellow-fleshed fruit but a distinct flavor, by crossing it with a male whose sister vines all bore large fruit. Two other DSIR breeders, Russell Lowe and Hinga Marsh, assessed vines in the family in 1991 and identified cv. Hort16A as a large-fruited, yellow-fleshed kiwifruit with superior flavor. As Zespri® Gold, cv. Hort16A established a new consumer category whose spectacular growth only ceased with the arrival of Psa. Others soon followed, including the aforementioned Chinese wild selection cv. Jintao and later cv. Soreli from breeders in Italy. These same breeders have also developed an early-harvest green *A. chinensis* var. *deliciosa* cultivar called Summerkiwi™.

There have been fewer generations of kiwiberry (*Actidinia arguta*) breeding, although a large number of wild selections and first-generation selections are available. Hybrids of *A. arguta* with other species have led to some commercial cvs. e.g. Hortgem Rua (Williams et al. 2003). Kiwiberries remain a small breeding effort relative to kiwifruit, concomitant with the size of the international market; this may change in the future.

10.2.2 Playing with Ploidy

Awareness of ploidy is essential in kiwifruit breeding, both as an opportunity and a hindrance. Exploiting ploidy alteration was the basis of the spectacular recovery of the yellow-fleshed kiwifruit export industry after Psa. Natural ploidy alterations have been observed. The Hort16A cv. infrequently produced numerically unreduced 2*n* gametes which allowed it to produce viable tetraploid offspring when crossed with a tetraploid pollinizer (Seal et al. 2012). One offspring of such a cross, the 4*x* cv. Zesy002, was found in 2010 to be tolerant to the Psa bacterium eliminating its mother cv. Hort16A. Zesy002 (also known as Gold3) went on to be marketed as Zespri® SunGold Kiwifruit, which in 2016–2017 resulted in 46 million TEs of exported yellow-fleshed kiwifruit from New Zealand. Within six years of the devastation of the Hort16A cv., its replacement was providing greater volumes, grower returns, and economic value than its Psa-susceptible predecessor. One lesson for breeders is to continue breeding despite success.

Interploidy crosses in the wild or during breeding have also led to successful cvs. such as Jinyan and HortGem Rua (Ferguson and Wu 2016). Such crosses can sometimes be wasteful, either failing as a result of embryo abortion or resulting in sterile (*odd-ploid*) seedlings. These obstacles may be overcome or avoided by embryo rescue techniques (Mu et al. 1990) and direct ploidy manipulation (see below), respectively. Tissue culture of *Actinidia* species is also used to accelerate clonal propagation of new cultivars; in some countries, e.g. Italy, growers establish entire orchards from seedlings produced by micropropagation.

10.3 Enhancing *Actinidia* Breeding

Since the advent of molecular genetics, richer information is now available to breeders on the nature of traits and the mechanisms underlying them. In particular, Next-generation sequencing (NGS)—a selection of high-throughput low-cost methods widely available from service providers competing on price and quality to deliver large-scale nucleic acid sequence information—has changed the field. Readily available DNA sequencing not only allows more rigorous pedigree analyses but also increases the scale of allele mining and transcript profiling. These assist and accelerate the discovery of molecular markers and the elucidation of the molecular mech-

anisms underlying traits of interest. The application of molecular genetics towards population improvement in kiwifruit has followed the development of molecular biology tools. But it is less advanced than in more widely-planted crops with transnational investment and greater roles in food security. Success in other species, albeit occasionally overhyped (Bernardo 2016), has meant their application and impact in kiwifruit is sure to increase.

10.3.1 Mutation Breeding

Sports arise spontaneously, infrequently, and occasionally beneficially in all species. In kiwifruit, several budsports of cv. Hayward have been found with potential advantages over existing cultivars e.g. hairless TopStar®, early-maturing Green Light® and cylindrical Wilkins Super (Ferguson and Seal 2008)—however, none have replaced Hayward. Most sports are no doubt overlooked and not everyone can wait, and so the deliberate induction of mutations into genomes, followed by selection, has been a mainstay of plant breeding since early twentieth-century experiments when X-rays produced useful wheat varieties (Kharkwal et al. 2004). The strategy has been successful in providing much-needed diversity to large-scale inbred crop plants such as wheat and barley, as well as a range of novel ornamentals (Broertjes 1966). Mutation breeding can theoretically also allow a breeder to rectify a single deficiency in an elite cultivar—for commercial kiwifruit, the most obvious deficiency target would be Psa susceptibility of cvs. such as Hongyang and Hort16A. However, the promise remains largely unfulfilled in kiwifruit after some early attempts e.g. Shen et al. (1990). There will be more progress now that genomic information and molecular biology techniques are available. To avoid the problem of chimeras in a vegetatively-propagated plant such as kiwifruit, Pathirana et al. (2016) used callus- and somatic embryo-based in vitro approaches, allowing regeneration from single mutagenized cells. Mutagens used included chemical (ethyl methanesulfoanate), physical (gamma irradiation), and biological (transposon) agents. A large population of cv. Hort16A kiwifruit were regenerated from treated callus tissue and cultivated in an open orchard in an epiphytotic Psa region of New Zealand. The survivors are being screened for their potential in breeding programs.

10.3.2 Marker-Assisted Selection (MAS)

The ability to genotype a plant—whether to the fullest degree with the complete genome sequence or with only a single identifier such as a random amplified polymorphic (RAPD) DNA pattern or a single nucleotide polymorphism—offers nothing by itself. The only useful genotype is one associated with a phenotype. In *Actinidiae*, where phenotypes of interest may take time to manifest, e.g. sex and fruit traits follow after a long juvenility period, or be unavailable, e.g. male contributions to the fruit

traits of daughters, genotypes have more value than in short-generation or monoecious plants. But the association of genotype with phenotype must be durable. This was initially challenging in *Actinidia* species because high intraspecific diversity meant that molecular markers such as RAPDs were not transferable between individuals or able to be ordered into linkage groups corresponding to chromosomes.

The first successful use of marker-assisted selection in kiwifruit followed the generation of sequence-determined marker from an RAPD marker segregating with the male sex-determining region (Gill et al. 1998). Male kiwifruit vines do not bear fruit but occupy economically-valuable canopy space, consume orchard management resources and mandate the use of honeybees. Unfortunately, they are morphologically indistinguishable from females until post-juvenility flowering at least 2–3 years after germination. They represent 50% of seedlings because the ratio of females to males in *Actinidia* crosses is 1:1 regardless of ploidy (Testolin et al. 1995). It is therefore desirable to identify males within a breeding program as early as possible, preferably while still in the nursery, and eliminate all but a few needed to develop new pollinizers or to pollinate the many females in the breeding program. Sex determination in *Actinidia* is located in a recombination-suppressed region of one autosome and several *Actinidia* sex markers are now available (Testolin and Cipriani 2016), including in kiwiberry (Gustafson 2016). However, the specific mechanisms for sex determination remain unresolved, although recent progress is promising (Akagi et al. 2018).

A genetic map allows a deeper understanding of the transmission of markers (and any traits that may be found associated with them) through the generations, uniting genotypes and pedigrees so that breeders can have more certainty and make progress more rapidly with less resources. Where traits result from few genes of large phenotypic effect, genotypes from a segregating family can identify the nearest markers on the map. Alternatively, a sufficiently diverse population structure (Burghardt et al. 2017), which can then be used to assay young plants (or, in dioecious species, males for fruit traits). After an early *Actinidia* map derived from interspecific cross (Testolin et al. 2001) an *A. chinensis* var. *chinensis* linkage map was generated from a large family using microsatellites (Fraser et al. 2009) harvested from expressed sequence tag and other libraries (Crowhurst et al. 2008). This has become the reference map used for discovering markers in kiwifruit and also seeking causal mutations, for example, to identify areas of the genome involved in Psa defense and resistance that can be selected during breeding (Fraser et al. 2015). It can also be used in wide crosses to assess intermixing and allow background selection during trait introgression from wild relatives (Tanksley and McCouch 1997). Again, Psa tolerance is the most promising trait available in undomesticated *Actinidia* germplasm in the form of mechanisms not present in cultivars (Datson et al. 2015). Some cultivars and species of *Actinidia* rarely or never show symptoms, for example, the *A. arguta* cvs. Tahi and Rua show leaf spots but seemingly shed infected leaves (Vanneste et al. 2011). Breeding efforts in this direction are taking place in programs around the world (Li and Liu 2016). Beyond the traits of sex determination and Psa tolerance, the opportunity of *Actinidia* genetic maps remains underexploited.

10.3.3 Genome Sequences

The ultimate end point of mapping and the tool most useful for both marker development and genetic analysis is the complete genome of a representative of the species. Genome sequences provide a resource for determining the genetic architecture of traits, the movement of haplotypes through generations, the physical locations of alleles and any constraints on breeding caused by genomic structures. The first *Actinidia* genome sequence was for the *A. chinensis* var. *chinensis* cv. Hongyang (Huang et al. 2013). Most published plant genomes are drafts, being assemblies of nucleic acid sequences subject to revision. With the exception of *Arabidopsis*, the functional assignments of genes are carried out via computational predictions. Draft genomes also typically have a considerable amount of sequences unassigned to chromosomes and predicted genes with no measure of confidence in their quality. In common with most draft genomes, the published cv. Hongyang genome contained a significant amount of unassembled sequence (164 Mbp or 18.7% of their estimated genome size). Many of the genes were predicted with low confidence and limited experimental evidence. To address these shortfalls, the genome of a more homozygous *A. chinensis* var. *chinensis* genotype Red5 was created by combining new sequencing and mapping data (Pilkington et al. 2018). All but 13 Mbp of nucleic acid sequence was able to be assigned to pseudochromosomes. More than 33,000 loci in the new genome were manually annotated, the vast majority differing from and improving on those in the Hongyang genome (as evidenced from cDNA resequencing). Not only is this a greatly improved resource for *Actinidia* breeding and the science community but it demonstrates a new standard for plant gene model quality (Crowhurst et al. 2016). This new genome can form a reference for all future trait dissection and molecular breeding in kiwifruit and reports will soon appear in the scientific literature. Other *Actinidia* genomes can be constructed using this one as a framework.

In the cases of both marker maps and genomes for *Actinidia*, all work has been carried out in diploids. Polyploids present problems. A genetic map cannot be constructed when high chromosome homology prevents separate linkage groups from resolving, while a genome cannot be assembled where allelic variants cannot be distinguished from homologues in other subgenomes. Even mapping in a diploid that is a palaeopolyploid can be difficult, where subgenome diversity is low due to a combination of functional and structural conservation and recent diploidization. NGS has overcome these problems in other species e.g. the hexaploid bread wheat genome (Zimin et al. 2017) but has yet to be reported successfully for kiwifruit or any other comparably diverse outcrosser.

10.3.4 Pooling Gene Pools

As noted above, the spontaneous ploidy change offered by the Hort16A cultivar's tendency to produce unreduced 2*n* gametes resulted in a commercially successful

breeding outcome: Zespri® SunGold Kiwifruit. Other genotypes can also produce 2*n* gametes. Deliberate ploidy manipulation offers breeders another route to cultivars. Induced chromosome doubling has successfully increased fruit size by creating autotetraploid *Actidinia chinensis* var. *chinensis* plants, demonstrating the potential to produce significant improvement in relevant fruit traits (Wu et al. 2012). However, the approach is empirical because the genetic basis of the variations in fruit size and shape are unknown. It is probably for this reason that artificial ploidy manipulation has yet to provide a commercial cultivar. However, it will be a powerful breeding tool—the several ploidy races in *Actinidia* species keep gene pools apart and artificial ploidy manipulation can mix them. Traits can be introgressed that have evolved in the different gene pools under different circumstances, creating new polyploids with novel trait combination that cannot be otherwise easily achieved.

Somatic hybridization provides another underexploited approach to move traits between gene pools, whether those pools are between different *Actinidia* species or ploidies, or even between individuals of the same sex. Methods of somatic embryogenesis followed by protoplast isolation from multiple *Actinidia* tissue types have been developed and successful fusion of *A. chinensis* var. *chinensis* with *A. chinensis* var. *deliciosa* was first reported by Xiao and Han (1996). One attempt to transmit a valuable trait into breeding material has been reported: chilling tolerance from *A. kolomikta* into *A. chinensis* var. *chinensis* (Xiao et al. 2004). Once again, to date no commercial cultivars have resulted from such work. The failure of these technologies to deliver marketable cultivars can and should be read as the obverse of the continuing success of large-scale conventional and marker-based breeding in this species—newer technologies must keep up with the value created by existing approaches.

10.3.5 Dealing with Dioecy

Dioecy complicates kiwifruit breeding. Although there have been successful commercialization of open-pollinated selections from wild seedlings, a multigenerational breeding program for trait improvement needs to quantify the breeding potential of male plants for fruit traits. This can be estimated through progeny tests (Cheng and Day 2013), in which the breeding value of males is estimated from performance of daughters in test crosses with several differing females. The costs of progeny testing are high, both financially (using land and other resources without directly advancing the chosen breeding strategy) and in time (long juvenility periods in *Actinidiae* mean that it may take four years or more to have reliable fruit phenotypes of daughters and thereby calculate a male's breeding value). Male kiwifruit vines deplete orchard space and resources, hence the priority given to the development of the molecular marker to identify and eliminate males from breeding programs. Orchard management practices that reduce the negative impacts of males such as spray pollination and interplanting are common. In the longer term, it would desirable to introgress the hermaphroditism trait into commercial varieties. This can occur by good fortune

(Sakellariou et al. 2016) or through extensive advanced breeding using techniques such as ploidy manipulation and somatic hybridization to transfer this rare trait into elite breeding materials. There are informal reports of work in *Actinidia* taking place and the results, when available, will be exciting.

10.3.6 Gene Editing

CRISPR-Cas-mediated editing of genomes is a breakthrough technology (Belhaj et al. 2013) that would be another way to achieve the introgression of traits such as hermaphroditism or Psa tolerance into elite germplasm, or to amend any other genetic characteristic where the molecular mechanism is known (a not insignificant caveat for this technology). The first reports in the scientific literature of gene editing in *Actinidia* species are appearing (Wang et al. 2018) and the nascent technology's promise across all breeding is of sufficient consequence that it must be included here. Other genomic approaches offer more immediate, applicable gains.

10.3.7 Genomic Selection

Many sought-after traits are determined by more than a few unlinked loci scattered throughout an *Actinidia* genome, each of which has a minor effect (Cheng et al. 2004). The development and validation of many single markers is cost-prohibitive, apparently ruling out MAS in these cases. Genomic selection (GS) is one approach that allows breeders to make progress in traits with this type of dispersed genetic architecture. It is an enhanced form of MAS that analyses in parallel markers distributed densely across the entire genome (Meuwissen et al. 2001) and has only become cost-effective with the advent of NGS (Bhat et al. 2016). With sufficient genotyping markers, every locus contributing variance to a trait will be in linkage disequilibrium with at least one marker; the sum of all these associations, whether positive, negative, or neutral, can derive a genome-estimated breeding value (GEBV) for the individual. This breeding strategy outperforms other MAS approaches (Liu et al. 2016) even when incorporating non-additive genetic effects. GS does not have to be used exclusively: GEBVs have been shown to be more accurate where markers (preferably those derived from the same phenotypic and genotypic information sets as for GS) are included as fixed effects (Spindel et al. 2016) or where deep pedigree relationships are included (Clark et al. 2012).

The application of GS in place of, or alongside, phenotypic selection is particular to the species and breeding program, being dependent upon the genetic architecture of traits within the species; the population structures within the breeding program; the resources available for genotyping and phenotyping the relevant populations; and the benefits of the gain from selection. Stating that the costs of NGS are falling has become a commonplace; it should always be accompanied by a note that the costs of

phenotyping are significant and steady. The practical challenges of measuring traits such as root architecture, disease response and lifelong yield affect the practical application of GS in all crops. GS must be tested in each species to identify whether and where it provides gains; it is only recently that it has been demonstrated in large-genomed polyploid perennials such as perennial ryegrass (Faville et al. 2016) and oil palm (Kwong et al. 2017).

In a typical GS implementation, a relatively small training population representative of the larger breeding effort is genotyped and phenotyped. The measured breeding values from this population are used to derive a statistical model to predict them from genotypes. This is then applied to a small phenotyped and genotyped validation population to evaluate the correlation between predicted-from-genotype and predicted-from-phenotype breeding values for these individuals beyond the training population. Once confidence is assured, it can be used to estimate breeding values from genotypes of the breeding population that is too large to phenotype cost-effectively. The genetic relationship between the breeding population and the training (and validation) population determines prediction accuracy (Desta and Ortiz 2014) and this genetic proximity must be maintained over multiple generations even as the breeding population shifts in response to successful selection. Phenotyping is never complete! Nor is modelling if non-additive genetic effects contribute significantly: here, the predictive models, even for recurring parents, must be continuously updated because gains from specific combining abilities do not accumulate over generations (Aliloo et al. 2017).

Some of the genus-specific challenges of kiwifruit breeding may plausibly be addressed by GS (Cheng et al. 2016). As noted above, progeny tests carried out to estimate the breeding value of males in terms of traits expressed only in females are currently an expensive and time-consuming necessity. Being able to derive GEBVs for males would be a significant gain. In exactly this way, GS has profoundly affected dairy cattle breeding, where the rate of genetic progress for traits of economic importance has doubled, generation intervals have decreased, selection accuracy has increased, the previous costs of progeny testing fallen and recessive lethals been identified (Wiggans et al. 2017).

Actinidia dioecy may offer an opportunity where the breeding value of males of unknown genetic merit produced as a consequence of breeding for females could be derived from their thoroughly-phenotyped fruit-bearing sisters. Importantly, many economically-important kiwifruit female traits, including Psa resistance, fruit quality, vine yield and flavor, appear late in the plant's life cycle. Being able to predict these traits from genotypes shortly after germination would reduce planting costs, decreasing generation intervals, and increase selection intensity, as has been demonstrated in several crops e.g. apples (Kumar et al. 2012). However, strong selection on relatively small gene pools will not lead to breeding success (Jannink 2010) and techniques to avoid inbreeding must be applied e.g. Lin et al. (2017). Negatively-correlated traits will also compromise progress and these have been observed in kiwifruit (Cheng 2014).

Among the many available genotyping technologies, kiwifruit GS requires one that is low-cost and high-throughput, owing to the number of markers required if they

are to be in linkage disequilibrium with traits in this polymorphic, polychromosomal and often-polyploid genome. Genotyping must also be sequence-based in order to capture the genomic context of a genetic marker and thus distinguish between alleles, translocations and polyploidal subgenome homologues. Genome reduction i.e. creating a representative subset of the full genotype, is also necessary to make the task of genotyping training, validation and breeding populations cost-effective. Coverage of the genome must be balanced with depth of sequencing or rare but consequential variants may be overlooked. Genotyping-by-sequencing (Elshire et al. 2011) has been widely used in plant breeding to achieve reduction and sequence-specific genotyping (Kim et al. 2016). An improved protocol with more representative sampling of the genome has even been devised and applied in kiwifruit (Hilario et al. 2015), as have some other genome reduction and marker-discovery approaches e.g. Scaglione et al. (2015). Methods that require reliable sequence data, e.g. capture-based will soon be applied now that a high-quality reference genome is available.

Prediction models for GS contain simplifying assumptions about population structure, training-breeding relatedness, marker distribution, diploidy and trait genetic architecture—they therefore vary in their applicability for a given trait in a given species (Daetwyler et al. 2010). It is essential to use several methods when calculating GEBVs from a training population dataset. Their predictive accuracy of the methods can then be compared by applying them to the genotypes of the validation population against the *true* values that can be derived from their phenotypes (Daetwyler et al. 2013). After iterative improvements to the prediction model—for example, by selecting a subset of the markers (Kwong et al. 2017)—it can then estimate GEBVs for the individuals in the breeding population.

Datson et al. (in press) have begun to apply GS in *Actinidia*, selecting males from a red-fleshed diploid *A. chinensis* var. *chinensis* population. Genotypes and phenotypes of 78 females were used to develop models that were validated on a further 10 individuals. Correlations varied in strength up to 0.85 and 0.91 for predicting fruit firmness at 60 and 120 days, respectively, suggesting that males could be selected from this population that would improve female fruit storage. The next step is to apply this in a related breeding population to identify best (and worst) males and confirm their breeding value with progeny tests. Extending the work to polyploid *Actinidia*, as has been seen in perennial ryegrass (Faville et al. 2016) and oil palm (Kwong et al. 2017), will follow.

GS is not a *silver bullet*. Complete phenotypic predictability solely from genotypic information is a practical impossibility. There are real-world limits to phenotypic measurement resolution and genotyping precision. Genotyping markers are usually chosen based on the available high-throughput genotyping technology, e.g. SNPs within targeted massively parallel short-read DNA sequences. They will not necessarily be informative or in perfect linkage with all trait-determining genetic differences. Assaying causal genetic variants directly would eliminate this source of inaccuracy (Goddard 2017). However, some traits may be determined by genome changes unamenable to the available genotyping technology e.g. structural variations, such as large deletions, inversions, translocations and copy number variations (Couldrey et al. 2017), or may be epigenetic. Environmental effects on genetic variance

in populations are even more difficult to measure accurately, precisely, and cost-effectively, especially in non-clonal populations; the first work incorporating these effects into cattle GS models is just beginning to appear e.g. Tiezzi et al. (2017). Any stochastic effects on trait expression cannot be accounted for. Elite genotypes selected via GS will still require clonal trials.

The foregoing notwithstanding, the gains that can be expected from eliminating progeny testing of most males and selecting parents and seedlings long before maturation offer the possibility that the application of GS in kiwifruit breeding promises gains of similar scale to those seen in some other species: faster generations, greater selection accuracy and accelerated genetic progress in commercially relevant traits.

10.4 Conclusions and Prospects

The genus *Actinidia* is underexploited by conventional breeding approaches, despite some notable successes. As a minor crop intended for premium markets, investment into innovative breeding strategies has been scarce until recent times. New molecular tools began to be applied two decades ago and, in combination with traditional plant breeding, are poised to deliver new cultivars with expanding consumer appeal. Several features of the genus and its most important-to-date species have held back breeding progress and an enormous setback was the arrival in key global production areas of the kiwifruit vine canker-causing bacterium Psa. Following the industry's recovery, a new accelerated phase of cultivar development is about to begin with a multi-disciplinary combination of traditional breeding, MAS, and GS. The future for kiwifruit is bright, and green, and gold, and red and more.

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Appendix: Genetic Resources of *Actinidia*

The following is a list of *Actinidia* cultivars mentioned in the text and/or of significance. A fuller treatment of characteristics, cultivation techniques, and other details can be found in Huang (2016a). There is little information on the under surveyed diversity available in China or the contents of repositories, botanic gardens, and private collections around the world.

Bounty71	A rootstock recently released in New Zealand that, although low vigor, results in better yields of cvs. Hayward, Hort16A and Zesy002 (commonly known as Gold3) when on heavier soils and under high levels of Psa than Bruno seedling rootstocks
Bruno	A cultivar of *A. chinensis* var. *deliciosa* selected in New Zealand by Bruno Just, a contemporary of Hayward Wright, using material derived from the same first introduction into New Zealand. Bruno was inferior to Hayward in fruit size and storage although the vine was more productive. Open-pollinated seedlings of Bruno are still widely used on New Zealand commercial orchards as a rootstock for Hayward and Gold3 scions
Chieftain	A pollinizer widely selected in 1989 from an orchard in New Zealand. It is a highly floriferous hexaploid *A. chinensis* var. *deliciosa* that flowers in New Zealand coincident with Hayward, hence its extensive use
Dorì	A yellow-fleshed tetraploid cultivar of *A. chinensis* var. *chinensis* bred in Italy (Universities of Udine and Bologna) by Raffaele Testolin and selected in 2003. Released to growers in 2014, it stores well and the vine is resistant to winter cold
Green Light®	A budsport of cv. Hayward identified in the 1980s in Brisighella, Italy and managed by Vivai Co. (Spada pers comm). This *A. chinensis* var. *deliciosa* matures earlier than its source and is cultivated in central Italy
Hayward	A hexaploid cultivar of *A. chinensis* var. *deliciosa* selected by Hayward Wright and first commercialized in New Zealand in the 1930s. In comparison to other contemporary varieties, it was larger, had a popular flavor, and stored for longer. The hirsute, green-brown fruit are large with green flesh and an acidic taste. After 1975, Hayward became the sole exported green-fleshed variety from New Zealand and therefore the world due to this superior storage
Hongyang	The most widely-planted red-fleshed kiwifruit cultivar. It is an *A. chinensis* var. *chinensis* selected in 1990 from multiple trials of open-pollinated seedlings of fruit collected from Henan Province, China. The fruit are sweet and yellow-fleshed with strong red coloration to the locules of the inner pericarp. Although highly susceptible to Psa and with flesh colour development affected by temperature and altitude, it is widely grown in China
Hort16A	A vigorous cultivar of *A. chinensis* var. *chinensis* that was commercialized as Zespri Gold and created the international market for yellow-fleshed kiwifruit. As described above, it was a large-fruited, yellow-fleshed kiwifruit with superior flavor bred in New Zealand at the end of the last century. The fruit are almost hairless ovoids with characteristic stylar beaks and an aromatic, tropical flavor. It proved vulnerable to the global Psa outbreak in the early twenty-first century and is no longer grown in commercially significant quantities
HortGem Tahi	A tetraploid cultivar of *A. arguta* resulting from breeding at Plant & Food Research in New Zealand, sometimes known as kiwiberry or K2D4 and producing fruit sold in Europe under the tradename Nergi®. Bred by Mark McNeilage and released to growers in 2003, the fruit are small (12–16 g) with glossy-to-dull smooth green edible skins

Jintao	An increasingly popular cultivar with Chinese growers. This gold-fleshed variety was selected from vigorous wild vines collected in Jiangxi province, China by the Wuhan Institute of Botany in 1981. It is also grown in Europe under an agreement between the Italian consortium Consorzio Kiwigold® and the Wuhan Botanic Gardens
Jinyan	The most commercially successful interspecific hybrid *Actinidia*, arising from an *A. chinensis* var. *chinensis* × *A. eriantha* cross made in Wuhan, China in 1984. This tetraploid is grown in China, Italy and Chile and its fruit are even higher in Vitamin C than Hayward
Kramer	A clonal selection from Hayward made by John Kramer and has been widely distributed in New Zealand because it is considered to have fewer fruit faults
Moshan No. 4	A long-flowering pollinizer cultivar selected in China in 1984 from the wild in Jiangxi Province, China. This *A. chinensis* var. *chinensis* has a flowering period which overlaps with other var. *chinensis* and some early var. *deliciosa* cultivars in China, hence its wide use
Qinmei	This cultivar was until recently the most widely planted kiwifruit cultivar in China. It is a cultivar of *A. chinensis* var. *deliciosa* selected from the wild in Shaanxi Province, China in 1979. This vigorous vine produces large, green-brown fruit that can shed their hairs on handling. The flesh is green
Soreli	An early-maturing cultivar of *A. chinensis* var. *chinensis* recently released by Raffaele Testolin and Guido Cipriani of the University of Udine, Italy. The fruit are strongly colored but storage life is short
Summer 3373, Summer 4605	Summer varieties were bred in Italy by crossing Hayward with inconstant male and are commercialized together as Summerkiwi™. They are green-fleshed *A. chinensis* var. *deliciosa* plants that can be harvested earlier than Hayward but do not store as long
Tomua	The first cultivar to emerge from a directed breeding program of kiwifruit. This early-maturing *A. chinensis* var. *deliciosa* with many fruit traits similar to its mother Hayward was briefly commercialized in the late 1990s from New Zealand
TopStar®	A budsport of cv. Hayward identified in 1985 and propagated by the Perotto Carla nursery, Italy. This *A. chinensis* var. *deliciosa* is a less vigorous, more easily managed vine than cv. Hayward and yields hairless fruit but is no longer cultivated in significant quantities
Wilkins Super	A budsport of cv. Hayward identified in 1979 on a New Zealand orchard. This *A. chinensis* var. *deliciosa* yields longer, larger, cylindrical fruit than its source
Zesh004 (Syn. Green14)	A green-fleshed sweet-flavored hybrid of *A. chinensis* var. *chinensis* and *A. chinensis* var. *deliciosa* bred by Alan Seal in New Zealand that can be harvested earlier than Hayward. Commercialized in the early 2010s as Zespri® Sweet Green, production levels remain low at the time of writing
Zesy002 (Syn. Gold3)	A large-fruited gold-fleshed cv. of *A. chinensis* var. *chinensis* that replaced Hort16A when Psa struck New Zealand. It is a tetraploid resulting from the combination by Russell Lowe of unreduced gametes of diploid cv. Hort16A and a tetraploid pollinizer

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Chapter 11
Citrus Genetics and Breeding

José Cuenca, Andrés Garcia-Lor, Luis Navarro and Pablo Aleza

Abstract Citrus is one of the most important fruit crops worldwide. It is grown in more than 130 countries, mainly in tropical and subtropical areas. Sweet orange represents about 60% of total citrus production, being marketed as fresh fruit or as processed juice. Mandarins represent about 21% of total citrus fruit production and are intended for the fresh market. Conventional breeding in citrus by hybridization is hampered by its complex genetics and reproductive biology (apomixis, partial pollen and/or ovule sterility, cross- and self-incompatibility and high heterozigosity). In addition, citrus have a long juvenile period and usually take several years for hybrids to set fruit. Despite these limitations, many citrus breeding programs exploiting both diploidy and polyploidy as well as mutation breeding exist worldwide. From them, very important advances in releasing new varieties adapted to new market demands, as well as achievements in gaining knowledge of citrus genetics and genomics. The development of molecular markers, the availability of a reference genetic map, the advances in biotechnological tools and the complete genome sequence of several citrus species allow the acceleration of key studies such as germplasm characterization, marker-assisted selection, gene function discovery and variety improvement.

Keywords Hybridization · Mandarin · Mapping · MAS · Mutation · Orange Polyploidy · Varieties

J. Cuenca · A. Garcia-Lor · L. Navarro · P. Aleza (✉)
Tissue Culture Laboratory, Center of Citriculture and Vegetable Production, Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, Spain
e-mail: aleza@ivia.es

J. Cuenca
e-mail: jcuenca@ivia.es

A. Garcia-Lor
e-mail: angarcia@ivia.es

L. Navarro
e-mail: lnavarro.ivia@gmail.com

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11.1 Introduction

Citrus is one of the most important fruit crops worldwide with more than 130 million mt produced in 2016. Citrus mainly includes oranges (with 55% of the world production), mandarins (25%), lemons/limes (13%) and grapefruits (7%) (FAO 2017). There are two clearly differentiated markets: fresh fruit and processed juice; about two thirds of the total production is destined to fresh consumption and the remaining third to the processing industry.

Citrus fruits are produced in more than 140 countries around the world, between approximately 40°N and 40°S latitudes. However, the main production concentrations are in certain geographical areas with about two thirds of the total citrus fruit production, such as China (24%), the Mediterranean Basin (25%) and Brazil (15%). In the Mediterranean Basin, citrus fruits are primarily produced for the fresh fruit market and Spain is the principal producer of the region, with a cultivated area of 360.000 ha and a production about 7 million mt.

Citrus fruits are mainly consumed within developed countries, with an increasing consumption in developing countries, especially of fresh oranges. By contrast, fresh mandarin and citrus juice consumption is increasing worldwide mainly due the improvements made in quality, and the competitiveness of prices associated with technological progress.

Fresh citrus fruit exports account for approximately 8% of the world production. The Mediterranean Region leads fresh citrus fruits exports, with 60% of total volume and Spain is the leading fresh citrus fruit exporter with 25% of the total. Countries of the Southern Hemisphere, such as Argentina, Australia and South Africa are reinforcing their contribution to world trade by providing out-of-season citrus fruits to the Northern Hemisphere, where imports account for approximately 65% of the world export of fresh citrus fruits. By contrast, in the Asian countries, citrus production is primarily consumed domestically and their contribution to world trade is limited. Notably, Brazil's production is mainly destined to juice production and, therefore, its export trading as fresh fruit is limited (Table 11.1).

Regarding citrus juice processing, sweet oranges represent more than 80% of the volume. The main orange producer regions for juice processing are the State of Florida in the United States and the State of São Paulo in Brazil, supplying approximately 85% of the world market. Brazil exports 99% of its production, whereas 90% of the production of Florida is consumed in the USA domestic market. The European Union is the largest orange juice importer with more than 80% of the world imports.

Citrus fruit production worldwide faces increasing biotic and abiotic constraints. They are affected by many pests and diseases caused by nematodes, fungi, bacteria, phytoplasmas, spiroplasmas, viruses and viroids. Important citrus diseases are the oomycete *Phytophtora* spp., the citrus tristeza virus (CTV), citrus canker, citrus variegated chlorosis, sudden death and, more recently, Huanglongbing (HLB) which is caused by the bacteria '*Candidatus Liberobacter* asiaticus', and has become the main limitation for citrus production in Asia, Brazil and Florida in the USA. Other diseases are also prevalent in some regions, such as the cercosporiosis in Africa and

Table 11.1 Data on the main citrus producer countries

Country	Production (mt)					Cultivated area (ha)	Exports (mt)
	Total citrus	Orange	Tangerine	Lemon/lime	Grapefruit		
World	132,401,431	73,187,571	32,792,532	17,447,154	9,074,174	8,011,950	15,769,220
China	32,451,471*1	8,419,881*2	17,155,493*1	2,289,576*3	4,586,521*1	2,428,043*1	1,039,339*6
Brazil	19,591,623*2	17,251,291*1	997,993*6	1,262,353*5	79,986**	759,951*2	102,449**
India	10,871,500*3	7,503,000*3	nd	2,978,000*1	390,500*4	852,850*3	49,891**
Mexico	7,938,600*4	4,603,253*5	467,451**	2,429,839*2	438,057*3	528,184*4	580,174*7
USA	7,489,787*5	5,160,000*4	779,787*8	822,000*8	728,000*2	298,185*6	1,042,656*5
Spain	7,008,590*6	3,137,546*7	2,941,971*2	857,754*6	71,319**	361,064*5	3,972,084*1
Egypt	4,802,111*7	3,438,030*6	1,020,492*5	341,451**	2138**	195,967*8	1,153,392*4
Turkey	4,290,757*8	1,850,000*10	1,337,037*3	850,600*7	253,120*6	135,453**	1,358,491*3
Argentina	3,281,320*9	1,032,446**	468,278**	1678,337*4	102,259*10	145,665*10	451,598*10
Iran	3,127,573*10	1,944,023*9	651,571*10	457,270*9	74,709**	187,881*9	41,784**

Note Superscripted numbers after the asterisks indicate the position of the country within the top ten list; countries not included in the corresponding top ten list are marked with "**". nd: no data available

the mal secco in the Mediterranean Basin. Since citrus are grown in a wide diversity of climatic and soil conditions, they are consequently affected also by several types of abiotic stress. Limited water quantity and quality (mainly salinity) are major constraints for many countries, while flooding is in others. Other limiting abiotic stresses in some regions are ferric chlorosis associated with calcareous and basic soils, whereas in other areas, acid soils become a problem. Freezing and very high temperatures also cause important losses in many producing areas.

The present chapter aims to review the state of the art of citrus taxonomy, genetics and breeding, from conventional techniques to the modern molecular tools, in rootstock and variety development. This includes sexual hybridization, mutation breeding, ploidy manipulation, molecular breeding and the recent use of biotechnology and genomic tools.

11.2 Origin, Diffusion and Botanical Classification of Citrus

Citrus genus is classified in the family Rutaceae (order Geraniales, suborder Geranineae). This family comprises six subfamilies, with the Aurantioideae being divided into two tribes, Clauseneae and Citreae. The latter is also subdivided into three subtribes, with the Citrinae subtribe comprising the *Eremocitrus, Microcitrus, Clymenia, Fortunella, Poncirus* and *Citrus* genera; all six genera are considered as the true citrus (Swingle and Reece 1967). The most commonly-grown citrus cultivars and rootstocks belong to the *Fortunella*, *Poncirus* or *Citrus* genera.

Citrus taxonomy remains controversial, due to its broad morphological diversity, total sexual interspecific compatibility and the partial apomixis of many cultivars. Two different classification systems are commonly accepted, i.e., the one proposed by Swingle (1943) and Swingle and Reece (1967), that recognize 16 species in the genus *Citrus* and the system by Tanaka (1977), that includes 162 species.

Currently, the most accepted origin for cultivated citrus, supported by several biochemical and genomic studies, is that they arose from the natural interspecific hybridization between only four ancestors: *Citrus medica* L. (citron), *C. reticulata* Blanco (mandarin), *C. maxima* (L.) Osb. (pummelo) and *C. micrantha* Wester (a Papeda cv. wild citrus) (Barkley et al. 2006; Barrett and Rhodes 1976; Federici et al. 1998; Garcia-Lor et al. 2013; Gulsen and Roose 2001; Herrero et al. 1996; Li et al. 2010; Nicolosi et al. 2000; Penjor et al. 2013; Scora 1975; Wu et al. 2018).

The differentiation among these four sexually-compatible ancestors results from the origin effect in four different geographic zones and an initial allopatric evolution (Ollitrault et al. 2012a). Using genomic, phylogenetic and biogeographic analyses of citrus germplasm, Wu et al. (2018) proposed that citrus diversified during the late Miocene epoch (10.4–5 million years ago) through a rapid Southeast Asian radiation that correlates with a marked weakening of the monsoons. Citron evolved in northeastern India and the nearby region of Myanmar and China; mandarins were diversified over a region including Vietnam, southern China, and Japan; pummelo originated in the Malay Archipelago and Indonesia; and *Citrus micrantha* originated in the Philippines. Other cultivated *Citrus* (secondary) species, such as sweet orange (*C. sinensis* (L.) Osb.), sour orange (*C. aurantium* L.), grapefruit (*C. paradisi* Macf.), lemon (*C. limon* (L.) Burm.) and lime (*C. aurantifolia* (Christm.) Swing.) resulted from reticulation events between the four ancestral species followed by a few interspecific recombinations and by subsequent natural mutations (Curk et al. 2016; Davies and Albrigo 1994; Garcia-Lor et al. 2012, 2013; Gmitter et al. 1992; Nicolosi et al. 2000; Ollitrault et al. 2012a; Oueslati et al. 2017; Uzun and Yesiloglu 2012; Wu et al. 2018). Recent studies reveal that the interspecific *Citrus maxima*/*C. reticulata* admixture along the genomes of modern mandarins of commercial importance, such as tangors (sweet orange × mandarin hybrids) and tangelos (mandarin × grapefruit hybrids), originated from hybridizations between ancestral or secondary species. Subsequently, nucellar polyembryony and horticultural vegetative propagation methods fixed these interspecific heterozygous structures (Oueslati et al. 2017).

Sour orange (*Citrus aurantium*) is a direct hybrid between mandarin and pummelo (Barrett and Rhodes 1976; Nicolosi et al. 2000; Scora 1975; Uzun and Yesiloglu 2012; Wu et al. 2014).

Sweet oranges (*Citrus sinensis*) are close to mandarins, but show features introgressed in their genomes from the pummelo ancestor (Nicolosi 2007). It was first believed that sweet oranges were not direct hybrids of mandarins, but backcrossed hybrids of first or second generations (Barrett and Rhodes 1976; Nicolosi et al. 2000). Later, it was suggested that sweet orange came from a backcross (BC1) [(*C. maxima* × *C. reticulata*) × *C. reticulata*)] (Roose et al. 2009; Xu et al. 2013). However, more recent studies propose that both parents of sweet orange were of interspecific hybrid origin (Curk et al. 2015; Garcia-Lor et al. 2013; Wu et al. 2014, 2018).

Grapefruit (*Citrus paradisi*) is a species close to *C. maxima* derived from a spontaneous cross between *C. maxima* and *C. sinensis* (Barrett and Rhodes 1976; Moraes et al. 2007; Ollitrault et al. 2012b; Scora et al. 1982; Wu et al. 2018). Diverse chloroplastic (Green et al. 1986; Nicolosi et al. 2000) and mitochondrial genome analyses (Froelicher et al. 2011) indicated that *C. maxima* provided the cytoplasm in the origin of these three secondary species (sweet orange, sour orange and grapefruit).

Lemons (*Citrus limon*) have a close genetic relationship with *C. medica* (Froelicher et al. 2011), which is their male parent (Curk et al. 2015, 2016). Molecular markers indicate that the genomes of *C. reticulata* and *C. maxima* also contributed to the origin of lemon. Nicolosi et al. (2000) proposed that lemons come from a direct cross between *C. aurantium* and *C. medica*. This hypothesis was confirmed by Gulsen and Roose (2001) and Ollitrault et al. (2012b). Curk et al. (2016) proposed two lemon groups, one from direct hybridizations between *C. reticulata* and *C. medica* (*C. limonia*, *C. karna* and *C. jambhiri*; cvs. Rangpur lime, Volkamer and Rough lemons), and another coming from crosses between *C. maxima* x *C. reticulata* and *C. medica* (hybrids like *C. limettioides*cv. Palestine sweet lime-types and *C. meyeri*). In this work it was also shown that sour orange may be the female parent of hybrid types Eureka (*C. limon*) and *C. limetta*, which are the yellow lemon types.

Mexican lime (*Citrus aurantifolia*) is a hybrid between *C. medica* and the papeda *C. micrantha* (Curk et al. 2016; Nicolosi et al. 2000; Ollitrault et al. 2012b). Triploid limes are divided in two groups: cv. Tahiti type limes probably resulted from a cross between a lemon type cv. Eureka and a diploid gamete of a Mexican lime, while the other group came from a backcross between *C. aurantifolia* (diploid gamete) and *C. medica* (Curk et al. 2016).

Mandarin germplasm was classified as *Citrus reticulata* by Swingle and Reece (1967), and also Mabberley (1997). Tanaka (1977) divided mandarins into 5 groups which included a total of 36 species, based on morphological changes of the tree, leaves, flowers and fruits.

Although mandarins have been widely studied using molecular markers (Barkley et al. 2006; Coletta Filho et al. 1998; Luro et al. 2004) and phenotypic characters (pomology, tolerance to biotic and abiotic stresses), there was little information about the intraspecific diversity of *C. reticulata* and the determinants of its phenotypic diversity. Garcia-Lor et al. (2015) did a broad study of the mandarin horticultural group and identified 7 groups at the nuclear level (N): N1 (all *C. deliciosa*), N2 (*C. nobilis*), N3 (mandarin hybrids), N4 (all *C. unshiu*), N5 (*C. reticulata*), N6 (*C. tangerina* and others) and N7 (*C. depressa*, *C. reshni*, *C. sunki*, *C. tachibana*). Some species defined by Tanaka as mandarins were identified as hybrids, like *C. indica* or *C. daoxianensis*, indicating the introgression of other ancestral genomes in the mandarin one. Curk et al. (2014, 2015), Wu et al. (2014, 2018) and (Oueslati et al. 2017) also revealed the existence of *C. maxima* introgressions in most of the old and in all recent selections of mandarins; in addition, Wu et al. (2018) suggested that *C. reticulata* × *C. maxima* reticulation and introgression processes were important in edible mandarin domestication. It seems that acid mandarins share the acidic mandarin mitotype defined previously (Froelicher et al. 2011; Garcia-Lor et al. 2015), while all mandarins found as introgressed by *C. maxima* share the mitotype of edible

mandarins. Therefore, the reticulation(s) event(s) between *C. reticulata* and *C. maxima* and further introgression processes appear to be important components of the mandarin domestication. It has also been observed by Butelli et al. (2017) studying the Ruby gene configuration, in which there is a pummelo allele in several mandarin genotypes. Going further at the genome level, Oueslati et al. (2017) defined the phylogenomic karyotypes of modern citrus varieties using genotyping by sequencing (GBS), displaying mosaics of large fragments of *C. reticulata* and *C. maxima* genomes. More recently, Wu et al. (2018) divided mandarins into 3 groups: type 1 including ancestral non-introgressed mandarins like Tachibana and Sun Chu Sha, type 2 including varieties with a low percentage of pummelo introgression in small segments (like cvs. Cleopatra, Willowleaf or Ponkan) and type 3 with high pummelo introgression (12–38%) in long segments (clementines, satsumas and other hybrids).

11.3 Germplasm Resources

To guarantee a diverse food production, it is essential to have the conservation and sustainable use of phytogenetic resources, along with their prospection, collection, characterization, evaluation and documentation. Human plant selection and vegetative propagation have produced a generation of elite citrus varieties, leading to the loss of many wild citrus genotypes. Moreover, the genetic diversity in the centers of origin is under threat due to the loss of habitat as a consequence of deforestation, population pressure, tourism, etc., as is happening in countries like India and China. In this context, germplasm banks (ex situ conservation) play a key role to protect and maintain the phytogenetic resources that form the source of the variability to obtain new varieties for a society in a constant state of evolution.

Citrus seeds are sensitive to desiccation and conservation at low temperatures (recalcitrant seeds, Chin and Roberts 1980); therefore, conservation must be accomplished using whole plants, explants or calli, in tissue culture or through cryopreservation.

The main citrus germplasm banks are located in Japan, China, USA, France and Spain. In Japan, there are 6 collections where more than 1200 genotypes are maintained; satsuma being the main group represented (Krueger and Navarro 2007). In the National Citrus Germplasm Repository of China, there are more than 1000 accessions maintained ex situ and around 100 calli of citrus accessions (Liu and Deng 2007). The Citrus Variety Collection of the University in California Riverside, USA contains more than 1000 genotypes of the *Citrus* genus and related genera (Barkley et al. 2006). In addition, there are approximately 350 accessions currently available as pathogen-free budwood at the National Citrus Germplasm Repository (USDA). The INRA-CIRAD collection of the Institut National de la Recherche Agronomique (INRA), San Giuliano, Corsica, France is rich in mandarin genotypes, and contains around 1100 *Citrus* accessions and related genera. The collection at the Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, Spain, includes

more than 700 genotypes including modern mandarin cultivars, *Citrus* species and also related genera of the subfamily Aurantioideae.

An alternative to the maintenance of germplasm is conservation in vitro (Engelmann 1997). In citrus, there exist some procedures for cryoconservation of embryogenic callus and embryos (Duran-Vila 1995; González-Arnao et al. 2003). For example, at IVIA there is a callus cryopreserved collection of around 60 genotypes. Recently, a new procedure for cryopreservation of shoot tips and the regeneration of plants by shoot-tip grafting in vitro has been developed, and it is widely used as backup for long-term conservation of the USDA Citrus Clonal Repository, Riverside, California (Jenderek and Reed 2017; Volk et al. 2012).

For the maintenance of citrus phytogenetic resources, there are certain steps that need to be followed (Krueger and Navarro 2007). First, it is necessary to identify and localize sources of new material to be introduced through the prospection of new diversity areas, new cultivated genotypes, or by exchanging material between germplasm banks. The material introduction is a very important step and it must be done carefully. Due to the presence in specific areas of diseases that severely affect citrus production, the exchange of plant material between different geographic areas poses a real danger of accidentally introducing new diseases. Citrus is the fruit crop with a large number of diseases that potentially can destroy the industry in an area. To avoid this problem, the introduction of citrus germplasm is legally regulated in most countries and must be done via quarantine stations. Furthermore, it is highly advisable to maintain all accessions of the germplasm banks as pathogen-free plants, including those that are present in the country where they are located. Some citrus germplasm repositories, such as the USDA repository located in California and the IVIA repository located in Valencia, Spain follow this policy. This is accomplished using the technique of shoot-tip grafting in vitro to eliminate the pathogens (Navarro 2005; Navarro et al. 1975). The information and characterization of a collection is an essential issue for the proper utilization of the germplasm resources. For morphologic characterization it is recommended to follow the *Citrus* descriptors developed by the International Plant Genetic Resources Institute (IPGRI 1999).

The evaluation of traits like resistance to biotic and abiotic stresses is almost impossible in all the genotypes of a germplasm collection in woody plants; therefore, it becomes necessary to establish a core collection (CC) to evaluate these types of characters. This can also reduce management costs, and make effective use of the main collection. Generally, it is agreed that a CC should contain around the 15% of the initial collection accessions and to have represented in it at least the 70–80% of the total number of alleles (Balas et al. 2014; Belaj et al. 2012; Brown 1989; Escribano et al. 2008; Hintum et al. 2000). Initially, the CCs were defined using passport and phenotypic data, but due to the lack of information decades ago and the differences caused by the environment, they were not reliable (Hu et al. 2000; Tanksley and McCouch 1998). Nowadays, the most common way to develop a CC is through the employment of molecular markers such as AFLPs (Fajardo et al. 2002; van Treuren et al. 2006), RAPDs (Ghislain et al. 1999; Marita et al. 2000), SNPs (Holdsworth et al. 2017; Mckhann et al. 2004; Muñoz-Amatriaín et al. 2014) or SSRs (Ellwood et al. 2006; Hao et al. 2006; Rao et al. 2012).

The uses of CCs are very diverse: maintenance of the global diversity of the initial collection (Escribano et al. 2008), evaluation of different characters with breeding purposes (Yan et al. 2009), resistance to diseases (Bernet et al. 2008; Pessoa-Filho et al. 2010) or genetic association studies (Muñoz-Amatriaín et al. 2014; Perseguini et al. 2015; Pino Del Carpio et al. 2011). For these types of studies, it is necessary to have a low population structure and linkage disequilibrium (LD) between loci (Breseghello and Sorrells 2006). In citrus, this is the reason it is not possible to perform these types of studies at the generic level (Garcia-Lor et al. 2012).

The genetic diversity of the USDA/UC citrus germplasm was reduced to a CC of 50 accessions using SSR markers (Barkley 2003; Barkley et al. 2006, 2009). Bernet et al. (2008) established a CC of sour orange (*C. aurantium*) to study the citrus tristeza virus (CTV). Recently, Garcia-Lor et al. (2017) developed core collections in mandarins with two purposes, a subset with 30 genotypes to study quality traits or stresses and another subset with 70 genotypes more appropriate for genetic association studies in order to avoid spurious associations, due to its lower genetic structure (Abdurakhmonov and Abdukarimov 2008; Yu and Buckler 2006). This CC reduced the LD over the distance and recovered most of the diversity with a lower number of samples than in the initial collection. However, any CC should be periodically revised, since other interesting genotypes or even new molecular markers could be included in the selected subsets in order to make the CC more dynamic.

11.4 Conventional Citrus Breeding Programs

Citrus breeding programs have different objectives for scions and rootstocks. For scions, breeding programs aim mainly to improve specific traits depending on the target group, i.e. oranges, mandarins, pummelos, grapefruits or lemons. Due to the low genetic diversity found within oranges, clementines, satsumas, lemons and grapefruits, detecting or inducing mutations in elite cultivars are the main source of variability. In these groups, seedlessness, expanding the harvest period and reducing the furanocoumarin content (for grapefruits) are principal breeding objectives. Regarding mandarins and mandarin-like hybrids destined for fresh consumption, breeding programs are directed to obtain easy-peeling, high-quality fruits (seedlessness, attractive fruit color, good sugar/acid ratio and flavor),good postharvest behavior, disease resistance and expanding the ripening season (earlier- and later-ripening cultivars). More recently, there is increasing interest in fruits with high levels of beneficial compounds such as the health-promoting anthocyanins, which has become an objective in some citrus breeding programs (Navarro et al. 2015; Rapisarda et al. 2003, 2008; Russo et al. 2014). Citrus growers are confronted worldwide with increasing biotic constraints, and therefore breeding programs are also focused on obtaining new cultivars resistant to diseases.

Alternaria brown spot (ABS) is an important fungal disease affecting fruit quality in many producing countries such as Brazil, Spain and Australia, among others, and resistance to ABS has become a major goal in mandarin breeding programs,

with some resistant cultivars already released (Aleza et al. 2010a; Cuenca et al. 2010, 2013a, 2016). Regarding HLB disease, the most devastating citrus disease that is currently threatening the very survival of citrus the world over (Duran-Vila et al. 2014), many efforts are under way to find genetic resistance. HLB disease is associated with the phloem-limited α-proteobacterium '*Candidatus Liberibacter*' and its linked insect vector. There are two psyllid vectors of HLB: *Diaphorina citri* and *Trioza erytreae*. Both species of the vector can transmit either the Asian form or the African form of HLB, and *D. citri* has been shown to transmit the American form in Brazil. *Trioza erytreae* occurs mostly in Africa, while *D. citri* occurs in northern Africa, Asia and the Americas, being a more efficient vector (Bové 2006; Lopes et al. 2010). In addition to causing the yellow color of young shoots, the leaves show blotchy mottling, yellow veins and mineral deficiency symptoms, like that induced by zinc in particular. Fruits are lopsided, with color inversion and aborted seeds (Bové 2006). Despite many studies, no natural resistance has been found within *Citrus* (McCollum et al. 2016; Ramadugu et al. 2016). Therefore, generating genetically-modified cultivars resistant/tolerant to the bacteria and/or their insect vectors seems the only effective, long-term measure to cope with HLB.

On the other hand, the main objectives in rootstock-breeding programs are resistance or tolerance to biotic (nematodes, *Phytophtora*, viruses, HLB) and abiotic stresses (flooding, drought, salinity, iron chlorosis), adaptation to different soil conditions (acid, calcareous) and the influence of early higher yield and fruit quality in the scions. In addition, some programs are aimed at obtaining dwarf rootstock to adapt citrus to modern intensive cultivation techniques (Forner et al. 2003; Forner-Giner et al. 2014; Grosser et al. 2015; Lliso et al. 2004).

11.4.1 Mutation Breeding

Selection of spontaneous mutants is the oldest and the most efficient breeding method in citrus, and most of the varieties cultivated worldwide arose from this process (Aleza 2015). Spontaneous mutations are identified on adult material and, therefore, the obtained genotypes do not display juvenile characteristics (Aleza 2015; Ollitrault and Navarro 2012). Additionally, those new cultivars usually have the general characteristics of the parent, with specific change in traits concerning harvesting period, size or fruit color.

The frequency of spontaneous mutations in the field is relatively high and easy to find, especially if the mutation affects fruit morphological characteristics (Henrique et al. 2016; Ollitrault and Navarro 2012; Vardi et al. 2008). In the Mediterranean area, many new clementine varieties have been selected in Spain for their harvesting period and fruit size and color, and Tarocco blood orange varieties for fruit quality, harvesting season and anthocyanin content in Italy. Similar results have been obtained for satsuma mandarins in Japan and sweet oranges in Australia and South Africa (Aleza 2015).

Since 1935, various mutagenesis agents, mainly gamma irradiation, have been used to obtain new cultivars (Aleza 2015). The main advantage of this method is the preservation of the genetic background of the original cultivar and the modification of only one or a small number of agronomical traits. Another advantage of this technology is its simplicity (it is not necessary to have previous knowledge of gene control traits), rapidity (resulting trees will not display juvenile phase) and inexpensiveness. The main disadvantages are the large populations needed to find desirable stable mutations and the frequent chimeric status of the mutations. Star Ruby cv. grapefruit was the first commercial cultivar obtained by irradiating seeds of cv. Hudson grapefruit; later, Rio Red cv. grapefruit was obtained by irradiation of Ruby Red cv. grapefruit (Hensz 1971). This technique is mainly used for obtaining diploid low-seeded genotypes and there are many examples of recently released seedless cultivars like Nulessin and Nero from cvs. Clemenules clementine (Asins et al. 2002; López-García et al. 2015); Mor and Murina (Bermejo et al. 2012; Vardi et al. 1993); Murcott tangor and Orri from Orah mandarin (Vardi et al. 2003); and Tango from Nadorcott tangor (Roose and Williams 2007).

11.4.2 Sexual Hybridization

There are several limitations to the establishment of citrus breeding programs based on sexual hybridization. Citrus presents a complex reproductive biology including apomixis, pollen and/or ovule sterility and self- and cross-incompatibility, which are relatively common among citrus genotypes. These aspects limit the possibilities to select parents for specific crosses. In addition, citrus are usually highly heterozygous, and this causes a strong variability in the progeny and makes necessary the evaluation of a large number of hybrids. Moreover, the hybrids obtained are characterized by a long juvenile period in which plants undergo a vigorous growth phase, with the presence of thorns and an absence of flowering. These limitations are common for all breeding programs based on sexual hybridization, at both the diploid and triploid level. Nevertheless, despite all these limitations, conventional sexual breeding programs, especially for mandarins, are routinely promoted in many producing countries.

Sexual breeding is mainly used for diversification in mandarins. The main limitation of this strategy is that most of the diploid hybrids are fertile and thus seedy, representing a very important economic problem mainly for new mandarin varieties. The selection of seedless cultivars displaying high quality and good yield requires the evaluation of extremely large numbers of progeny. Moreover, if seedlessness of these new hybrids is based on self-incompatibility or male sterility, important problems of cross-pollination would be encountered in areas where self-incompatible varieties such as clementines are the predominant production, as occurs in the Mediterranean Basin.

Chance seedlings are diploid hybrids with unknown parentage that have produced very important varieties in the past. This is the case of clementines, satsumas, cvs.

Ponkan, Ellendale, Ortanique, Murcott, Imperial or Nadorcott mandarins. Several diploid hybrids have been obtained in breeding programs with importance at the commercial level in different countries, such as cvs. Fortune, Kinnow, Minneola, Nova, Kara, Primosole, Gold Nugget and Kiyomi mandarins.

Several rootstock programs have been also carried outin recent years. The USDA program early released Carrizo and Troyer hybrids (Savage and Gardner 1965), which are among the most widespread rootstocks in the world due to their tolerance to citrus tristeza virus (CTV), such as cvs. Swingle citrumelo and C-35. IVIA in Spain has released several rootstock cultivars obtained by sexual hybridization, such as Forner Alcaide 5 (FA5), FA418, FA13, FA517, V17 and F17, which are resistant or tolerant to CTV, and to salinity and some are resistant to nematodes, have good tolerance of calcareous soils and flooding, or induce dwarfism (Forner et al. 2003; Forner-Giner et al 2009a; Legua et al. 2011).

11.5 Ploidy Manipulation

Diploidy is the general rule in *Citrus* and its related genera with the basic chromosome number x = 9 (Krug 1943). However, some polyploid genotypes were detected early in citrus germplasm, such as triploids (Frost and Soost 1968; Lapin 1937) and tetraploids (Cameron and Frost 1968; Lapin 1937). The importance of ploidy manipulation in citrus is reflected in three practical applications: (i) haploids for genome sequencing and obtaining double haploids; (ii) triploids for obtaining seedless cultivars; and (iii) tetraploid to be used as parents in triploid breeding programs and for rootstock tree-size control.

In recent years, the development of structural genomics has generated a growing interest in obtaining haploid plants. Haploid and double haploid lines also play an important role in genomics for whole genome sequencing (WGS) projects, where homozygosity is of particular advantage. Heterozygosity is a major challenge to an efficient and high-quality genomic assembly in whole genome sequence projects due to polymorphism, displaying lower quality and assembly contiguity which results in more incomplete sequences than would be expected in homozygous lines.

The recovery of haploid and double haploid plants from gametic embryogenesis enables homozygous lines to be isolated in a single step, especially important in citrus, which are highly heterozygous and have long juvenile phases, requiring several decades to obtain a near-homozygous plant (Aleza et al. 2009a). In addition, many citrus species are self-incompatible, which hampers obtaining a near-homozygous plant from selfing.

Androgenesis has been the most commonly employed approach to obtain haploid, double haploid and trihaploid plants in citrus. This methodology requires complex culture media with several growth regulators, formation of calli and long culture periods. Moreover, the callus stage has generally been proved to generate somaclonal variation in citrus due to the regeneration methods.

Gynogenesis is an alternative technique for producing haploid plants, and also occurs in citrus. A haploid line of Clementine obtained at IVIA, Spain (Aleza et al. 2009a) derived from a single ovule after parthenogenesis in situ induced by irradiated pollen was selected among other candidate haploid lines (Germana et al. 2013) for the whole citrus genome sequencing project (Wu et al. 2014). Other haploid lines have been obtained for several citrus species, such as orange (Cao et al. 2011), pummelo (Wang et al. 2017; Yahata et al. 2010) and mandarin cvs. like Fortune, Ellendale (Froelicher et al. 2007) and Wilking cv. (Jedidi et al. 2015).

Seedlessness is a major objective in breeding programs aiming to develop fruits for the fresh market. Some diploid citrus varieties are usually seedless, such as Navel cv. orange, clementine and satsuma mandarins and pummelos, due to either male/female sterility or self-incompatibility. However, some of them produce seeds in cross-pollination conditions. The selection of triploid lines constitutes a very interesting way to develop seedless cultivars, due to both male and female sterility generally associated with triploidy. Indeed, obtaining triploid hybrids has become an important breeding strategy to develop new seedless citrus commercial varieties (Navarro et al. 2015; Ollitrault et al. 2008; Starrantino 1992). Many triploid varieties have been released from citrus breeding programs worldwide, including cvs. Oroblanco and Melogold grapefruits (*C. grandis* × *C. paradisi*) (Cameron and Bernett 1978; Soost 1987), and cv. Winola (Spiegel-Roy and Vardi 1992). More recently, cvs. Shasta Gold®, Tahoe Gold® and Yosemite Gold® triploid mandarins were released by the USA program in California (Williams and Roose 2004); cvs. Tacle, Clara, Mandared, Mandalate and other triploid mandarins were released by the Italian program (Recupero et al. 2005) and cvs. Garbí (Aleza et al. 2010a) and Safor mandarins (Cuenca et al. 2010) were released by the triploid breeding program in Spain.

Several strategies have been developed for triploid citrus breeding, with exploitation of the natural formation of unreduced (2n) gametes and the utilization of tetraploid parents in interploid crosses being the most remarkable.

In sexual polyploidization (2x × 2x crosses), spontaneous triploid hybrids are produced at a relative low frequency (1–20%), usually from the union of an unreduced (2n) megagametophyte with haploid pollen (Aleza et al. 2010b; Cameron and Frost 1968; Chen et al. 2008; Cuenca et al. 2011, 2015; Esen and Soost 1971, 1973; Geraci et al. 1975; Luro et al. 2004; Ollitrault et al. 1996).The triploid embryos are found in small seeds which require in vitro culture techniques for plant regeneration.

More recently, tetraploid hybrids arising from diploid unreduced pollen have been reported in 4x X 2x crosses, using a mandarin X orange hybrid as a diploid male parent (Rouiss et al. 2017a), indicating 2n gamete formation from the male parent. First-division restitution (FDR) and second-division restitution (SDR) mechanisms are considered the principal mechanisms of 2n gamete formation (Bretagnolle and Thompson 1995; Cai and Xu 2007; Tavoletti et al. 1996). FDR gametes are produced if an equational mitosis of all chromosomes occurs in the first division instead of a reductional mitosis. As a result, the non-sister chromatids are included in the same gamete (Cuenca et al. 2011; Gallais 2003; Park et al. 2007). On the other hand, if the first mitosis occurs normally, but an omission of the second meiotic division occurs, an SDR 2n gamete will be produced with sister chromatids included in the same

gamete (Cuenca et al. 2011; Gallais 2003; Park et al. 2007). The different mechanisms of 2n gamete formation have different genetic consequences and particularly affect the transmission of the parental heterozygosity in relation to centromere distance. FDR 2n gametes contain non-sister chromatids, which in the absence of crossover, maintain the parental heterozygosity. When crossover occurs, the parental heterozygosity restitution (PHR) rates vary from 100% for loci close to the centromere to 50–66% for loci far from the centromere, depending on the level of chromosome interference (Cuenca et al. 2011). For SDR, the 2n gametes contain two sister chromatids, which reduces the parental heterozygosity level (Bastiaanssen et al. 1998; Cuenca et al. 2011; De Storme and Geelen 2013). When crossover occurs, the PHR rate varies from 0% for loci close to the centromere to 66–100% for loci far from the centromere, depending on the level of chromosome interference (Cuenca et al. 2011).

The SDR mechanism is considered as the main mechanism of 2n megagametophytes in citrus (Aleza et al. 2016a; Cuenca et al. 2011, 2015; Esen et al. 1979; Luro et al. 2004). Later, Cuenca et al. (2015) reported that FDR 2n gametes originated triploid hybrids at a very low frequency (0.6%) using mandarins as female parents. In lemon, Rouiss et al. (2017b) also reported that SDR is the main mechanism of unreduced female gametes (88%), but they also reported for the first time a mixture of other mechanisms implied in the unreduced gamete formation, such as FDR or pre-meiotic doubling (7%) and post-meiotic genome doubling (PMD) mechanisms (5%). In the case of unreduced pollen formation, Rouiss et al. (2017a) reported the coexistence of SDR and FDR meiotic restitution mechanisms producing unreduced pollen leading to tetraploid plants, although FDR seems to be prevalent in unreduced pollen formation.

Triploid hybrids can also be recovered from interploid crosses, i.e. 2x × 4x and 4x × 2x, which have been early exploited by breeders (Cameron and Frost 1968; Esen et al. 1979; Oiyama et al. 1981; Starrantino and Recupero 1982).In these hybridizations, the tetraploid parent used is usually a doubled diploid (DD) obtained by nucellar doubling or colchicine treatments of diploid genotypes, although tetraploid hybrids can also be used. The pollen viability of the DD genotypes is generally reduced compared to diploids, but sufficient for successful pollination. The majority of embryos in interploid crosses are triploid, which are found mainly in undeveloped seeds, unable to germinate under greenhouse conditions (Aleza et al. 2012). The PHR transmission from the DD parent to the diploid gametes depends on the rate of preferential chromosome pairing and, thus, the proportion of disomic versus tetrasomic segregations, and on the double reduction rate. A recent study comparing the genetic structure of diploid gametes arising from DD clementine and SDR unreduced gametes from diploid clementine shed light on DD clementine largely exhibiting tetrasomic segregation, but some intermediate segregation and disomic segregation was also observed in some linkage groups. Moreover, significant double reduction rates were observed in six of the nine LGs (Aleza et al. 2016a). Differences of PHR transmission between SDR 2n gametes and diploid gametes from the DD are highest in the centromeric region and progressively decrease toward the distal regions. Over all markers, PHR wastwo-thirds lower in SDR 2n gametes than in DD-derived diploid gametes. The

two strategies appear complementary in terms of genotypic variability. Interploid hybridizations are potentially more efficient for developing new cultivars that are phenotypically closer to the DD parent than sexual hybridization through SDR 2n gametes. Conversely, 2x × 2x triploidization has the potential to produce novel products with new characteristics for market segmentation strategies.

Scarcity of tetraploid parents represents a major limitation to create triploid hybrids from interploid crosses. DDs are mainly obtained either by selection of spontaneous tetraploid plants or by induction with antimitotic chemicals. Spontaneous tetraploidization seems to occur relatively frequently in apomictic citrus genotypes by chromosome doubling of nucellar cells (Aleza et al. 2011; Frost and Soost 1968; Kobayashi et al. 1981). At IVIA in Spain, many DD plants have been identified by analyzing a high number of seedlings by flow cytometry (Aleza et al. 2011). In non-apomictic citrus genotypes spontaneous tetraploidization is not common, so it requires in vitro shoot-tip grafting combined with colchicine or oryzalin treatments and selection of non-citochimeric plants by flow cytometry (Aleza et al. 2009b). Moreover, this methodology allows recovering plants without juvenile characters permitting, consequently, its use for triploid hybrids production through 4x × 2x and 2x × 4x sexual hybridizations (Navarro et al. 2015). At IVIA, within the triploid breeding program, a large number of hybrids have been obtained using both 2x × 2x and interploid hybridizations, with more than 5500 triploid hybrids from 130 parental combinations by 2x × 2x crosses, more than 4300 triploid hybrids from 100 parental combinations by 2x × 4x pollinations, and more than 5600 triploid hybrids from 103 parental combinations by 4x × 2x pollinations (Fig. 11.1)

Somatic hybridization is an important tool in citrus breeding programs (Aleza 2015; Dambier et al. 2011; Grosser and Gmitter 2005; Grosser et al. 2010; Navarro et al. 2015; Ollitrault et al. 2008). Somatic hybrids can be obtained by protoplast fusion of two non-sexual cells (even if sexually incompatible) using electrical, chemical or electro-chemical protocols. Somatic hybrids are allotetraploid plants possessing the entire genetic configuration of their parents (Aleza et al. 2016b; Dambier et al. 2011; Grosser and Gmitter 2005; Grosser et al. 2010; Louzada et al. 1993). Several citrus tetraploid genotypes arising from somatic hybridization have been obtained from different combinations, and evaluated for their use as rootstock or as tetraploid parents in interploid sexual hybridizations for triploid breeding (Aleza et al. 2016b; Grosser and Gmitter 2005; Grosser et al. 2010; Ollitrault et al. 2008).

11.6 Molecular Breeding

Several types of molecular markers in citrus have been developed for genetic studies, such as isozymes (Roose 1988; Torres et al. 1978, 1982); random amplified polymorphic DNA (RAPDs) (Luro et al. 1994); sequence characterized amplified regions (SCARs) (Nicolosi et al. 2000); restriction fragment length polymorphism (RFLPs) (Federici et al. 1998); simple sequence repeats (SSRs) (Barkley et al. 2006; Chen et al. 2008; Cuenca et al. 2011; Froelicher et al. 2008; Garcia-Lor et al. 2012,

Fig. 11.1 Some promising new triploid hybrids from the IVIA breeding program

2015; Kijas et al. 1997; Luro et al. 2001, 2008; Terol et al. 2007, 2008); intersimple sequence repeat (ISSRs) (Fang et al. 1997); amplified fragment length polymorphism (AFLPs) (Liang et al. 2007; Pang et al. 2007); cleaved amplified polymorphic sequences (CAPs) (Lotfy et al. 2003); insertion-deletion (Indel) and diversity arrays technology (DArT) (Curtolo et al. 2017); and single nucleotide polymorphism (SNPs) (Chen and Gmitter 2013; Cuenca et al. 2013b; Garcia-Lor et al. 2012, 2015; Ollitrault et al. 2012a, b).

Molecular markers have been used in citrus for phylogenetic analysis, germplasm and new variety characterization, discrimination between zygotic and nucellar seedlings, analysis of the origin of unreduced gametes, linkage mapping and marker-assisted selection in breeding (Aleza et al. 2016a; Chen et al. 2008; Cuenca et al. 2011, 2013a, 2016; Curk et al. 2016; Curtolo et al. 2017; Froelicher et al. 2007; Garcia-Lor et al. 2015; Luro et al. 2004; Ollitrault et al. 2012a; Oueslati et al. 2017; Rouiss et al. 2017a, b; Ruiz and Asins 2003; Ruiz et al. 2000). More recently, high-throughput techniques, which allow the analysis of a high number of markers such as genotyping-by-sequencing (GBS) (Elshire et al. 2011) or DArT markers, have been used in citrus for phylogenetic analysis and genome-wide association studies (GWAS) of fruit quality traits (Gois et al. 2016; Minamikawa et al. 2017; Oueslati et al. 2017; Yu et al. 2016).

A very important breakthrough for efficient use of molecular markers in genetics and breeding is the availability of genetically mapped codominant markers anchored with the physical sequence. In 2012, a clementine reference genetic map was published (Ollitrault et al. 2012a) based on segregating populations genotyped with SNP, SSR and Indel markers. The map comprises 961 markers spanning 1084.1 cM in all 9 linkage groups. The availability of a saturated genetic map of clementine was identified as an essential prerequisite to assist the whole genome sequence assembly (Wu et al. 2014) of a haploid clementine (Aleza et al. 2009a). Later, Aleza et al. (2015) located the centromere positions for all LGs, which constitute valuable information for further genetic studies based on recombination patterns, such as polyploid genetics.

At the polyploid level, SSR markers have been used in citrus to infer the genetic origin and allelic configurations of triploid and tetraploid hybrids. In the case of shared alleles between parents for a given marker, the allele dosage of the obtained triploid and tetraploid hybrids could be estimated by the MAC-PR method (Esselink et al. 2004) validated in citrus by Cuenca et al. (2011). SNPs are also very useful for the estimation of allele doses in heterozygous triploid and tetraploid hybrids as described by Cuenca et al. (2013b), comparing allele signals from competitive allele-specific PCR.

The development of molecular markers in citrus helped in construction and integration of genetic and physical maps of important traits, such as RAPD markers linked to dwarfing (Cheng and Roose 1995), RAPD markers linked to a gene controlling fruit acidity (Fang et al. 1997), SSR markers linked to CTV resistance from *Poncirus trifoliata* (Asins et al. 2004; Deng et al. 1997; Gmitter et al. 1996; Yang et al. 2003)and the dominant PCR assay for the anthocyanin content of pulp from blood orange due to a transposable element in the 5' extremity of the Ruby gene (Butelli et al. 2012, 2016).Regarding nucellar embryony, AFLP and Indel markers have been reported as linked to this trait (Kepiro and Roose 2010). More recently, Wang et al. (2017) reported a gene which is potentially responsible for the nucellar embryony, although they did not prove its functionality. Alternaria brown spot (ABS), produced by the fungus *Alternaria alternata*, is an important disease which hampers citrus cultivation in most growing areas. RAPD and SSR markers linked to ABS resistance have been developed both at the diploid (Dalkilic et al. 2005; Gulsen et al. 2010) and triploid levels (Cuenca et al. 2013a). More recently, Cuenca et al. (2016) performed a fine mapping of the region linked to ABS resistance, which is located near the centromere in chromosome III. In this work, they developed SNP markers flanking the ABS resistance locus at 0.4 and 0.7 cM, spanning a region of 366 kb, and also reported candidate genes responsible for the resistance.

Other characters of interest have been tagged to quantitative trait loci (QTLs). Linkage mapping and genetic association studies are used to find candidate genes or genome regions that contribute to a specific phenotypic trait, by testing for a correlation between phenotypic diversity and genetic variation based on the linkage disequilibrium between closely linked loci (Lewis and Knight 2012). In citrus, QTLs have been detected in several traits using RAPDs or AFLPs markers, such as salinity tolerance (Moore et al. 2000) and nematode resistance (Ling et al. 2000).

With the development of new techniques for high-throughput genotyping, such as SNP-arrays and GBS, many studies have been carried out in order to detect QTLs mainly associated with fruit quality and resistance to diseases. Sugiyama et al. (2011, 2014) performed QTLs and expression-QTL analysis of carotenoid content and genes related to carotenoid biosynthesis. Yu et al. (2016) detected several QTLs associated to fruit quality traits such as peel or juice color and aroma volatiles; Curtolo et al. (2017) used DArTseq markers to analyze fruit traits and detected QTLs associated to fruit size and internal quality. On the other hand, many QTLs have been detected as associated with *Phytophthora* and CTV resistance (Asins et al. 2004, 2012; Siviero et al. 2006).

However, despite the development of such molecular markers, only Alternaria resistance, the anthocyanin content in fruits, and the monoembryony/polyembryony trait are currently applied for marker-assisted selection in breeding programs.

11.7 Genomic Tools and Biotechnology

11.7.1 Genomic Tools

Techniques for sequencing DNA have enabled the development of databases containing annotated gene sequences. Genomics has provided new tools for crop improvement, helping to identify and select candidate genes responsible for agronomic characters of interest, and allowing the development of fast methods to incorporate these characters into crop plants (Terol et al. 2007). *Citrus*, with a basic chromosome number of 9, has a relatively small genome size. The size of haploid genomes of *C. sinensis* and *C. clementina* were estimated, respectively, at 380 and 370 Mb (Arumuganathan and Earle 1991). As a step towards genome sequencing, clones from BAC libraries of sweet orange, clementine and satsuma were sequenced in the last few years in the USA, Spain and Japan. A low-coverage (1.2X) shotgun sequence of the *C. sinensis* genome revealed the difficulties related to high heterozygosity, and led the International Citrus Genomic Consortium to select a haploid clementine (Aleza et al. 2009a) as the reference for whole citrus genome sequencing. Meanwhile, Xu et al. (2013) sequenced and assembled a dihaploid genome of sweet orange and mapped the parental diploid genomic DNA sequence reads to the haploid reference genome to complete the construction of the heterozygous genome map. The International Consortium for Citrus Genomics released the whole genome sequence of the haploid clementine, assembled in pseudo-chromosome sequence (Wu et al. 2014). The sequencing, assembly and annotation of this haploid genome were the result of an international collaboration between research centers, private companies and researchers from France, Italy, USA, Spain and Brazil. This genome assembly is currently used as a template to organize sequences of the highly heterozygous species such as *C. sinensis, C paradisi* and *C. limon*. Recently, Wang et al. (2017) published data on resequencing of several primitive, wild and cultivated species. The availability of all these genome sequences provide a valuable genomic resource for citrus

genetics and breeding improvement. In addition, the complete chloroplast genome sequence of *C. sinensis* was published by Bausher et al. (2006). It is 160.129 bp in length and contains 133 genes (89 protein-coding, 4 rRNAs, and 30 distinct tRNAs).

The analysis of the transcriptome is a crucial step to characterize any species genome, allowing the identification of candidate genes related to agronomic traits and the development of molecular markers for large-scale genotyping of many plants (Terol et al. 2016). In recent years, expressed sequence tags (EST) collections and microarrays have been developed (Forment et al. 2005; Martinez-Godoy et al. 2008) as resources for genomic and transcriptomic studies. Microarray analysis has been used to identify gene-expression patterns in different tissue or at different developmental stages (Agustí et al. 2008; Alós et al. 2008; Cercós et al. 2006; Huerta et al. 2008), in various genotypes (Ancillo et al. 2007; Aprile et al. 2011), in response to abiotic or biotic stress situations (Gandía et al. 2007; Gimeno et al. 2009) or the influence of mitochondria on gene expression in somatic hybrids (Bassene et al. 2011).

Subsequently, high-throughput technologies for RNA-seq permitted the development of many transcriptomic analyses, such as those focused on fruit characteristics in *Citrus sinensis* (Shalom et al. 2014; Sun et al. 2014; Yun et al. 2012), *C. paradise* (Patel et al. 2014) and *C. grandis* (Wang et al. 2017). RNA-seq was also usedto study the level of heterozygosity of sweet orange and the effect of the ploidy level in clementine on gene expression (Jiao et al. 2013; Niñoles et al. 2015). Other transcriptomic analyses in vegetative traits have been done, such as the comparison of flower development in fertile/sterile genotypes (Zheng et al. 2012), the apomixis trait (Long et al. 2016), the seed formation in seedy/seedless genotypes (Zhang et al. 2017) and the analysis of seedling etiolation (Xiong et al. 2017).In relation to response to infection, analysis of the transcriptome profilingin response to HLB infection was done in *C. sinensis* (Albrecht and Bowman 2008; Martinelli et al. 2016) and in response to *Xylella fastidiosa* bacterial infection in *C. reticulate* (Rodrigues et al. 2013). Terol et al. (2016) developed a RNA-seq-based reference transcriptome for citrus from the analysis of flower, leaf, root and phloem organs in 12 citrus species, providing a comprehensive view of the citrus transcriptome.

Regarding rootstock, several differential gene expression analyses have been done. Forner-Giner et al. (2009b) analyzed differential expression of *Poncirus trifoliata* in response to iron deficiency; Mouhaya et al. (2010), Allario et al. (2011) and Tan et al. (2015) evaluated the effect of genome duplication of a rootstock by transcriptional analysis in relation to some anatomical and physiological changes.In addition, Souza et al. (2017) evaluated the interactomics in scion/rootstock combinations with different ploidy levels in response to drought stress using microarray data.

11.7.2 Biotechnology

For tree crops such as citrus, the length of the juvenile phase hampers conventional breeding. Genetic transformation has the advantage of potentially reducing breeding time, particularly important in the case of a perennial crop such as citrus, and also

facilitating the introduction of traits not readily available in the particular species (Febres et al. 2011). However, citrus have usually been considered as recalcitrant to transformation, limited by the low transformation efficiency and the difficult regeneration of transformed plants (Peña et al. 2007). Since 1989, many efforts have been done to overcome these issues (Hidaka et al. 1990; Kobayashi and Uchimiya 1989; Moore et al. 1992; Peña et al. 1995, 1997, 2001; Vardi et al. 1990). Peña et al. (2007) established an efficient and reliable transformation systems from adult material for many economically-important citrus species, based on appropriate *Agrobacterium* strains, the most appropriate infection and cocultivation conditions, as well as adequate selection conditions and culture media. Since then, many successful transformation events have been achieved and different genes have been introduced into citrus, which comprise antibiotic and reporter genes, genes shortening the juvenile phase, genes conferring stress tolerance and disease resistance and fruit-quality related genes (Alquézar et al. 2008, 2017; Dutt et al. 2015; Peña et al. 2001; Pons et al. 2014).

In particular, many efforts have been carried outon conferring disease resistance to citrus plants and more specifically, to HLB (Dutt et al. 2015; Hao et al. 2016; Riera et al. 2017). Transgenic citrus varieties expressing genes for antibacterial peptide have already been obtained and tested for HLB-resistance in the field (Hao et al. 2016). On the other hand, chemical volatiles repelling the vector have been identified, which can be an efficient way to avoid HLB infection (Alquézar et al. 2017).

Shortening the juvenile phase through transgenics is mainly achieved by introducing genes responsible for early flowering, such as *Leafy* (LFY), *Apetala1* (AP1) and *Flowering Locus T (FT)* in citrus plants, whose constitutive expression results in early flowering and fruiting (Endo et al. 2005; Peña et al. 2001). Recent advances in the identification and isolation of genes responsible for carotenoid accumulation in citrus fruits enable the production of increased β-carotene levels in orange fruits via metabolic engineering of carotenoid biosynthesis genes (Alquézar et al. 2008; Pons et al. 2014).

Viral vectors can be a helpful tool to study the function of host genes by reverse genetics. When a viral vector carries a sequence of an endogenous plant gene, virus infection triggers degradation of the cognate plant mRNA through a homology-dependent RNA degradation mechanism known as virus-induced gene silencing (VIGS) that results in a loss of function phenotype of the host (Ruiz et al. 1998). Also, VIGS can be used in forward genetics, by the viral-induction of an exogenous gene. The use of viral vectors to evaluate plant gene functions is particularly attractive for woody species such as citrus, in which analysis of certain traits like flowering and fruiting by conventional breeding is hampered by their long juvenile period, and genetic transformation of adult plants is difficult (Cervera et al. 2008). Viral vectors based on the citrus leaf blotch virus (CLBV) were developed to be used in functional analyses (Agüero et al. 2012, 2013, 2014). These vectors proved to be useful in inducing precocious flowering in juvenile plants by expressing the *FT* gene (Velázquez et al. 2016). Hajeri et al. (2014) also performed reverse genetics using CTV-based RNAi technology (Folimonov et al. 2007) to induce gene silencing in the HLB-vector *Diaphorina citri*. The use of viral vectors, together with the new genetic

and genomic tools available, facilitates genetic studies, allowing the development of markers for focused introgression and marker-assisted early selection.

Despite the importance of the conventional genetic engineering (plant transformation) strategy, it still presents several issues and limitations (technical, ethical and legal). More recently, several tools that help to solve these problems using precise genome editing of plants are available.

The use of the clustered regularly interspaced short palindromic repeats (CRISPR) coupled with the CRISPR-associated proteins (Cas, specially Cas9 and Cpf1 proteins) system, has become widely popular (Jinek et al. 2012). This strategy is based on the CRISPR/Cas type II-A system from *Streptococcus pyogenes*, and is composed by three genes coding for a CRISPR-RNA (crRNA), a crRNA trans-activator (tracrRNA) and its associated protein (CRISPR-associated -Cas-). This system recognizes a genome region defined by base complementarity, based on the interaction between a single-guide RNA (sgRNA) and the DNA sequence of interest. Then, the complex CRISPR/Cas9 produces double-strand breaks within the DNA sequence. This genome editing tool has been successfully applied for gene mutation or regulation in many plant species (Nishitani et al. 2016; Ren et al. 2016; Song et al. 2016). In citrus, this strategy has been used for the modification of the expression of the CsPDS gen (Jia and Wang 2014) as a proof of concept of its applicability. Later, the CsLOB1 gene was modificated, conferring some degree of resistance to citrus canker (Jia et al. 2017).

The release of cultivars obtained using the CRISPR genome-editing strategy is under analysis in most countries. The USDA deregulated the commercialization of two crops obtained through CRISPR/Cas9 (Waltz 2016), while Argentina announced that CRISPR-obtained plants will be outside the regulation for transgenic crops (Whelan and Lema 2015). In Europe, an expert commission concluded that organisms obtained using CRISPR cannot be differentiated from those obtained through other mutation methods and, therefore, would not be considered as genetically modified organisms according to the European Directive2001/18/EG (Abbot 2015; Cyranoski 2015).

11.8 Conclusions and Prospects

The advances in citrus genetic and genomic resources and new experimental developments, such as the reference map (Ollitrault et al. 2012a) and various genome sequences (Wang et al. 2017; Wu et al. 2014, Xu et al. 2013) have contributed to decipher genome structures of most commercial and ancestral citrus as well as in assessing molecular mechanisms underlying agronomically-important traits, with some responsible genes characterized. For citrus-breeding programs, marker-assisted selection is a key step in the process of obtaining new varieties, and this strategy needs to be further developed and applied as new gene functions are discovered. These new varieties should maintain the established high fruit quality, with seedlessness being a crucial trait. In this context, triploid breeding has proved to be a very adequate strategy, in which advances in basic and applied knowledge has been done. In addition to

these traditionally-important breeding traits, new varieties should be enriched with health-promoting compounds, like anthocyanins, which will bring an added value to citrus fruits.

Despite the importance of transgenics, the existence of some legal, ethical and experimental issues hampers its commercial utilization, limiting its use mostly to research. In contrast, CRISPR/Cas system seems to be the most promising strategy for both discovering new gene functions and directed mutation to improve cultivars, overcoming legal constraints.

Appendix 1: Research Institutes and Online Resources

Institution	Contact information and website
Instituto Valenciano de Investigaciones Agrarias (IVIA). Spain	CV-315 km 10,7 46113 Moncada, Valencia (Spain) www.ivia.es
Institut National de la Recherche Agronomique/La Recherche Agronomique pour le Développement (INRA/CIRAD). France	20230, San-Giuliano (France) www.inra.fr www.cirad.fr
Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA). Italy	Corso Savoia 190 95024Acireale(Italy) http://sito.entecra.it
Agricultural Research Organization (ARO)—Volcani Center. Israel	Derech HaMaccabim 68, Rishon LeTsiyon, (Israel) www.agri.gov.il
Citrus Research and Education Center (CREC). USA	700 Experiment Station Rd. Lake Alfred, FL 33850 (USA) http://www.crec.ifas.ufl.edu
Agricultural Experiment Station-Citrus Research Center. University of California, Riverside. USA	900 University Avenue, Riverside, CA 92521 (USA) www.citrusvariety.ucr.edu
Centro de Citricultura 'Sylvio Moreira'. Brazil	Rodovia Anhangüera, Km 158, Cordeirópolis, SP (Brasil) http://ccsm.br
Brazilian Agricultural Research Corporation (EMBRAPA)	Parque Estação Biológica, Brasília, DF (Brazil) https://www.embrapa.br
Instituto Nacional de Tecnología Agropecuaria (INTA). Argentina	Av. Rivadavia 1439 (C1033AAE) Buenos Aires (Argentina) https://inta.gob.ar

Institution	Contact information and website
Instituto Nacional de Investigación Agropecuaria (INIA) EE Salto Grande. Uruguay	Camino al Terrible, Salto (Uruguay) www.inia.uy
Citrus Research Institute (CRI). Chinese Academy of Agricultural Sciences. China	Xiema, Beibei, Chongqing (China) http://www.southfruit.com.cn
Institute of Citrus Science (ICS). HZAU. China	Huazhong Agricultural University, Wuhan 430070 (China) http://ics.hzau.edu.cn
NARO Institute of Fruit Tree Science.Citrus Research Station. Japan	485-6 Okitsunaka-cho, Shimizu, Shizuoka City, Shizuoka 424-0284 (Japan) www.naro.affrc.go.jp
Commonwealth Scientific and Industrial Research Organization (CSIRO)	Gate 13, Kintore Avenue University of AdelaideAdelaide SA 5000 (Australia) www.csiro.au

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Chapter 12
Advanced Innovative Tools in Lemon (*Citrus limon* L.) Breeding

Ilknur Polat

Abstract Lemon (*Citrus limon* L. Burm. F.; Rutaceae) is one of the most important commercial and nutritional fruits in the world. Therefore, it needs to be improved for the diverse needs of consumers and crop breeders. Breeders have attempted to eliminate undesirable characteristics in plants and develop new varieties with desired characteristics that meet the needs of farmer and consumer by using different breeding techniques. In lemon breeding, seedlessness or with fewer seeds, resistance or tolerance to biotic and abiotic stress, acidity, yield, quality and early- or late-ripening are the most desired features and topics. Lemon improvement through conventional breeding is time consuming and cumbersome. Moreover, it has obstacles such as the high degree of heterozygosis due to frequent gene mutations either in reproductive or somatic cells and long juvenility in conventional breeding. In recent years, both conventional and molecular breeding techniques have been used to obtain new lemon varieties. In particular, the use of molecular techniques is rapidly increasing in an attempt to reduce the difficulties faced in conventional breeding like specific reproductive physiology of lemon and protracted length of time. In this chapter, conventional and advanced lemon breeding techniques are evaluated and discussed. In addition, emergent methods and technologies are evaluated to advance basic and applied lemon-breeding methods and to suggest directions for future research.

Keywords Genomic selection · Mapping · MAS · Molecular markers
Mutations breeding · Polyploidy · Transgenic

12.1 Introduction

Lemon, *Citrus limon* L. Burm. f., is a small tree in the Rutaceae family that is thought to have been first grown in Asia (likely India, Pakistan, Myanmar and China) and is now cultivated commercially worldwide in tropical and subtropical countries (Gulsen and Roose 2001). Nowadays, the top ten countries for lemon and lime production

I. Polat (✉)
Bati Akdeniz Agricultural Research Institute (BATEM), Antalya, Turkey
e-mail: i_polat@hotmail.com

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Table 12.1 Major countries exporting lemons and limes

Rank	Exporter	Lemons and limes exports (USD million)	World total (%)
1	Spain	803.1	23.2
2	Mexico	425.3	12.3
3	Netherlands	387.5	11.2
4	Turkey	304.6	8.8
5	Argentina	289.4	8.4
6	South Africa	264.9	7.7
7	United States	207.7	6.0
8	Chile	115.5	3.3
9	China	103.3	3.0
10	Brazil	89.9	2.6

are China, USA, Israel, Argentina, Spain, Lebanon, Turkey, Brazil, South Africa and Australia, respectively (FAO 2014). Among continents, Europe accounts for the highest dollar value of exporting lemons and limes during 2016 with shipments valued at USD1.5 billion or 42.6% of the global total. In second place were North American exporters at 18.3%, while 15.5% of worldwide lemons and limes shipments originated from Latin America excluding Mexico. Smaller percentages were sourced from Africa (8.7%) as well as Australia and other Oceania nations (0.4%). A list of countries exporting lemons and limes is given in Table 12.1 (Workman 2017).

Citrus limon L. Burm. f. is a *Citrus* species with a very difficult and complex taxonomy. Initially, it was accepted as a species by Swingle and Reece (1967) and Tanaka (1977). Afterwards, it was considered a hybrid (Torres et al. 1978). In recent studies, lemon is accepted as a hybrid of citron (*C. medica*) and sour orange (*C. aurantium* L.) (Gulsen and Roose 2001; Nicolosi et al. 2000).

Lemon varieties are obtained via natural mutations, nucellar variation or the result of hybridization (Nicolosi et al. 2000). Although most lemons are derived via clonal selection these have low-level polymorphism, whereas hybrid origin lemons have high genetic diversity (Uzun et al. 2011).

Sexual breeding of lemon is a difficult task mostly because of their highly heterozygous genetic structure as a result of interspecific hybridization (Nicolosi et al. 2000). Therefore, conventional techniques of genetic improvement using hybridization and recurrent selection always encounter genetic challenges like incompatibility, long-selection phase and botanical obstacles like adventitious nuclear apomixis and long juvenility (Hossain and Rabbani 2011; Machado et al. 2011; Murkute and Singh 2015; Tusa et al. 2002).

Plant breeding can be accomplished through many different techniques including simply selecting plants with desirable characteristics for propagation (Sleper and Poehlman 2006). Although many lemon varieties exist, breeders are still working intensively to further improve lemon fruit quality and yield to satisfy the demand of consumers and growers. Seedless (Del Bosco et al. 1999; Gulsen et al. 2007; Viloria

and Grosser 2005), thornless (Turgutoglu et al. 2015), diseases resistances/tolerant (Gulsen et al. 2007; Tusa et al. 1990) and early maturing (Uzun et al 2008b) lemon varieties have been developed from breeding programs. Especially in recent years, efforts have been improved through the possible combined use of biotechnological and conventional techniques. Thus, the following approaches have been used to overcome the problems in lemon breeding. For this purpose, new biotechnology tools like embryo rescue, protoplast fusion, mutation, mapping, molecular techniques and genetic transformation are being used to save time and are more productive and reliable because rapid results can be attained in a short time period (Machado et al. 2011; Pons et al. 2012).

The aim of this chapter is a review advanced breeding techniques in lemon by providing examples of successful uses. In addition, new and useful technologies will be described as well as key differences of advantages and disadvantages.

12.2 Genetic Markers

Major activities of plant breeding involve the collection of variation, selection, evaluation, release, multiplication and distribution of new varieties (Sleper and Poehlman 2006). Specific traits are generally described in plant breeding using genetic markers including morphological, biochemical and molecular.

Morphological markers are traditional markers in a plant breeding program. However, there are several undesirable factors that are associated with morphological markers. First, they are affected by high dependency on environmental factors. Often the conditions that a plant is grown in can influence the expression of these markers and lead to false determination. Second, it takes a long time to identify some traits of fruit pomology because of protracted juvenility and harvest time. And last, performing breeding experiments with these markers is labor intensive and large populations of plants require extensive plots of land and/or greenhouse space in which to be grown (Stuber et al. 1999).

Protein and isoenzymes are extensively used as biochemical markers. These can be used as genetic markers to map other genes in plant breeding. Although used in most crop species, some can be identified only with a specific strain because of there are only a small number of isozymes. Therefore, the use of enzyme markers is limited (Jiang 2013).

Molecular markers are specific fragments of DNA that can be identified within the whole genome. Markers based on DNA have been introduced over the last two decades and have revolutionized the entire scenario of biological sciences. Major DNA markers used for identification, diversity, taxonomy, relationship, fingerprinting, breeding, diagnostics and mapping are compared in Table 12.2, following Barkley et al. (2006), Distefano et al. (2009) and Datta et al. (2011).

No single genetic marker is inherently *good* or *bad*; markers vary in attributes and in their most appropriate application. Some of the desirable attributes of a genetic marker are inexpensive to develop and apply, unaffected by environmental and devel-

Table 12.2 Comparison of the widely used markers in plants

Marker	PCR-based	Polymorphism	Inheritance	References*
Morphological	No	Low	Dominant Recessive Codominant	20
Allozymes/Isozymes	No	Low-Medium	Codominant	11
STS	Yes	High	Codominant Dominant	20
EST-SSR	Yes	High	Codominant Dominant	7, 22
SNP	Yes	High	Codominant	2, 5, 8, 19
SCAR	Yes	High	Codominant	20
CAPS	Yes	High	Codominant	20
RFLP	No	Low-Medium	Codominant	2, 20
RAPD	Yes	Medium-High	Dominant	2, 17, 20, 21
SSR	Yes	High	Codominant	2, 8, 10, 11, 16, 22
ISSR	Yes	High	Dominant	6, 11, 17, 21
AFLP	Yes	High	Dominant	2, 10
IRAP/REMAP	Yes	Medium-High	Codominant	1, 12
SRAP	Yes	High	Dominant	1, 14, 15, 21
RGA	Yes	High	Codominant	1
cpDNA/mtDNA	Yes	High	Codominant	1, 3, 4, 9
SSAP	Yes	High	Codominant	1, 10, 12
SAMPL	Yes	High	Codominant	10
TRAP	Yes	High	Codominant	1
SSCP	Yes	High	Codominant	18

References* 1: Poczai et al. (2013), 2: Kordrostami and Rahimi (2015), 3: Li et al. (2010), 4: Araújo et al. (2003), 5: Curk et al. (2015), 6: Meziane et al. (2016), 7: Chen et al. (2007), 8: Garcia-Lor et al. (2013), 9: Garcia-Lor et al. (2015), 10: Biswas et al. (2011), 11: Gulsen and Roose (2001), 12: Kalendar et al. (2011), 13: Kalendar et al. (1999), 14: Li and Quiros (2001), 15: Uzun et al. (2009a), 16: Golein et al. (2006), 17: Uzun et al. (2009b), 18: Luro et al. (2011), 19: Cuenca et al. (2016), 20: Datta et al. (2011), 21: Robarts and Wolfe (2014), 22: Liu et al. (2013)

opmental variation, highly robust and repeatable across different tissue types and different laboratories, polymorphic and codominant in its expression (Datta et al. 2011; Jiang 2013). A marker is deemed to be dominant if only one form of the trait is associated with the marker, whereas the other form of the trait is not associated with any marker. Such markers cannot discriminate between the heterozygote and homozygote marker allele. A marker is designated as codominant if both forms of the trait are associated with the marker. It can discriminate between the heterozygote and homozygote marker allele (Datta et al. 2011). There are many types of molecular markers, each with particular limitations and strengths, as shown in Table 12.2.

12.3 Germplasm Identification, Evaluation and Conservation

First of all, it is very important to have a rich and varied gene pool and known morphological and genetic characteristics of the plant and parents before initiating a breeding program. However, in citrus origin centers (China, Myanmar, Pakistan and India), there are no more native plants to collect to introduce new genetic variability. Therefore, genetic variability can only be achieved in germplasm collections around the world (Machado et al. 2011). The USA, with approximately 1200 citrus accessions, has one of the most important and largest genepools. The Citrus Variety Collection and Citrus Experiment Station were established in Riverside, California in the early 1900s. The Citrus Experiment Station later became the foundation for the University of California Riverside (Khan et al. 2001). In China, there are about 1000 accessions preserved ex situ in the National Citrus Germplasm Repository (Yong-Zhong and Xiu-Xin 2007). On the other hand, conservation of lemon genetic resources is becoming more important than ever because of the increasing threat from urbanization, climate changes, outbreak of new diseases and pests and the frequent occurrence of natural disasters due to global warming and rapid industrial growth. For this purpose, in situ, ex situ, in vitro and DNA banking techniques are used extensively.

Most lemon cultivars originated from nucellar seedlings or budsports. Therefore, lemon genetic diversity is relatively low, in spite of many named varieties with important differences in horticultural traits (Herrero et al. 1996; Krueger and Navarro 2007). Therefore, most lemons varieties have highly similar morphological and biochemical characters (Gulsen and Roose 2001). Studies on genetic identification have been carried out mainly using morphological and biochemical characteristics. Recently, genetic diversity and relationship studies have gained further support from various studies using molecular techniques based on DNA. Utilized in many studies were different molecular markers such as restriction fragment length polymorphism amplified (RFLP) (Federici et al. 1998; Kordrostami and Rahimi 2015), amplified fragment length polymorphism (AFLP) (Biswas et al. 2011; Hussein et al. 2003; Li et al. 2010), randomly amplified polymorphic DNA markers (RAPD) (Aka Kacar et al. 2005; Federici et al. 1998; Tripolitsiotis et al. 2013; Uzun et al. 2008a, 2009b), inter simple sequence repeat (ISSR) (Araújo et al. 2003; Capparelli et al. 2004; Gulsen and Roose 2001; Hussein et al. 2003; Shahsavar et al. 2007; Tripolitsiotis et al. 2013; Uzun et al. 2008a, 2009b), sequence-related amplified polymorphisms (SRAP) (Uzun et al. 2009a, 2011), telomeric repeat amplification protocol (TRAP) (Poczai et al. (2013), SSR (Biswas et al. 2011; Gulsen and Roose 2001; Hussein et al. 2003; Snoussi et al. 2012; Uzun et al. 2011), sequence-specific amplified polymorphism (SSAP) (Biswas et al. 2011), cleaved amplified polymorphic sequences (CAPS) (Datta et al. 2011), chloroplast DNA (cpDNA), mitochondrial DNA (mtDNA) (Araújo et al. 2003), inter-retrotransposon amplified polymorphism (IRAP), retrotransposon-microsatellite amplified polymorphism (REMAP) (Poczai et al. 2013), selectively amplified microsatellite polymorphic loci (SAMPL) (Biswas

et al. 2011) to determine genetic diversity and phylogenetic relationships among lemons and related genera in the genepool. For example, the origin of the Interdonato lemon was determined by cpRFLP, SSR and ISSR markers and found to be a hybrid between lemon and citron (Gulsen and Roose 2000). To perform extended analyses of the diversity, genetic structure and origin of lime and lemon cytoplasmic and nuclear markers were used (Curk et al. 2016).

Internal transcribed spacer (ITS) and three plastid DNA regions of accessions of the cultivated citrus and their putative wild ancestors were analyzed in an attempt to determine their paternal and maternal origins (Hynniewta et al. 2014; Li et al. 2010). ITS analysis was used in Indian taxa of *Citrus* to differentiate the true species and species/varieties of probable hybrid origin in distinct clusters or groups (Kumar et al. 2013). *Citrus limon* and *C. aurantifolia* have multiple maternal cytoplasmic origins using mitochondrial sequences or markers (Froelicher et al. 2011). On the other hand, expressed sequence tag (EST) datasets and a number of microarray platforms for exploring the transcriptomic responses of citrus species and hybrids to a wide range of conditions have been shared (Gmitter et al. 2012).

12.4 Polyploidy Breeding

Although most *Citrus* species are diploid (2n = 2x = 18), triploid (2n = 3x = 27) and tetraploid (2n = 4x = 36) plants were developed sexually through the fusion of unreduced gametes or somatically by chromosome duplication in nuclear cells and the non-division of these cells in two during mitoses (Lee 1988). The phenomenon of polyembryony was first noticed by Leeuwenhoek in 1719, who observed the formation of two plantlets from the same citrus seed (Batygina and Vinogradova 2007). On the other hand, most lemons of commercial importance are polyembryonic (Frost and Scoost 1968). Polyploidy can give citrus fruit interesting characteristics. Thus, genetic lemon breeding programs aim to create triploid and tetraploid varieties, which are sterile, produce seedless fruit, tolerant/resistance to diseases and tolerant to some abiotic traits (Andrade-Rodríguez et al. 2004; Usman et al. 2008).

To reduce total seed numbers or achieve seedlessness in citrus fruits, various useful breeding approaches have been used by researchers. These consist of both conventional and modern molecular breeding tools. Conventional techniques have encountered many problems because of the specific reproductive physiology of citrus and are very time consuming. However, modern molecular techniques like embryo rescue, protoplast fusion, irradiation, inter specific or generic crosses and citrus transformation are less time consuming and more productive, and as well rapid results can be achieved in a short time period (Ali et al. 2013).

To achieve seedless fruit setting, self-incompatibility may be used successfully (Hossain and Rabbani 2011). However, an efficient strategy to generate seedless cultivars in citrus is by the production of triploids from interploid crosses (Singh and Rajam 2009). In recent years, most triploid and tetraploid citrus varieties were developed from polyploidy lemon breeding programs. Triploid (3x) fruits are usually seedless due to abnormal meiosis and embryo abortion. To create a triploid, a sexual

cross is made by hybridization between a diploid (2x) and a tetraploid (4x) (Zhu et al. 2009). Besides, triploid hybrids can be obtained by means of 2x × 2x hybridizations through the production of unreduced gametes by the female parent (Esen and Soost 1971). Triploid progenies usually have lower variability than diploids which is why many triploid hybrid characteristics are more like those of the male tetraploid parents than the female diploid ones (Reforgiato Recupero et al. 2005). Tetraploid hybrids were obtained from different interploid crosses (2x × 4x) between diploid and allotetraploid somatic hybrid ($2n = 4x = 36$) (Aleza et al. 2009; Del Bosco et al. 1999; Çimen et al. 2016; Tusa et al. 1992).

Polyploidy breeding in citrus employs different techniques. Protoplast fusion and embryo culture are the most common auxiliary techniques used to make polyploidy varieties in lemon breeding.

12.4.1 Protoplast Fusion

Protoplast fusion is a powerful tool to achieve somatic hybridization in genetic improvement (Mendes et al. 2001). This technique can be used to facilitate conventional breeding, gene transfer, and cultivar development by overcoming some challenges associated with traditional sexual hybridization including sexual incompatibility, nucellar embryogenesis and sterility. Also, it is used to produce somatic hybrids that combine complementary diploid rootstocks, which have shown good potential for tree size control to reduce harvesting costs, maximize the efficiency of modern cold protection methodology, and facilitate the adaptation of new fruit production systems (Grosser and Gmitter 2011).

Although protoplast fusion techniques have many advantages, they also have some disadvantages. Application of the methodology requires an efficient plant regeneration system from isolated protoplasts. Protoplasts from two species can be fused, however, production of somatic hybrids is not easy. Lack of a proper selection method for fused products poses a problem. Also, the products of somatic hybridization are often unbalanced (sterile, misformed or unstable). Therefore, it is not guaranteed that a character will completely express after somatic hybridization. The regeneration products of somatic hybridization are often variable due to somaclonal variation, chromosome elimination or organelle segregation. All diverse intergeneric somatic hybrids are sterile and, therefore, have limited chances of developing new varieties (Ollitrault et al. 2007).

Protoplast fusion has not been used extensively in lemon breeding programs and few studies have been done. Initial research about isolation and culture of lemon protoplast were started by Vardi and Galun (1989). An interspecific tetraploid citrus somatic hybrid between Valencia sweet orange x Femminello lemon was produced in an effort to combine the good quality and performance of Femminello lemon with the cold hardiness and tolerance to mal secco infection from Valencia (Tusa et al. 1990). Allotetraploid somatic hybrid plants of Hamlin sweet orange x Femminello lemon and Milam lemon (purported hybrid of *C. jambhiri* Lush) x Femminello lemon were regenerated by somatic embryogenesis following protoplast fusion (Grosser

and Gmitter 1990). The somatic hybrids were used in interploid crosses with lemon in an effort to generate seedless triploid lemon types with improved tolerance to mal secco disease (Tusa et al. 1992). Therefore, protoplast fusion is a very useful technique to create new lemon varieties for different purposes such as biotic and abiotic resistance/tolerance, quality, yield, seedlessness, thornless, short juvenile and long harvest period. In particular the development of molecular technology and protoplast fusion in recent years will enhance use of this technique.

12.4.2 Embryo Rescue Technology

Embryo rescue, sometimes called embryo culture, is an in vitro technique that has been used to save the hybrid products of fertilization when they might otherwise degenerate (Bridgen 1994; Pérez-Tornero and Porras 2008). The success of embryo rescue depends on specific ingredients of the medium and the embryo developing stages (Andrade-Rodríguez et al. 2004). The technique usually has been employed to produce interspecific and intergeneric hybrids in breeding programs. On the other hand, this technique has been utilized to obtain progeny from intraspecific hybridizations that do not normally produce viable seed (Pérez-Tornero and Porras 2008). The embryo rescue technique was utilized following novel genetic combination with interploid hybridization (diploid x tetraploid crosses using lime, lemon and orange) to obtain seedlessness, cold tolerance, acidness and diseases resistance (Viloria and Grosser 2005).

However, this technique has not been used as extensively in lemon breeding programs as it has in protoplast fusion. Likewise, the embryo rescue technique is very useful to create new lemon varieties for different purpose such as biotic and abiotic resistance/tolerance, quality, yield and seedlessness. Advances in molecular technology and tissue culture will enhance use of this technique.

12.4.3 Determination of Zygotic and Nucellar Genotypes

One of the major problems in citrus breeding is the competition between zygotic and nucellar embryos (Soost and Roose 1996). Lemons, like many other citrus types, have a long juvenile period, and most cultivars take at least six years to fruiting (Aleza et al. 2010b). Nucellar embryony has long been an obstacle for hybrid breeding of citrus, as hybrids are morphologically indistinguishable from the clonal offspring derived from one polyembryonic seed (Long et al. 2016). Histological studies were carried out to try to identify the initiation process underlying polyembryony. Likewise, regenerated from nucellus culture in vitro have been systematically analyzed in different non-apomictic citrus genotypes (Aleza et al. 2010b). Determination of genetic differentiation between nucellar and zygotic seedlings is difficult (Aleza et al. 2010b; Deng et al. 1995). Conventional techniques of off-type rouging and other mor-

phological identification techniques are not always a success (Mondal et al. 2015). For this purpose, molecular markers are available to distinguish the zygotic plants at an early stage in a breeding program (Deng et al. 1995; Mondal et al. 2015).

Flow cytometry, estimation of nuclear DNA content, has been a major application in research, breeding and production (Doležel and Bartoš, 2005; Koç et al. 1999; Loureiro et al. 2007; Tusa et al. 2002; Zhu et al. 2009). Pérez-Tornero et al. (2010) made several sowings of seeds and about 1800 seedlings of different lemon cultivars were analyzed by flow cytometry. On the other hand, chromosome counting is another method to determine the ploidy variation and genetic composition of the progenies (Zhu et al. 2009).

In recent years, the use of molecular marker technologies is also increasing for early selection of zygotic seedlings from controlled crosses has resulted in the possibility of selection of a high number of new combinations for the establishment of a great number of hybrids in field experiments (Machado et al. 2011). Isozymes analysis also has some limitations (Tusa et al. 2002). However, molecular techniques RAPD (Bastianel et al. 1998; Koç et al. 1999), inter-simple sequence repeat (ISSR) (Golein et al. 2011; Tusa et al. 2002), simple sequence repeat inter SSR (Mondal et al. 2015; Yildiz et al. 2013; Zhu et al. 2009) were successfully used to identify zygotic and nucellar genotypes in citrus interspecific crosses. Development of biotechnology such as next generation sequencing (NGS) will be used for more reliable and faster determination of nucellar/zygotic genotypes in future lemon-breeding programs.

12.5 Mutation Breeding

Improved lemon cultivars have also been produced through natural as well as induced mutations. Natural mutations have occurred spontaneously over time. Trees bearing natural mutant fruits observed to be very different from the parent have been selected, evaluated and mass-propagated. Tissue culture-induced cell mutations or irradiation of budwood to induce mutations, can also result in the development of improved cultivars. These processes can cause bits and pieces of DNA to break away and rearrange themselves differently, or a point mutation that is a single nucleotide change in the DNA's sequence, producing changes that can result in an improved cultivar to increase the genetic variability (Dutt and Grosser 2015; Latado et al. 2012). The FAO/IAEA (International Atomic Energy Agency) Mutant Variety Database or MVD collects information on plant mutant varieties (cultivars) released officially or commercially worldwide.

Research has shown that irradiation of fruit trees, with fast neutrons, X rays, or gamma rays (^{60}Co), is an efficient way of inducing mutations (van Harten and Broertjes 1989). Irradiation by gamma rays on budwood can produce higher frequencies of mutation, leading to the creation of new variants compared to the control (Piri et al 2011). Radio sensitivity (LD 50) of acute exposure of lemon ranges from 40 to 100 Gy (Gulsen et al. 2007; Turgutoglu et al. 2015) depending on varieties. On the other hand, gamma rays were used on immature fruits in citrus. In general,

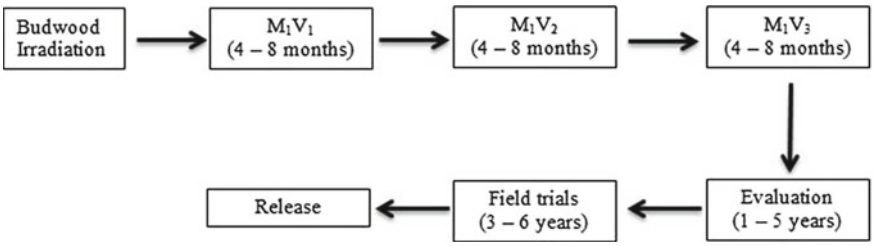

Fig. 12.1 Induced mutation breeding stages

there was suppression of embryogenesis at 90 and 120 Gy. After exposed immature fruits of 36 *Citrus* cultivars, the healthy embryos with normal balanced germination were ex-vitro grafted on rough lemon seedlings for growth and development. Since embryos are from single cell radiation of pro-embryogenic nucellus they can give solid mutants which can provide improvement within *Citrus* cultivars (Altaf 2006). In contrast, the use of chemical mutagens has been found to be inefficient (van Harten and Broertjes 1989). Induced mutation breeding stages are shown in Fig. 12.1.

The first induced mutation was performed on citrus seeds using X-rays by Haskin and Moore in 1935 (Cameron and Frost 1968). Many projects were conducted to improve some properties of citrus such as the seedless mandarin (Bermejo et al. 2012; Goldenberg et al. 2014; Montañola et al. 2015; Starrantino et al. 1988; Sutarto et al. 2009; Williams and Roose 2010), seedless pumelo (Huang et al. 2003; Sutarto et al. 2009), fruitful and compact canopy in orange (Donini 1982), fruit peel color in grapefruit (Chapot 1975; Hensz 1985), early-bearing grapefruit and orange varieties (Donini 1982; Tang et al. 1994), all through artificial mutation. Likewise, some varieties of lemon were obtained in this way. Seedless mutants of Eureka cv. and Villafranca cv. lemon (Spiegel-Roy et al. 1985, 1990) were obtained with budwood gamma ray irradiation in lemon breeding. Cultivars Eureka and Villafranca, were subjected to a mutation-inducing process, which led to the production of seedless mutants in both cultivars. Seedless mutations were induced by exposing buds (M_1V_1) to ^{60}Co gamma irradiation (13.3 Gy/min) (Spiegel-Roy et al. 1985, 1990). That study successfully produced a seedless cv. of Eureka, named Galya, and a seedless cv. of Villafranca, named Ayelet (Spiegel-Roy et al. 2007). Likewise, seedless mutations with mal secco tolerance (Gulsen et al. 2007; Uzun et al. 2008b) and early maturing (Uzun et al. 2008b) lemon varieties were obtained by using ^{60}Co radiation. Table 12.3 lists desirable traits that have been produced in various commercial lemon varieties through induced mutation. Photographs of Alata, Gülşen and Uzun seedless mutant lemon varieties are presented in Fig. 12.2. Also, some mutation breeding programs are trying to obtain low-seeded (Montañola et al. 2015) and thornless (Turgutoğlu et al. 2015) lemon varieties.

Although mutation breeding is widely used to develop new varieties, there are some disadvantages. Mutagenesis is not targeted to a specific locus and therefore requires laborious screening of large populations to obtain individual plants express-

Table 12.3 Commercial mutant lemon varieties

Mutant variety	County of release	Year	Main character
Eureka 22 INTA	Argentina	1987	Fruit set
Alata	Turkey	2007	Seedlessness
Gülşen	Turkey	2007	Seedlessness
Uzun	Turkey	2007	Seedlessness
Eylul	Turkey	2007	Early maturing
Galya	Israel	2007	Seedlessness
Ayelet	Israel	2007	Seedlessness

Fig. 12.2 Alata, Gülşen and Uzun seedless mutant lemon varieties. Photos by Aydin UZUN

ing the desired traits (Dutt and Grosser 2015; Vardi et al. 2008). In addition, determination of a stable mutant genotype requires considerable time; especially to determine fruit traits such as seedlessness which takes a long time due to juvenility (van Nocker and Gardiner 2014). Therefore, recent advances in molecular biology techniques should aid in the development of faster, targeted, and more efficient tools to expedite lemon breeding. For example, DNA markers for traits such as seedlessness, could be used as tools for directed breeding (Machado et al. 2011; Vardi et al. 2008). Molecular markers were used to determine the mutant individuals after mutation breeding in few studies such as mandarin (Distefano et al. 2009; Polat et al. 2015; Sülü et al. 2016) and lemon (Polat et al. 2015; Sülü et al. 2016).

In lemon breeding, RAPD markers were used to identify in vivo and one in vitro lemon mutants in comparison with a known zygotic origin genotype. The results showed that mutant genotypes could be discriminated from their parents (Deng et al. 1995). Polat et al. (2015) determined the genetic diversity within stable mutant Yerli Yuvarlak lemon genotypes obtained through ^{60}Co gamma radiations using SSR and sequence-related amplified polymorphisms (SRAP) markers. Although six SSR primers exhibited polymorphism and were closely linked to shoot tip color, SRAP primer combinations did not produce any scorable polymorphic bands. Sülü et al. (2016) used the same stable mutant Yerli Yuvarlak lemon genotypes to determine genetic diversity by using SSR, ISSR and single-strand conformation polymorphism (SSCP) markers. ISSR markers were effective in determining genetically different

mutant Antalya Yerli Yuvarlak lemons, both their own and control parent. However, SSR and SSCP markers could not be used to effectively separate some genotypes.

Over the past decade, a variety of knowledge-based mutational breeding tools, including oligonucleotide-directed mutagenesis (ODM) and site-directed nucleases (SDNs), have been developed that result in plant varieties with desirable characteristics in a more precise and efficient manner than the classical breeding and mutagenesis approaches (Tzfira et al. 2012; Xie and Yang 2013).

12.6 Genetic Mapping and Marker-Assisted Selection

Genomic science and its powerful new tools are increasing rapidly for citrus improvement to develop and utilize critically-significant traits (Talon and Gmitter 2008). Especially in this decade, genetic linkage maps have been produced with increasing value and resolution, as the evolution of new marker systems has taken place for advanced studies in genetics, both for the identification and isolation of genes, and for the study of their structure, expression and function (Machado et al. 2011; Talon and Gmitter 2008). Genetic mapping and linkage analysis were developed more than a century ago in 1911 by D. H. Morgan and his graduate student Alfred H. Sturtevant and are still used today in much the same way, but with far more advanced techniques (Gupta et al. 1999). Some of these maps in different *Citrus* spp., allowed the mapping of genes associated with agronomic characteristics of interest, such as salinity (Tozlu et al. 1999), nematode resistance (Ling et al. 2000), resistance/tolerant to tristeza (Cristofani et al 1999; Fang et al. 1998), alternaria (Cuenca et al. 2016; Dalkilic et al. 2005; Gulsen et al. 2010), citrus variegate chlorosis (Oliveria et al. 2004), male sterility (Dewi et al. 2015), seedlessness (Aleza et al. 2010a; Chavez and Chaparro 2011) and the citrus genome (Ollitrault et al. 2012; Roose et al. 2000; Şahin-Çevik and Moore 2007).

Most of traits have complicated genetic regulation as quantitative traits loci (QTLs) regulated by several genes with an accentuated effect of environment. The improvement of quantitative traits has been an important aim for many plant breeding programs, genetic and genomic (Machado et al. 2011). Most linkage maps in citrus correspond to segregating populations. Information on genomic regions showing segregation distortion is important in breeding, particularly if these regions contain genes or QTLs of agronomic interest and candidate gene discrimination in citrus, where breeding populations are expensive to maintain due to the size and long juvenility period of the plant (Bernet et al. 2009; Gulsen et al. 2016).

Genes can be assessed for linkage with the closest markers on a map and indirect selection performed using them. Mapping must be saturated with markers of several different types so that a variety of markers can be tried and assessed for their usefulness in detecting a specific trait (Bernet et al. 2009). Thus, it is possible to construct highly-saturated genetic maps, which can be associated with cloning of large genome fragments in bacterial artificial chromosome (BAC) libraries to trace and locate genes and their regulation pathways (Jarrell et al. 1992). Combining different

marker systems in linkage mapping studies may provide better genome coverage due to their chromosomal target site differences (Gulsen et al. 2010).

DNA markers have enormous potential to improve the efficiency and precision of conventional plant breeding by marker-assisted selection (MAS) (Collard and Mackill 2008). The established experimental tools and resources used in genome analysis are expected to accelerate the discovery of the loci associated with various quantitative traits and contribute to the design of breeding programs supported by MAS, including genomic selection in citrus breeding. Efficiency of breeding is affected by long juvenility, heterozygosis, gametophytic cross-incompatibility, male sterility, apomixis, seedlessness and instability of characteristics under different environmental conditions. To produce new attractive and promising cultivars in the future, it is expected that the use of MAS will overcome these obstacles and improve the efficiency of conventional breeding programs (Omura and Shimada 2016). With the advent of MAS, a new breeding tool is now available to make more accurate and useful selections in breeding populations (Datta et al. 2011). For example, Chae et al. (2011) developed a sequence-characterized amplified region (SCAR) marker linked to male fertility traits was constructed from a cross between Kiyomi cv. (*C. unshiu* × *C. sinensis*) carrying the male sterility trait and Jinkyool cv. (*C. sunki*), for MAS breeding.

New technology will continue to enhance progress toward high resolution and highly informative maps of citrus genomes in the future. Nowadays, studies to utilize microarrays for mapping single-nucleotide polymorphisms (SNPs) in various families and genetic backgrounds, and, as a full genome sequence comes forward for citrus, followed by additional resequencing of other genomes of interest, the genomic identification and locations of thousands of trait relevant SNPs will become known and exploited for genetic improvement of the crop (Talon and Gmitter 2008). Microarray-based genomic selection (MGS), capable of selecting and enriching targeted sequences from complex eukaryotic genomes without the repeat blocking steps necessary for bacterial artificial chromosome (BAC)-based genomic selection have been developed (Okou et al. 2007). Development of NGS and technology for assembly of sequence data from NGS has made whole genome sequencing of horticultural crops possible (Shiratake and Suzuki 2016).

However, such work in support of lemon breeding programs remains inadequate. It is believed that new research areas will involve mapping fruit characteristics (such as seedlessness, nutrient component, etc.) resistance or tolerant to abiotic (salinity, coldness, etc.) and biotic stress (mal secco disease, coldness, etc.) and tree characteristics (such as thornless, dwarfness, etc.) for lemon breeding. For this purpose, most of the important breeding objects such as fruit quality and fruit yield are quantitatively inherited, and the development of QTL markers is urgently needed. Discovery of the loci associated with various quantitative traits and contributing to the design of breeding programs must be supported by MAS. And also, molecular markers could be taken for MAS to overcome many obstacles and improve the efficiency of conventional and molecular breeding programs.

12.7 Metabolomics and Proteomics Tools

In recent years, the use of the omics approach has increased strikingly in plants. Omics are powerful means to identify key genes for important traits, to discern physiological mechanisms and to reveal unknown metabolic pathways in crops (Shiratake and Suzuki 2016). A schema of omics studies is given in Fig. 12.3. Although the most reported omics studies on citrus is genomics, transcriptomics and phenomics, and a few are reported on proteomics and metabolomics.

Proteomics aims to separate, identify and profile proteins that play a role in determining traits and specific processes. These also include those not regulated at gene expression or mRNA levels (Anonymous 2013; Shiratake and Suzuki 2016). Metabolomics is one of the newest omics sciences, aiming to separate, identify and profile compounds which determine traits, such as flavor and color. Metabolomics and proteomics of the model genotype would allow validating results derived from DNA microarrays and EST studies, by confirming protein expression, and allow the subsequent coordination of gene transcription with protein expression and changes in metabolite level. In addition, these studies would validate the effect of newly introduced or modified genes in the model genotype on protein expression and metabolite level and profile (Anonymous 2013). Metabolomics has been suggested to have the potential to generate metabolite-based *biomarkers* of important traits of crops, such as citrus greening (HLB) resistance in citrus (Chin et al. 2014).

In research on lemons, enormous undiscovered treasures, genomics, important genes, transcriptomics, metabolites, metabolic pathways, hormonnomics, ionomics

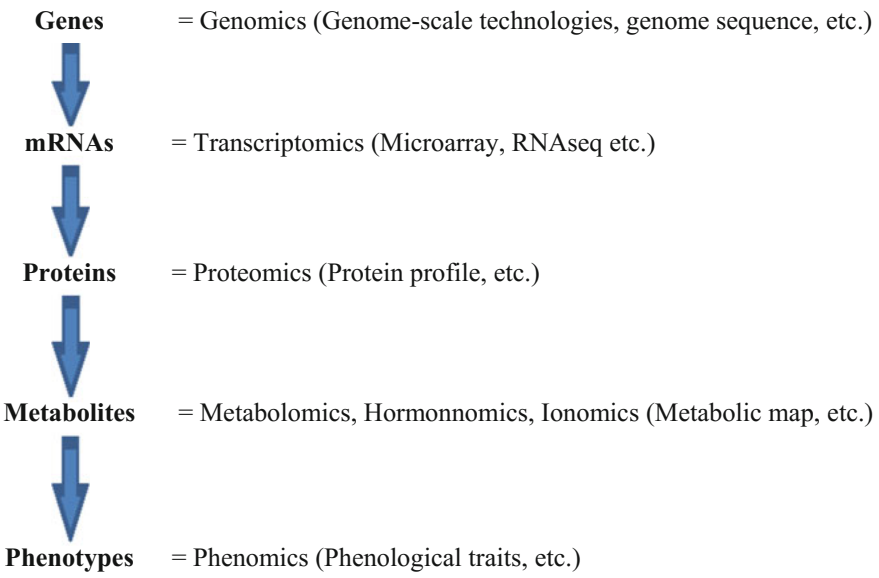

Fig. 12.3 Schema of omics studies

and unknown physiological processes, lie hidden. Treasure hunting using omics technology has just begun to help breeders.

12.8 Genetic Transformation

Genetic engineering is a powerful tool for plant improvement and has the potential to achieve the integration of desirable characteristics into existing genomes (Dönmez et al. 2016). Currently, genetic modification (also called transgenic or genetically-engineered plants) as a tool for citrus breeding programs has been gaining popularity as in many plants (Pons et al. 2012).

This method is especially useful in cases where it is impossible to introduce a particular trait of interest to another elite cultivar using conventional breeding. Recent developments in gene transfer techniques via the classical regeneration method have been applied to the *Citrus* genus and have opened the way to induce a specific genetic change within a shorter period of time than using the classical genetic selection method (Donmez et al. 2013; Pons et al. 2012). Citrus transformation has now been achieved in a number of laboratories; for example, *Agrobacterium tumefaciens* and *A. rhizogenes,* particle bombardment, electroporation and RNA interference are used in citrus transformation (Bespalhok Filho et al. 2003; Donmez et al. 2013; Pons et al. 2012). On the other hand, interfering RNAs (iRNA) have been gaining popularity among scientists due to their potential use for targeting specific genes (Machado et al. 2011). Among molecular techniques, sequencing technology has been used for many years and recently a new concept titled RNA-Seq has been introduced to perform and understand molecular mechanisms in fruits. RNA-Seq analysis is an effective tool to understand which genes are involved and expressed in different mechanisms and organs/cells of a plant (Simsek et al. 2017).

The first transgenic citrus plants were obtained using sour orange and Mexican lime, expressing the coat protein gene of citrus tristeza virus (CTV), while the first transformed plant from adult tissue was produced using sweet orange cv. Pineapple. Other studies of genetic transformation on citrus include sweet orange, sour orange, grapefruit, mandarin and *Poncirus trifoliata* (Machado et al. 2011). Genetic transformation of *Citrus paradisi* was achieved with antisense and untranslatable RNA dependent RNA polymerase genes of citrus tristeza closterovirus (Çevik et al. 2006). Plant bHLHs functioning in cold tolerance and the underlying mechanisms remain poorly understood. Identification and functional characterization of PtrbHLH isolated from trifoliate orange (*P. trifoliata*). Overexpression of PtrbHLH in tobacco (*Nicotiana tabacum*) or lemon (*C. limon*) conferred enhanced tolerance for cold under chilling or freezing temperatures, whereas down-regulation of PtrbHLH in trifoliate orange by RNA interference (RNAi) resulted in elevated cold sensitivity (Huang et al. 2013).

Velázquez et al. (2016) developed a new biotechnology tool to promote transition from the vegetative to the reproductive phase in juvenile citrus plants by expression of the *Arabidopsis thaliana* or citrus flowering locus T (*FT*) genes using a citrus

leaf blotch virus-based vector (clbvINpr-AtFT and clbvINpr-CiFT, respectively). Overexpression of native *FT*s in many different plants has led to early flowering. Precocious flowering of juvenile citrus plants after vector infection provides a helpful and safe tool to dramatically speed up genetic studies and breeding programs. Likewise, Moore et al. (2016) carried out a project to develop and use early flowering citrus types to speed up citrus improvement, making it possible to rapidly introduce valuable traits from citrus relatives into high quality commercial citrus types. When the citrus *FT3* gene was overexpressed in citrus (Carrizo citrange, Hamlin sweet orange and Duncan grapefruit), it surprisingly worked too well.

Although the perspective for genetic transformation depends on the country, it is a very important technique to obtain new varieties in plant breeding. These efforts should be increased in lemon breeding.

12.9 Database and Bioinformation

A cultivar identification diagram (CID), as a novel analysis approach, was developed that can realize the utility of DNA markers in the separation of plant individuals much better, more efficiently, practically and referable for fingerprinting. In China, 47 important cultivated lemon cultivars were successfully separated for fingerprint analysis using the CID strategy by ten RAPD primers (Mu et al. 2012). Coalescence analysis conducted and combined with the phylogenetic tree data and STRUCTURE results may be useful to unravel many phylogenetic questions pertaining to citrus (Ramadugu et al. 2013).

The National Center for Biotechnology Information (NCBI), Bethesda MD, USA, provides access to genomic information on advances in science and health. NCBI comprises the DNA DataBank of Japan (DDBJ), the European Nucleotide Archive (ENA) and GenBank. It is designed to provide and encourage access within the scientific community to the most up-to-date and comprehensive DNA sequence information (Anonymous 2013, 2017).

It is very important to obtain as much information as possible before starting a breeding project. Plant genome resources are available through publicly available web portals such as the USDOEJGI (https://phytozome.jgi.doe.gov) and Tree Fruit Genome Database Resources (tfGDR, https://www.citrusgenomedb.org). However, there are no data about *Citrus limon* at the USDOEJGI web page. Therefore, the tfGDR Citrus Genome Database is a very important establishment and houses the genomics, genetics and breeding data for sweet orange, trifoliate orange, grapefruit, mandarin, tangerine, pumelo, lemon, lime and other related species of the *Citrus* genus to facilitate efficient application in molecular breeding programs. However, the information at the website and related studies about lemon are very scarce because of little information and data transfer. Therefore, there is a need to increase the studies and transfer of information (Gmitter et al. 2012; Kumar et al. 2013). On the other hand, The International Citrus Genome Consortium (ICGC) was established to develop useful genomic resources for citrus genetics and breeding research exceeded

the capacity of any of the individual laboratories or national groups, and sought therefore to develop a set of global and freely-available, genome-based tools (Gmitter et al. 2012).

Meanwhile, genomic determination of germplasm should be made in depth using the new marker techniques. Likewise, development of bioinformatics for the management and analysis of biological information arising from genomics and high-throughput experiments has been very important in assessing our work at the Bati Akdeniz Agricultural Research Institute.

12.10 Conclusions and Prospects

Lemon as a crop is about 800 years old; breeding began many years ago by farmers selecting the best fruit-producing plants in the field, leading to domestication (Kahn et al. 2001). The discovery of the laws of genetics by Gregor Mendel about 150 years ago enhanced the speed of plant breeding considerably. The invention of crossbreeding was followed by hybrid breeding in the 1930s, tissue and cell culture methods in the 1960s and recombinant DNA techniques and genetic engineering in the 1980s. So-called *smart breeding* started in the late 1990s with the use of molecular markers, genome mapping and sequencing. The progress made in biotechnology and genomics is preparing the way to meet the challenges ahead; besides that new genes for resistance to major biotic and abiotic stresses are constantly being identified using DNA markers (Kordrostami and Rahimi 2015).

The development of new techniques in plant breeding has not led to the replacement of the older methods. The use of all available technologies is essential for plant breeding. Conventional breeding techniques, transgenesis and new plant breeding techniques are essential components of what could be called the plant breeders' toolbox (Lusser et al. 2011).

In the future, it is expected that the use of marker assisted selection will overcome some obstacles and improve the efficiency of conventional breeding programs. Development of technologies included cisgenesis, intragenesis, emerging techniques to induce controlled mutagenesis or insertion will be used in future lemon breeding programs. It is hoped that this study contributes to the advance of lemon breeding.

Acknowledgements We thank Ertuğrul Turgutoğlu for his critical reading of this work and for helpful suggestions.

Appendix 1: Research Institutes and Online Resources

List of major Institutes engaged in research on lemon (*Citrus limon* L. Burm. F.)

1. Citrus Variety Collection. University of California Riverside, 900 University Ave, Riverside, CA 92521, USA. e-mail: academicpersonnel@ucr.edu

2. National Clonal Germplasm Repository for Citrus, USDA, ARS Natl Germplasm Repos, 1060 Martin Luther King Blvd. Riverside, CA 92507, USA. e-mail: marylou.polek@ars.usda.gov
3. Citrus Research and Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850, USA
4. University of California Davis, 1 Shields Avenue, 3100 Dutton Hall Davis, CA 95616 USA.
5. Alata Horticultural Research Institute, Alata Bahçe Kül. Araş. Enst. 33740, Erdemli, Mersin, Turkey. e-mail: alata@tarim.gov.tr
6. Batı Akdeniz Agricultural Research Institute (BATEM), Demircikara Mah. Paşa Kavakları Cad. No: 11 Pbox: 35, Muratpaşa, Antalya, Turkey. e-mail: batem@tarim.gov.tr
7. University of Cukurova, Faculty of Agriculture, Ziraat Fakültesi Dekanlığı, Sarıçam, Adana, Turkey. e-mail: bahce@mail.cu.edu.tr
8. Citrus Research Institute (CRI), Chinese Academy of Agricultural Sciences, No. 12 Zhongguancun South St., Haidian District Beijing P.R.China. e-mail: diccaas@caas.cn
9. Southwest Minzu University, #16, South Section, 1st Ring Road, Chengdu, Sichuan, 610041 China. e-mail: WJ@swun.edu.cn
10. National Citrus Engineering Research Center, Xiema, Beibei, Chongqing, 400712 P.R.China. e-mail: cric@163.com; www.cric.cn
11. Huazhong Agricultural University, Wuhan Hubei 430070, People's Republic of China.
12. Sylvio Moreira Citrus Center, Centro de Citricultura Sylvio Moreira, Instituto Agronômico, Cordeirópolis São Paolo, Brazil. e-mail: https://www.givaudan.com
13. Valencian Institute of Agricultural Research (IVIA), Ctra. Moncada-Náquera, km. 4, 5, 46113, Valencia, Spain. e-mail: www.ivia.es
14. Central Citrus Research Institute (CCRI), Nagpur, Post Box No. 464, Amravati Road, Nagpur—440033, Maharashtra, India. e-mail: director.ccri@icar.gov.in
15. University of Catania, Piazza Università, 2-95131 Catania—Partita IVA 02772010878, Italy. e-mail: protocollo@pec.unict.it
16. Centro di Studio per il Miglioramento Genetico degli Agrumi, Consiglio Nazionale delle Ricerche, Male delle Scienze, Palenno 90128, Sicily, Italy. e-mail: www.cnr.it
17. Centro di Ricerca per l'Agrumicoltura e le Colture Mediterranee, Corso Savoia 190 95024—Acireale, Italy. e-mail: acm@crea.gov.it
18. University of Palermo, Università degli Studi di Palermo Piazza Marina, 61 90133—Palermo, Italy. e-mail: www.unipa.it
19. Citrus Research International, South Africa. e: www.citrusres.com
20. National Institute for Agricultural Research (INRA), Avenue Lucien Brétignières 78850 Thiverval-Grignon, France. e-mail: www.inra.fr
21. Tel Aviv University, P.O. Box 39040, Tel Aviv 6997801, Israel. e-mail: https://tau.ac.il

22. Institute of Horticulture, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel. e-mail: http://www.moag.gov.il
23. Buenos Aires University, Buenos Aires, Argentina. e-mail: http://www.uba.ar

Appendix 2: Genetic Resources

A list of some of cultivars, their important traits and country of release

No.	Variety	Selection and breeding method	Year	Country of release
1	Eureka 22 INTA	Mutation breeding	1987	Argentina
2	Alata	Mutation breeding	2007	Turkey
3	Gülşen	Mutation breeding	2007	Turkey
4	Uzun	Mutation breeding	2007	Turkey
5	Eylul	Mutation breeding	2007	Turkey
6	Galya	Mutation breeding	2007	Israel
7	Ayelet	Mutation breeding	2007	Israel
8	Feminello Siracusano 2KR	Mutation breeding	1990	Italia
9	Lisbon	Clone	1824	Portugal
10	Limoniera 8A Lisbon	Clone	1966	USA
11	Frost Lisbon	Nucellar selection	1950	USA
12	Corona Foothills Lisbon	Nucellar selection	1980	USA
13	Rosenberger Lisbon	Hybridization breeding	1995	USA
14	Limoneiro Fino 49	Clone		Spain
15	Eureka	Selection from local variety	1850s	Italy
16	Frost nucellar Eureka	Budwood		USA
17	Taylor Eureka	Nucellar selection	1989	Australia
18	Variegated Pink	Bud sport	1931	USA
19	Meyer	Selection from local variety	1908	China
20	Improved Meyer	Clone	1950	USA
21	Santa Teresa	Selection from local variety		Italy
22	Ponderosa	Hybridization breeding	1887	USA
23	Yen Ben	Selection from local variety	1930	New Zealand

No.	Variety	Selection and breeding method	Year	Country of release
24	Villafranca	Selection from local variety	1875	Italy
25	Genoa (Genova)—Italian	Selection from local variety	1875	Italy
26	Feminello	Budwood	1966	USA
27	Santa Teresa Feminello	Selection from local variety	1960s	Italy
28	Lamas	Selection from local variety	1990	Turkey
29	Yediveren	Selection from local variety	2014	Turkey
30	Kütdiken	Selection from local variety	1990	Turkey
31	Interdonato	Selection from local variety	1875	Italy
32	BATEM Pınarı	Selection from local variety	2011	Turkey
33	BATEM Sarısı	Selection from local variety	2011	Turkey
34	Italyan Memeli	Selection from local variety	1990	Turkey
35	Karalimon	Selection from local variety	1990	Turkey
36	Kıbrıs Limonu	Selection from local variety	1990	Turkey
37	Lapithkiotiki	Selection from local variety		North Cyprus
38	Volkamer	Hybridization breeding	1955	Italy
39	Baboon	Selection from local variety		Brazil
40	Cameron Highlands	Selection from local variety		Malaysia

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Aleza P, Juárez J, Ollitrault P, Navarro L (2010b) Polyembryony in non-apomictic citrus genotypes. Ann Bot 106:533–545
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Chapter 13
Mandarin (*Citrus reticulata* Blanco) Breeding

Muhammad Usman and Bilquees Fatima

Abstract Mandarins including clementines, tangerines, satsumas, willowleaf, tangors and tangelos are the second largest cultivated group of citrus after sweet oranges and provide about 25% of world citrus production. Classical breeding has limited potential in citrus crop improvement and is handicapped mainly by nucellar embryony, long juvenility and self-incompatibility. Molecular biology tools have revealed mandarins as one of the primary citrus species and the ancestor of secondary species. A better understanding of genomics, valuable bioinformatics databases and recent advances in molecular breeding have shortened the breeding cycle and accelerated the breeder's productivity towards improvement in economically-important traits. This chapter elucidates the economic significance, botanical classification and leading cultivars, global distribution of varieties, floral biology and functional genomics, germplasm biodiversity and conservation, origin and genetic backgrounds of polyembryony, haploids and polyploids and their significance in structural genomics, mutation breeding, seedlessness, scope of conventional and somatic hybridization, applications of molecular markers, bioinformatics databases and transgenics. Enhancing international collaboration, accelerated germplasm conservation and exchange programs, integration of classical breeding and the molecular biology tools discussed could enhance the pace of development of high-yielding cultivars with better resistance against changing climatic conditions, emerging biotic and abiotic stresses and help to ensure food security.

Keywords Citrus · Seedlessness · Mutation · Ploidy · Genomics
Nucellar embryony

M. Usman (✉) · B. Fatima
Plant Tissue Culture Cell, Institute of Horticultural Sciences,
University of Agriculture, Jail Road, Faisalabad 38040, Pakistan
e-mail: m.usman@uaf.edu.pk

B. Fatima
e-mail: drfatimausman@gmail.com

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13.1 Introduction

13.1.1 Origin, Botanical Classification and Domestication

Mandarins are generally referred as easy-peeling, small-sized citrus fruits having an orange color. A highly diversified group of citrus, they include different species; however, generally mandarins are referred as *Citrus reticulata* Blanco. Among commercial citrus fruits, mandarins rank second to sweet oranges in terms of cultivation. Mandarins are known under different names in different parts of the world like *Mandarino* (Italian and Spanish), *Chu, Ju, Chieh* (Chinese), *Mikan* (Japanese) and *Santara* (Indian). Mandarins are a member of genus *Citrus*, family Rutaceae, sub-family Aurantioidae and sub-tribe Citrinae (Swingle and Reece 1967). Mandarins have been cultivated in China and Japan for millennia and were introduced in Europe during modern times. Mandarins ($2n = 2x = 18$) originated in Cochin China, the southern region of the Indo-Chinese Peninsula (Tolkwosky 1938) and traders carried different selections to Eastern India. Traditionally, mandarins have been produced in the Asian region. The first mandarin plant, probably cv. Willowleaf (*C. deliciosa* Tenol), was introduced from China to England in 1805 and later spread to the Mediterranean region. *Citrus reticulata* was introduced later to this region (Davies and Albrigo 2006). Most commercial cultivars have been developed through selection of bud sports from introduced cultivars. Most existing mandarin cultivars originate from China, Japan and Southeast Asia, while Satsuma (*C. unshiu* Marc.) was developed in Japan. Recently, the center of origin of *Citrus* species has been proposed as southeast of the Himalayas including east Asam, north of Myanmar and western Yunnan on the basis of genomic, biogeographic and phylogenetic analysis of 60 different citrus and related strains (Wu et al. 2018). The importance of mandarins in the global citrus industry and the world economy is very well demonstrated by its wide distribution, high production and ever-rising market demands. Usually mandarin cultivars are cold-resistant among commercially grown citrus, as well as tolerant to biotic and abiotic stress (Ben Yahmed et al. 2015). Its long history of cultivation, natural interbreeding, mutations and genomic introgression with other citrus ancestors have developed the currently available broad diversity of fruit shape, color, taste and quality.

13.1.2 Economic Importance and Global Distribution

Mandarins are grown throughout the tropical, subtropical and Mediterranean regions where suitable soils having enough moisture content exist with little or no cold conditions. Commercial producing areas are mainly in the subtropical regions at latitudes above 20°N or S of the equator. Dissemination took place to different regions such as the Mediterranean, Central America, Asian, South Africa, Australia and New Zealand. Citrus is being cultivated on an area of 8.01 million ha, with

annual world production of 132.40 million mt. Mandarin's share in the global citrus area and production is 32.5 and 24.7%, respectively and average global yield is 12.5 mt ha^{-1}. Citrus cultivation is widespread throughout the Northern Hemisphere (NH) and Southern Hemisphere (SH). Major citrus producing countries are situated in the NH and cover 56.9% of global citrus area and 54.4% of annual global citrus production. The share of NH is much higher in world mandarin area (86.8%) and production (83.7%); however, the average yield is lower (12.1 mt ha^{-1}) compared with SH. About 49.6% of the NH citrus area is under mandarin cultivation, providing 38.1% of total citrus production. In the NH, China is the leading citrus and mandarin producing country and 70.5% of its total citrus area and 52.4% production is from mandarins. The United States and Turkey are leaders in mandarin yield 28 mt ha^{-1} (Table 13.1). In Pakistan, 25% of citrus area and 26% citrus production is provided by mandarins, mainly cv. Kinnow. The mandarin yield of Pakistan is the lowest (11.5 mt ha^{-1}) in the NH except for China (FAO 2016).

The Southern Hemisphere accounts for 14.6% of world citrus area and 21.2% of annual production. Only 5.7 and 7.4% of global mandarin area and production is contributed by major citrus producing countries in the SH. Average yield of mandarins is much higher, 16.23 mt ha^{-1}, compared with yield in the NH of 12.13 mt ha^{-1}. Mandarins account for 12.7% of area and 8.8% of production in total citrus cultivation, respectively. Brazil and Argentine are leading mandarin producers and the proportion of Argentine's mandarin production is higher than in Brazil. South Africa is the leader in average yield 31.2 mt ha^{-1} followed by Australia (Table 13.1). Mandarins are being produced at a large scale in China comprising many local selections including the Satsuma mandarin. Mandarins are a major citrus species being produced and exported to different regions from the Mediterranean countries, particularly Spain.

Seedless selections play a major role in the mandarin market, including cvs. Clementine, Satsuma and recently developed low-seeded Kinnow mandarin. Mandarin production in Spain increased many fold in the early 1990s. In the USA, mandarin production declined during 1990s due to poor handling and harvesting practices. The main cultivars in Florida include Dancy, Murcott, Orlando, Sunburst, Robinson and Nova; these have not gained more consumer acceptance in the European markets due to their seediness. The US mandarin imports have increased manyfold during the last two decades due to rising demands and a decline in domestic production. In Australia, mandarins, mainly cv. Ellendale, are being produced; however, citrus is dominated there by sweet oranges. In India, oranges, mandarins (cv. Coorg) and lime dominate the citrus industry. The citrus industry in Pakistan is dominated by sweet oranges, cv. Kinnow mandarin and lime. Recently, low-seeded Kinnow mandarin has been developed by the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad (Altaf 2007); University of Agriculture, Faisalabad (UAF), and a few selections are also available at the Citrus Research Institute (CRI), Sargodha (Altaf et al. 2014). The world market share proportions of mandarins for Tangerines, Satsuma and Clementine are 4:3:2, respectively. The citrus industry in Japan is dominated by cv. Satsuma mandarin which is good for canker tolerance and freeze hardiness (Kitagawa et al. 1988).

Table 13.1 An overview of leading citrus and mandarin producers in the Northern and Southern hemispheres

Location	Total citrus		Mandarins, Tangerines, Clementines and Satsumas			Mandarin share (%) in total citrus	
	Area (000, ha)	Prod. (000, mt)	Area (000, ha)	Prod. (000, mt)	Yield (tons/ha)	Area (%)	Production (%)
World	8011.95	132,401.43	2609.12	32,792.53	12.56	32.56	24.76
Northern hemisphere							
China	2440.34	32,698.23	1722.16	17,155.49	9.961	70.57	52.46
Spain	361.06	7008.59	164.04	2941.97	17.93	45.43	41.97
Turkey	465.69	1337.03	46.56	1337.03	28.71	100	100
Egypt	195.96	4802.11	44.82	1020.49	22.76	22.87	21.25
Morocco	121.00	2023.72	62.19	1077.62	17.32	51.39	53.24
Japan	43.53	828.86	41.50	805.10	19.40	95.33	97.13
United States	298.18	7489.78	27.04	779.78	28.83	9.06	10.41
Italy	145.41	2528.35	35.40	649.14	18.33	24.34	25.67
Iran	187.88	3127.57	36.19	651.57	18.00	19.26	20.83
Pakistan	197.91	2270.136	51.45	592.13	11.50	25.99	26.08
Mexico	528.18	7938.60	33.60	467.45	13.91	6.36	5.88
Southern hemisphere							
Brazil	759.95	19,591.62	49.23	997.99	20.27	6.47	5.09
Argentina	145.66	3281.32	41.10	468.27	11.39	28.21	14.27
South Africa	66.72	2230.82	5.75	180.09	31.29	8.61	8.07
Australia	28.87	578.82	5.40	125.23	23.16	18.70	21.63
Venezuela	38.49	548.39	9.81	158.49	16.14	25.48	28.90
Thailand	72.77	970.63	10.23	139.66	13.64	14.05	14.38
Nepal	24.14	212.20	16.24	146.69	9.028	67.27	69.12

Source FAO production year book (2016), www.faostat.org

13.1.3 Reasons for Low Productivity

Many tropical countries have low mandarin production due to periodic dry and wet cycles and the increasing incidence of citrus canker, citrus tristeza virus (CTV) and citrus huanglongbing (HLB; greening) disease. A few mandarin cultivars also have specific climatic requirements for quality production such as Ponkan, Ellendale and Dancy, which are more suitable to be grown under subtropical conditions and cannot be cultivated along with Satsuma, which loves cold winters. Generally, mandarins have a short harvest season, are susceptible to injury during picking and

transportation, have a higher tendency to puffiness, the juice becomes insipid and loses acidity if allowed to overmature on the tree. Hence early and timely harvest is recommended. Mandarins also show great variability in skin color, size, fruit shape, fruit maturity and tree size. Despite having tremendous variability, there are many barriers faced by citrus breeders that hinder proper exploitation of this variability, which needs to be addressed in mandarin crop improvement programs. Advances in mandarin breeding and biotechnology are discussed in the following sections.

13.2 Reproductive Biology and Pollination

In subtropical zones, mandarins flower once a year in early spring. Flower induction starts in early December–January. In tropical and coastal areas, mandarins may bloom several times a year and the blooming season may be prolonged depending upon the area of cultivation and chilling received. Flowers are usually born singly in the leaf axil as a corymbose raceme type of inflorescence. Axillary flower buds develop later than the terminal buds. Flowers have 4–8 thick linear petals, 4–5 lobed calyx, the petals imbricate in buds but strongly reflexed at maturity. Stamens and petals are borne on the receptacle. The number of stamens is 4 times the number of petals. Anthers surround the pistil near the level of stigma, consist of 2 locules and dehisce longitudinally. The ovary contains 6–14 carpels and a placental layer is present inside each carpel. At flowering, the ovary shape is subglobose or subcylindrical while the style is cylindrical in shape. Both perfect and staminate flower types are present with up to 50% fruit set, depending upon the availability of pollinators and genotype. Pollen is sticky in nature and flowers are mainly entomophillic. Pollen viability varies among species and cultivars. Controlled pollination is relatively easy. Seed and pollen flowers should be protected against contaminating foreign pollen grains. Flowers from pollen parents should be collected just after opening of the petals at the *popcorn* stage or one-petal-open stage.

Androceium position in the flower enables self-pollination; however, cross-pollination is also facilitated by insects (honey bees, thrips and mites). Under suitable environmental conditions, fertilization occurs within 8 days of pollination; however, it may be delayed by up to 4 weeks. Most mandarin cultivars have a higher percentage of functional pollen grains. In cv. Satsuma a small number of pollen mother cells are produced and most of the pollens produced degenerate. The frequency of meiotic abnormalities increases at lower temperatures. Pollen sterility increases below 19 °C and may be associated with ovule abortion.

Controlled pollination is easy in mandarins and flowers may also be protected to avoid foreign pollen contamination. Trees with higher bloom should be avoided as these set poor fruit. Early and large flowers should be selected for pollination in the cluster of flowers. Anthers usually dehisce in 12–24 h at room temperature which could be used for pollination. Pollen can be stored for 4–6 weeks at 4 °C and for longer at lower temperature or after freeze drying. Ready-to-open flowers should be emasculated, pollinated with collected pollen and covered with paper bags. Fruit set

is better on leafy inflorescences; hence, leaves should not be removed. Covering of the flowers is sufficient for self-pollination. Pollination can be done immediately or several days after anthesis. Unpollinated flowers remain receptive for several days. In self-incompatible cultivars, selfing may be successful 3–4 days before opening. Paper bags could be removed 2 weeks after pollination. After fruit set, they may be covered with open mesh bags to avoid fruit drop. Aborted pollen frequency could be checked using acetocarmine staining. Pollen viability may be determined by dyeing with 1% acetic acid magenta (w/v) and may also be assessed by in vitro germination on Murashige and Skoog (MS 1962) media containing 10–20% sucrose and by the hanging-drop technique.

13.3 Taxonomy and Breeding Behavior of Cultivars and Rootstocks

The mandarin group consists of several species and hybrids, both interspecific and intergeneric, having unique traits. The term *mandarin* is used in the citrus-growing regions including China, Japan, Italy, Spain, Pakistan and India while *tangerine* is mostly used for the deeply-pigmented mandarins in the USA, China and Australia. In South Africa, mandarins are known as *soft citrus*. Citrus classification has been controversial and several classifications are available. Mandarin cv. Ponkan has been classified as *Citrus reticulata* by all taxonomists. Clementine and Dancy cvs. have been classified under *C. reticulata* by all taxonomists except Tanaka. Satsuma and Willowleaf cvs. have been classified as *C. reticulata* by taxonomists other than Tanaka and Hodgson while cv. King Siam was classified under species *reticulata* by Swingle and Hodgson. A detailed overview of different classifications proposed by different researchers at different times is presented in Table 13.2.

*13.3.1 Satsuma Mandarins (*Citrus unshiu *Marc.)*

Satsuma progenitors originated in China and were transported to Japan where it is now a major citrus crop. Cultivar Satsuma originated in Japan as a nucellar seedling from cv. Tsao Chieh mandarin and has about 100 varieties which vary in important different traits (Saunt 1990). Satsuma cv. is well adapted to cool conditions of the subtropical regions of China, Japan, Spain and South Africa. Its foliage and wood is highly freeze hardy (down to −9 °C) compared with other citrus species. Its fruit is large in size and oblate in shape. The flesh color changes before peel color and ovule sterility leads to seedlessness. Satsuma has a narrow harvest period and at maturity its segments start drying; hence, breeders are interested to develop early maturing Satsuma to extend the harvest span. A few early varieties including Okitsu Wase and Miyagawa Wase have been selected in Japan. The fruit may be harvested in September

Table 13.2 Mandarin classifications, interspecific and intergeneric hybrids

Common (cultivar) name	Scientific/botanical names				
	Swingle	Tanaka	Hodgson	Scora	Bailey and Bailey
Clementine	*Citrus reticulata*	*C. clementina*	*C. reticulata*	*C. reticulata*	*C. reticulata*
Dancy	*C. reticulata*	*C. tangerine*	*C. reticulata*	*C. reticulata*	*C. reticulata*
King (Siam)	*C. reticulata*	*C. nobilis*	*C. nobilis*	*C. reticulata*	*C. nobilis*
Ponkan	*C. reticulata*	*C. reticulata*	*C. reticulata*	*C. reticulata*	*C. reticulata*
Satsuma	*C. reticulata*	*C. unshiu*	*C. unshiu*	*C. reticulata*	*C. reticulata*
Temple	*C. reticulata*	*C. temple*	*C. temple*	*C. reticulata*	*C. tangor*
Willowleaf	*C. reticulata*	*C. deliciosa*	*C. deliciosa*	*C. reticulata*	*C. reticulata*
Ananas	*C. reticulata*	*C. reticulata*	–	–	–
Beauty	*C. reticulata*	*C. reticulata*	–	–	–
Citrandrin B47	*C. reticulata* ×*Poncirus trifoliata*	*C. reticulata* x*P. trifoliata*	–	–	–
Cleopatra	*C. reticulata var. astera*	*C. reshni*	–	–	–
Commune Clementine	*C. reticulata* x C. sinensis	*C. clementina*	–	–	–
Ellendale tangor	*C. reticulata* ×*C. sinensis*	*C. reticulata* ×*C. sinensis*	–	–	–
Feutrell's early	*C. reticulata*	–	–	–	–
Fortune	*(C. reticulata* ×*C. sinensis)* × *C. tangerina*	*C. clementina* ×*C. tangerine*	–	–	–
Fuzhu	*C. reticulata*	*C. erythrosa*	–	–	–
Kinnow	*C. reticulata*	–	–	–	–
Murcott tangor	*C. reticulata* ×*C. sinensis*	*C. reticulata* ×*C. sinensis*	–	–	–
Nan Feng Mi Chu	*C. reticulata*	*C. kinokuni*	–	–	–
Nasnaran	*C. reticulata hybrid*	*C. amblycarpa Hassk.*	–	–	–
Ougan	*C. reticulata*	*C. suavissima Hort.*	–	–	–

(continued)

Table 13.2 (continued)

Common (cultivar) name	Scientific/botanical names				
	Swingle	Tanaka	Hodgson	Scora	Bailey and Bailey
Pectinifera or Shekwasha	*C. reticulata hybrid*	*C. depressa Hayata*	–	–	–
San Hu Hong Chu	*C. reticulata*	*C. erythrosa*	–	–	–
Sunki	*C. reticulata*	*C. sunki Hayata*	–	–	–
Tankan	*C. reticulata hybrid*	*C. tankan Hayata*	–	–	–

Sources Davies and Albrigo (2006), Froelicher et al. (2011), Hu et al. (2007)

Table 13.3 A list of mandarin groups, few scion varieties and rootstocks

Mandarin types		Cultivars
Satsumas (*Citrus unshiu*)		Tsao Chieh, Okitsu, Miyagawa, Nangan, Gongchuan, Xinjin, Owari, Okitsu
Common mandarins (*C. reticulata*)		Ellendale, Murcott, Ponkan, Dancy, Clementine, Monreal, Oroval, Marisol, Changsha, Sunki
Mediterranean mandarins (*C. deliciosa*)		Willowleaf
Mandarin hybrids	Spontaneous	Temple, Sue Linda, Honey Murcott, Honey Orange
	Developed	Minneola, Orlando
	Others	Osceola, Lee, Robinson, Nova, Albir, Safor, Page, Sun Brust, Alborea, Fallglo, Kinnow
Rootstocks		Cleopatra mandarin, Sun Chu Sha Kat, Rangpur mandarin

when trees are grown in open fields. The Chinese have also selected early maturing varieties including Nangan Gongchuan and Xinjin. In Spain, cv. Owari is most widely cultivated. In Japan, due to development of highly sophisticated postharvest storage systems, Satsumas are stored for several months and are available in the Japanese markets for 6–8 months. A brief list of mandarin groups and included scion and rootstock varieties is provided in Table 13.3.

*13.3.2 Common Mandarins (*Citrus reticulata *Blanco)*

This group is highly diversified and include cultivars like cv. Ellendale tangor and Murcott (Honey) which are spontaneous hybrids. The plants of common mandarins

grow upright compared with Satsuma, have small flowers and fruit. Mandarin fruit is easy to peel compared with Satsuma and other mandarins of the Mediterranean region; however, these are easy peelers compared with oranges. Mandarins have better handling, storing and shipping life compared with cv. Satsuma except cvs. Ponkan and Dancy which are difficult to ship to distant markets. Clementine originated in China and is the most widely planted highly economical mandarin cultivar in the Mediterranean region along with North and South Africa (Saunt 1990). Clementine is also known as *Algerian tangerine*. Its trees are large in size and high yielders of excellent seedless fruit except cultivar Monreal. Clementine is more adapted to the Mediterranean climates compared with humid climate of the subtropical and tropical regions. Oroval and Marisol cvs. are early-maturing while Nules and Fortune are late-maturing cultivars.

Tangerine cv. Dancy was discovered in Florida in 1857, and has been most widely cultivated there. Dancy has a very high rate of nucellar embryony hence it comes true to type from seed and starts bearing in 4–5 years. Like other tangerines, Dancy also shows the alternate bearing habit and has limb breakage. Its fruit size remains small during *on* years and use of rough lemon rootstock induced early segment drying. Dancy is highly suitable for areas having hot and humid climates and the best fruit color develops in full sun. Its fruit holding on the tree is less hence it can be harvested and marketed after attaining market maturity. Its trees are relatively freeze hardy; however, it regrows slowly after freeze damage. It blooms late and may not bloom during *off* years, hence it cannot be used as a pollinizer for tangerine cultivars. It is a profitable cultivar if properly managed and marketed. Ponkan cv. also called *Nagpuri Sangtra* in Southeast Asia, produces high-quality and early-maturing fruit. It also exhibits alternate bearing and limb breakage due to a heavy crop load.

*13.3.3 Mediterranean Willowleaf Mandarins (***Citrus deliciosa** *Ten.)*

The Mediterranean mandarin originated in China and is commonly called cv. Willowleaf due to its leaf shape resemblance to that of *Salix* (willow tree). It was the first mandarin introduced to Europe from China in 1805. Mediterranean mandarin is known under different cultivar names in different regions like *Mediterranean* (England), *Ba Ahmed* (Morocco), *Bodrum* (Turkey), *Avana* and *Palermo* (Italy), *Valencia* (Spain), *Baladi* (Egypt and Middle East) and *Willowleaf* (USA). It is also freeze hardy, the fruit is medium sized, seedy and medium to good flavored. Its cultivation and popularity is decreasing due to alternate bearing and more fruit damages during transportation.

13.3.4 Mandarin Hybrids

13.3.4.1 Spontaneous Hybrids

Temple cv. is a natural hybrid (tangerine × sweet orange), called *Tangor*, and was found in Jamaica in late 1800s. It is the most widely cultivated mandarin in Florida and the crop matures in January–March. Unlike true tangerines, the tree is dense with a spreading growth habit. Like true mandarins, the leaves are lanceolate and have thin petioles. The fruit is seedy (20–30 seeds), quality is excellent with deep orange color juice, having high total soluble solids (TSS). The fruit is an easy peeler compared with oranges. Seedless selections of cv. Temple include Sue Linda from Florida and a few selections from South Africa. Due to monoembrony, its plants are not virus free and carry different viral diseases including citrus tristeza virus (CTV). It is less freeze hardy and is highly susceptible to citrus scab.

Murcott cv. (tangerine × sweet orange) is also called *Honey Murcott*, *Honey* and *Honey Orange*. It is a mandarin hybrid that originated in a USDA nursery in 1916. It is not a true tangerine, rather a tangor. Its tree and fruit characteristics are like mandarins. Trees are vigorous and freeze hardy. Fruits are medium in size, not easy peelers like mandarin and seedy. Like cv. Temple, Murcott matures in January–March in Northern Hemisphere climatic conditions and is the latest maturing cultivar. It is not suitable for processing due to its higher content of limonin. Fruit quality is the best with excellent flavor. Peel and juice is deep reddish orange in color at full maturity. Murcott also shows alternate bearing. The tree may collapse due to consistent heavy bearing (on years), higher production and root depletion. Higher fertilization including nitrogen and potash is recommended compared with other mandarins.

13.3.4.2 Developed Hybrids

Webber and Swingle developed mandarin hyrbids tangelo (*Citrus reticulata* × *C. paradisi*), the most important hybrids including cvs. Minneola and Orlando by crossing cvs. Dancy mandarin and Duncan grapefruit. Tangelos are freeze hardy, vigorous plants but develop weak parthenocarpic fruit requiring cross-pollination or spraying of growth regulators like gibberellic acid (GA_3) to get higher fruit setting. Orlando is a vigorous large tree and highly freeze hardy. It develops seedless to seedy (10–20 seeds) fruits depending upon the availability and activity of the pollinators. Fruit matures in December–January having bright orange peel color. Its juice flavor is a blend of mandarin and grapefruit. Its cross-pollination with Robinson tangerine or Temple cvs. is recommended for higher fruit yield. Minneola is cross-incompatible to Orlando. Parthenocarpic fruit may be developed for markets preferring seedless fruit. It shows winter chlorosis more frequently due to nitrogen deficiency. Minneola is a sister seedling of Orlando. Minneola is freeze hardy; however, it is less productive in areas with higher spring temperatures. The fruit has a prominent neck on

the stem end. Deep orange red color develops at maturity in the fruit, peel and juice as well. Like Orlando, seed may range from 0 to 20 depending upon the frequency of cross-pollination. Minneola, like Orlando also needs cross-pollination for higher fruit setting and parthenocarpic fruit may be developed using GA_3 for fresh fruit markets.

13.3.4.3 Other Hybrids

The higher frequency of nucellar embryony has been a considerable problem for citrus breeders. Selection of seed parents that produce only zygotic embryos is the best possible approach. Gardener and Bellow started using monoembryonic (ME) seed parents and crossed cv. Dancy with Clementine in 1942. In the late 1960s, four selections were released including Osceola, Lee, Robinson and Nova. Robinson cv. resembles true mandarin in morphology and requires cross-pollination by parents like Temple or Orlando. The leaf and wood of Robinson is freeze hardy. Excellent quality fruit matures in October–December with deep orange internal color. The fruit is seedy (0–20 seeds) depending upon the frequency of cross-pollination. Fruit having a greater number of seeds is usually larger in size. Limb breakage is common in Robinson and the fruit does not hold well. In 1963, cv. Page orange was released from progeny of a Minneola tangelo and Clementine cross. Sunburst cv. was released by the USDA in 1982 from a cross of Robinson × Osceola. Tree characteristics are similar to Robinson and are tolerant to freeze damage. Fruit matures in October–November before Dancy. Sunburst ships better than fruit of Dancy and Robinson. Sunburst needs pollinators like Orlando, Nova or Temple for higher fruit yield and produces polyembryonic (PE) seeds (Davies and Albrigo 2006). The Safor cv. triploid hybrid was obtained from embryo rescue of small seeds of a cross between Fortune and Kara mandarin cultivars (2x × 2x) during 1996. The fruit is seedless, having higher soluble solid contents (15.1%) and other fruit quality attributes and is a mid-late season hybrid (Cuenca et al. 2010). Alborea and Albir are two triploid hybrid cultivars developed in the Valencian Institute of Agricultural Research (IVIA) triploid breeding program. Cultivar Alborea was obtained from Fortune; the fruit matures in early January, is easy to peel and the rind is deep orange red in color. Albir cv. was developed from a cross between cvs. Fortune and Kara. The fruit matures in early February and its fruit rind is orange-red in color. The Albir and Safor cvs. could expand the harvest season span from January to March (Cuenca et al. 2015a).

Cultivar Fallglo (Bower × Temple) was released for commercial propagation in 1987. It matures early, from late October to November. Plant growth resembles Temple. These are more freeze sensitive and should not be planted in areas prone to freeze damage. Fallglo is a highly-seeded (30–40) cultivar and seed frequency depends upon cross-pollination. Cultivar Ambersweet was released in 1989 from a cross between hybrid mandarin (Clementine × Orlando) × sweet orange 15-3. It matures early, from October to December. The tree is relatively vigorous and is freeze hardy. Fruit is large in size and the stem end is similar to Temple orange. Juice color is deep orange under Florida climatic conditions. Fruit seediness (seedless to 15 or more

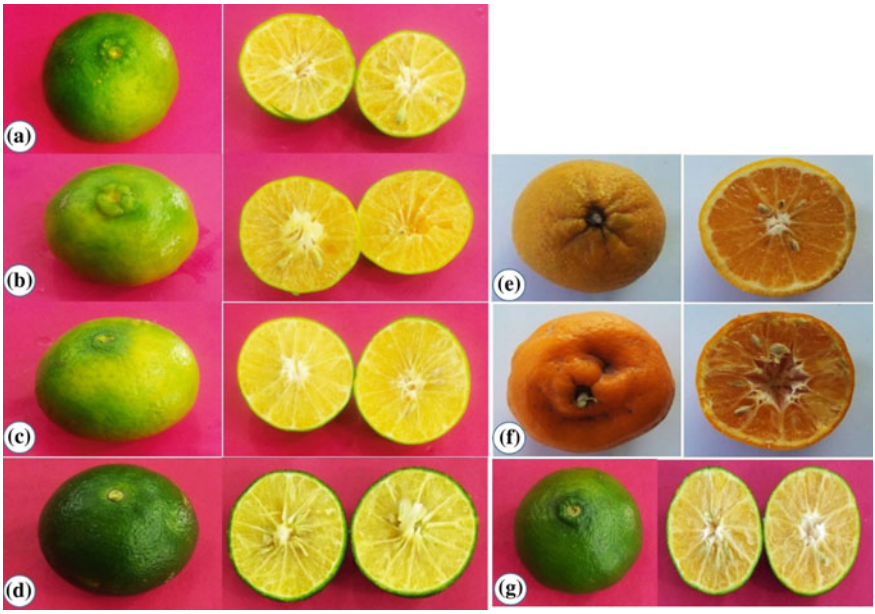

Fig. 13.1 Mandarin germplasm in Pakistan. **a** Dancy, **b** Mangal Singh, **c** Fremont, **d** Wilking, **e** Honey mandarin, **f** Ponkan, **g** Natal Nartjei

seeds) depends upon the frequency of cross-pollination. Seeds have zygotic embryos. Juice quality is similar to oranges with better fruit color. This hybrid cultivar is more utilized as a source of frozen concentrated orange juice. The parentage of cvs. Kinnow and Wilking is the same; however, Kinnow has large-sized tree, vigorous growth and higher tendency towards alternate bearing. It is a late maturing variety, holds well on the tree without puffiness and has smooth skin. The fruit is highly seedy (<30 seeds) with tender segments and highly juicy. Kinnow has been commercially cultivated in Pakistan and India only and never planted commercially in the Mediterranean region. Low seeded Kinnow has been selected as a spontaneous mutant (bud sport) and is now being commercially planted in the Punjab–Pakistan on a large scale.

Most mandarin cultivars and hybrids need higher amounts of cross-pollination for better yields. Selection of the pollinizer depends upon overlaps in blooming periods with a greater number of flowers attractive for the pollinizing agents (bees), self- and cross-compatibility with the main variety, production of quality fruit for marketing, freeze hardiness and resistance to major diseases and pests. Pollination problems have not been reported in Kinnow mandarin under the climatic conditions of the central Punjab and its bearing has been moderate to high compared with cv. Feutrell's Early. Pictures of fruits of a few mandarin varieties available in Pakistan are provided in Figs. 13.1, 13.2 and 13.3. A detailed list of important varieties with their salient traits and location of cultivation is provided in Appendix 2.

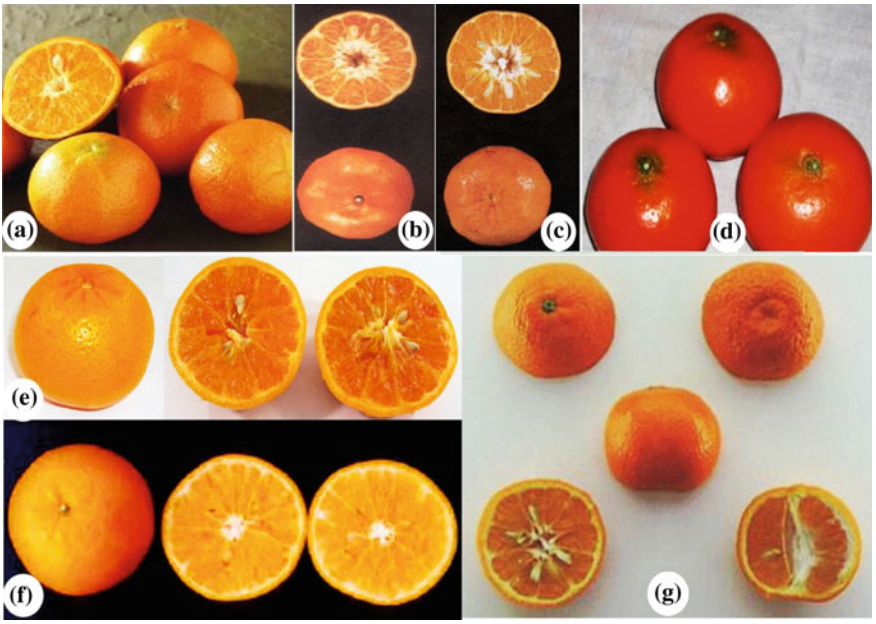

Fig. 13.2 Mandarin germplasm in Pakistan. **a** Fremont (CRI), **b** Dancy (CRI), **c** Robinson, **d** Fairchild, **e** Kinnow seeded, **f** Kinnow seedless, **g** Clementine

13.3.5 Rootstocks

13.3.5.1 Rangpur mandarin (*Citrus reticulata* Hybrid)

This cultivar is tolerant to drought conditions and citrus tristeza virus (CTV), hence it is used as an important rootstock in Brazil. In other citrus-producing areas of the world, it is not a widely planted rootstock. Scions planted on Rangpur grow and produce vigorously like Rough Lemon and higher than other rootstocks including cvs. Trifoliata, Citranges and Cleopatra mandarin (Castle 1987; Davies and Albrigo 2006). It is also tolerant of higher calcareous and saline soil conditions. It has intermediate freeze tolerance and fruit quality compared with Sour Orange and Lemon rootstocks. Rangpur is susceptible to citrus exocortis virus (CEV), foot rot, blight and citrus nematodes. Its utilization as a commercial rootstock is concentrated only in Brazil and it is unlikely to spread to other parts of the citrus world.

13.3.5.2 Cleopatra mandarin (*Citrus reticulata* Blanco)

The Cleopatra variety has not been an important citrus rootstock in the past, however, it has gained some popularity in the last few years. Scions planted on Cleopatra

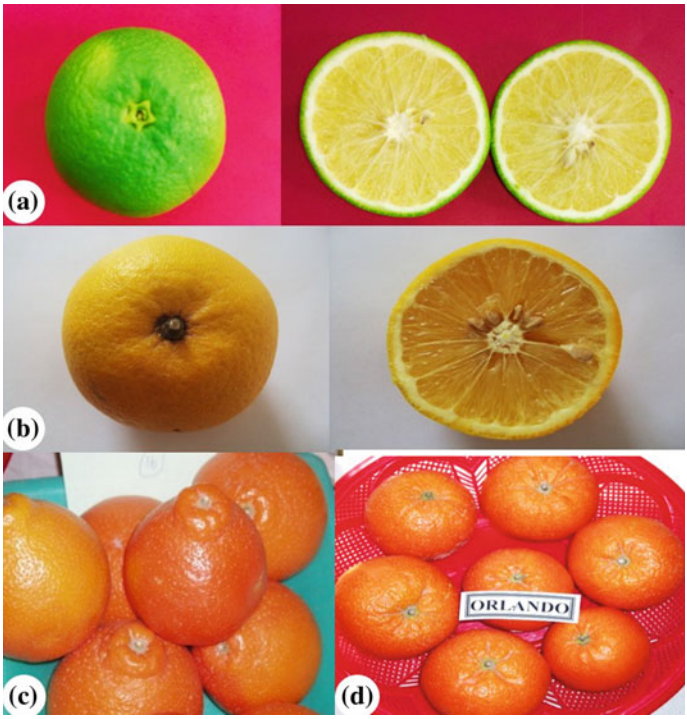

Fig. 13.3 Mandarin hybrid germplasm in Pakistan. **a** Dweet, **b** Pearl, **c** Minneola, **d** Orlando

mandarin rootstock grow vigorously and produce well branched deep root systems enabling the plant to survive under drought conditions. Ruby Red grapefruit grafted on Cleopatra gave fruit yield comparable to plants on sour orange and lesser yield compared with plants on rough lemon. The major drawback in this rootstock has been its long juvenile phase and delayed yield; however, this could be improved by modifying irrigation and fertilization practices. Trees developed on Cleopatra mandarin are equally freeze hardy like *Poncirus trifoliata*, cv. Swingle citrumelo trifoliate hybrid and sour orange and are better than plants developed on cv. Carrizo citrange or rough lemon. Cleopatra is tolerant to many viruses and viroids including CEV, CTV and Xyloporosis. However, it is susceptible to burrowing nematodes and shows moderate tolerance to foot rot caused by Phytophthora. It is also highly susceptible to blight after 10–12 years of age (Davies and Albrigo 2006; Young et al. 1982).

Cultivar Cleopatra is very productive on heavy soils, is tolerant to higher pH and calcareous soils and resistant to salinity. Cleopatra is an excluder of sodium and chloride and hence offers better salt tolerance compared with other citrus species. Fruit size is usually small compared with other commercial rootstocks. Fruit juice quality and TSS is moderately better compared with rough lemon and sour orange (Castle 1987). It is not a recommended rootstock for grapefruit, sweet oranges and

small fruited mandarins including cv. Dancy. It was found as better rootstock for commercial hybrids like cvs. Murcott (Honey), Nova, Orlando, Minneola, Robinson and Sunburst. However, yield of these hybrids was low unless a compatible cultivar was planted (Davies and Albrigo 2006; Hearn and Hutchinson 1977). Cleopatra mandarin rootstock is known to induce low levels of mineral elements including NPK, S, Ca and Cl and higher levels of Mg, Zn and B in the scion (Wutscher 1979). In areas having higher incidence of CTV and blight like Brazil, Cleopatra could be a suitable alternate compared to sour orange which is susceptible to CTV.

Other mandarins and their hybrids tested as rootstocks include cvs. Changsha, Sunki, *Citrus depressa*, Orlando tangelo and Sun Chu Sha Kat. The Hongju cv. known as *red tangerine* is being widely used as a popular rootstock in central China for different sweet orange cultivars. Cultivar Sun Chu Sha Kat is also tolerant to Phytophthora, blight, CTV and to calcium-rich soils. Rangpur × Troyer hybrid rootstock budded with sweet orange scions induced 30–50% dwarfness and fruit yield and quality as compared to plants budded on Poncirus (Castle 1987) indicating suitability of this rootstock for high density plantations. Furthermore, the scions budded on cv. Troyer hybrid started bearing in the second year of planting (Davies and Albrigo 2006). However, this hybrid is susceptible to Phytophthora, blight and sensitive to salinity.

13.4 Germplasm Biodiversity and Conservation

Germplasm collections, identification, characterization and conservation are a primary source of genetic diversity and act as useful tools for plant breeders. Ideally germplasm collections should be made in situ. However, ex situ collections are easy to maintain, have more accessibility for the researchers, and are less vulnerable to threats like diseases and climate change (Garcia-Lor et al. 2017; Krueger and Navarro 2007). Many countries have developed ex situ citrus conservation centers and mostly have named cultivars with little genetic diversity and lacking true representative taxa of the wild relatives (Ford-Lloyd et al. 2011; Krueger and Navarro 2007). Many genebanks have the history and other passport information related to each accession conserved, which other centers might lack. Hence, there is dire need to encourage national and international collaboration and partnerships for the exchange of germplasm to enrich global collections at different regional germplasm repositories and develop multiple backups for better conservation and utilization of the plant material (Hummer et al. 2015).

13.4.1 Germplasm Collections

Globally about 6000 accessions of *Citrus* are maintained, including a mixture of wild species, old and advanced cultivars and different breeding lines as reported

in the first report of the State of the World Plant Genetic Resources for Food and Agriculture, 1996 (http://www.fao.org/3/a-w7324e.pdf). A global citrus network has been established for germplasm collections by merging the ESCORENA Inter-Country Collaboration Network on Citrus Improvement for the Mediterranean Region and the Inter-American Citrus Network (IACNET). The Russian Research Institute of Floriculture and Sub-tropical crops in Sochi, Russia has 132 citrus accessions representing 50 different taxa including cv. Satsuma and other mandarins. The National Clonal Germplasm Repository of Citrus and Date (NCGRCD) of United States Department of Agriculture (USDA) and University of California, Riverside (UCR) maintains 1328 accessions of citrus including 164 mandarin species at NCGRCD and 60 mandarin accessions are maintained at UCR (Volk et al. 2017a). In Russia, breeders have mainly focused on cold hardiness and the germplasm collections offer great diversity to address Russian mandarin production challenges (Kulyan 2014). However, no publically available database of the Russian collections is available except printed catalogs. In the USA, California is known for production of quality mandarins including clementines and tangerines. The plant material is freely available to researchers worldwide. NPGS maintains enormous data of quality related attributes. The INRA-CIRAD citrus germplasm collection center of San Giuliano, Corsica, France has 408 accessions of mandarins (340), tangors and tangelos (68) maintained as ex situ collections. In Italy, CRA-ARC has maintained 548 accessions of citrus having 158 of Italian origin. However, no data about mandarin accessions is available.

Asia's largest citrus collections are at the NARO Institute of Fruit Tree Science, Tsukuba, Japan, maintaining about 1200 collections. In Australia, Commonwealth Scientific and Industrial Research Organization (CSIRO) and various research and university plant collections are maintaining about 500 accessions at different locations. In India, about 627 accessions of citrus are maintained at different germplasm collections centers. Among Asian countries including Pakistan, the West Asia and North Africa Plant Genetic Resources Network (WANANET) has played an important role in strengthening the national plant genetic resource documentation and conservation programs. Indonesia and Thailand have 498 and 585 citrus accessions, respectively. In China, Chongqing citrus nursery maintains 683 accessions of citrus representing 16 different species. In Pakistan, about 150 accessions of citrus including 33 accession of mandarins and mandarin hybrids are being maintained at different research institutes, particularly the Citrus Research Institute (CRI), Sargodha, Horticultural Research Institute (HRI), Sahiwal, Ayub Agriculture Research Institute (AARI), Faisalabad, experimental gardens of the University of Agriculture, Faisalabad (UAF) and Plant Genetic Resource Institute (PGRI), National Agriculture Research Council (NARC), Islamabad. Wild *Citrus reticulata* genotypes are under threat of extinction and the clonal repositories currently available need to be strengthened. The germplasm collections are not satisfactory and have not been utilized for morphological and physico-chemical or genetic characterization and in breeding programs. The genetic diversity in these collections is low for breeding and other crop improvement programs. Hence there is need to accelerate germplasm exchange programs with the regional and other major genetic resource centers in the

leading citrus producing countries. The list of *Citrus* germplasm collection centers or sources available in Pakistan and other important countries is provided with their web links for further information (Table 13.4). A list of mandarin varieties, hybrids and rootstocks available at different research institutes and universities in Pakistan is provided (Table 13.5). A detailed list of important genetic resource centers, institutes and universities working on breeding and biotechnology with their contact is presented in Appendix 1.

13.4.2 Core Germplasm Collections and Biodiversity

In woody perennials like citrus, it is difficult to maintain large germplasm collections. The cost of germplasm collections and maintenance could be reduced by creating core collections (CC) to facilitate utilization of genetic resources taken from germplasm banks (Frankel and Brown 1984; Pessoa-Filho et al. 2010). However, one has to ensure the prevalence of maximum genetic diversity (80%) in the collected and maintained plant material (Balas et al. 2014; Belaj et al. 2012). The size of the core collections should be 15–20% of the genetic resources and for selection of plant material molecular markers can be used to analyze genetic diversity including RAPD (Marita et al. 2000); isoenzymes (Chandra et al. 2002), SSRs (Djè et al. 2000), AFLP (van Treuren et al. 2006) and SNPs (McKhann et al. 2004). The CC should be randomly stratified and different characters including phenotypic traits based on standard descriptors (IPGRI 1999) and genetic structure using molecular markers with agronomic and geographic traits considered. Citrus CC for the mandarin group available at the germplasm collection maintained by IVIA (Moncada, Spain) and INRA-CIRAD (Corsica, France) have been developed using previous as well as new datasets using different molecular markers including 50 SSR markers, 19 indels and 61 SNP markers (Garcia-Lor et al. 2012, 2013; Ollitrault et al. 2012b).

Among different methods used for creating a CC, genetic diversity is analyzed, linkage disequilibrium (LD) noted and the maximum length subtree (MLsubtree30) method was found best due to reduced LD with higher recovery of genetic diversity in a markedly reduced number of samples. Based on the genetic data and phenotypic characterization it was found that 15% of the CC size could be optimal for initial collections. The selected genotypes include hybrids like cvs. Nova, Fortune and others that have exhibited higher introgression from the ancestral species (Garcia-Lor et al. 2015; Wu et al. 2014) including different nuclear and cytoplasmic genomes as described by Garcia-Lor et al. (2015). The ML subtree sampling method may also be applied to other citrus species for developing a CC and to facilitate better management of the genetic resources present in different citrus species at different genetic resource centers.

Table 13.4 List of important citrus genetic resource centers

Countries	Germplasm Resource Centre	Web links
Italy	CRA, Consiglio per La Ricerca e La Sperimentazioni In Agricoltura	http://sito.entecra.it/portale/cra_dati_istituto.php?id=209
China	Fruit Research Institute, Fujian Academy of Agricultural Sciences	http://www.cgris.net/China/CHINA%20ex%20situ.htm
	Citrus Research Institute Chongqing citrus nursery	http://www.caas.cn/en/administration/research_institutes/research_institutes_out_beijing/chongqing/77925.shtml
USA	USDA Germplasm Collection Center for Plant Conservation	http://www.ars.usda.gov/Main
	Citrus variety collections, University of California, Riverside	http://www.citrusvariety.ucr.edu/citrus/mandarins.html
	USDA-ARS National Clonal Germplasm Repository (NCGRCD)	https://www.genesys-pgr.org/wiews/USA129 https://www.ars.usda.gov/pacific-west-area/riverside-ca/national-clonal-germplasm-repository-for-citrus/
Russia	Russian Research Institute of Floriculture and sub-tropical fruit crops (RRIFSC)	https://www.greencom.ru/en/firm_info.html/fID/4277
Japan	NARO Institute of Fruit Tree Science, Tsukuba	https://www.naro.affrc.go.jp/org/fruit/eng/index-e.html
South Africa	Citrus and Subtropical Fruit Research Institute; Agriculture Research Council	http://www.arc.agric.za/arc-itsc/Pages/ARC-ITSC-Homepage.aspx
Spain	Citrus Germplasm Bank, Instituto Valenciano de Investigacions Agrarias, Valencia	http://www.ivia.gva.es/en/banco-de-germoplasma-de-citricos
France	INRA-CIRAD citrus germplasm collection, Corsica	https://urgi.versailles.inra.fr/siregal/siregal/welcome.do
Pakistan	Citrus Research Institute, Sargodha	http://aari.punjab.gov.pk/institutes-sections/citrus-research-institute
	Horticulture Research Institute, Sahiwal	http://aari.punjab.gov.pk/institutes-sections/citrus-research-institute/ciri-allied-stations/citrus-gpu-muhammad-nagar/contacts
	PGRI, National Agriculture Research Center, Islamabad	http://parc.gov.pk/index.php/en/pgrp-facilities
	Institute of Horticultural Sciences, University of Agriculture, Faisalabad (UAF)	http://uaf.edu.pk/faculties/agri/insts/horti_sciences

Table 13.5 List of Mandarin and Mandarin hybrids available at different research institutes/universities in Pakistan

Sr. no.	Varieties	Citrus Research Institute, Sargodha	Horticulture Research Station, Sahiwal	Ayub Agriculture Research Institute, Faisalabad	Institute of Horticultural Sciences, UAF
	Mandarins				
1	Kinnow 2N (seeded)	√	√	√	√
2	Kinnow 4N (seeded)				√
3	Kinnow (low seeded)	√	√	√	√
4	Murrcot	√			
5	Feutrell's Early	√		√	√
6	Dancy	√	√		√
7	Sunburst	√	√		√
8	Daisy	√			√
9	Nagpuri Sangtra (Ponkan)	√			√
10	Honey mandarin	√	√		√
11	Kala Sangtara	√			
12	Kishu	√			
13	Fallglo	√	√		
14	Clemenule	√			
15	Clementine	√			
16	King				√
17	Temple				√
18	Nules				√
	Satsumas				√
1	Satsuma				√
2	Natal Naartjei				√
	Tangerines				
1	Fremont	√			
2	Clementina	√			
3	Fairchild	√			

(continued)

Table 13.5 (continued)

Sr. no.	Varieties	Citrus Research Institute, Sargodha	Horticulture Research Station, Sahiwal	Ayub Agriculture Research Institute, Faisalabad	Institute of Horticultural Sciences, UAF
4	Ambersweet	√			
5	Robinson	√			√
	Tangelos				
1	Minneola	√	√		
2	Pearl	√	√		√
3	Orlando	√	√		
	Tangor				
1	Dweet				√
	Rootstocks				
1	Cleopatra	√			
2	Cox mandarin hybrid	√			√
3	Changsha mandarin				√
4	Sun Chu Sha Kat				√
33	Total accessions	24	9	3	21

13.4.3 Cryopreservation

Cryopreservation is the storage of biological material at ultra low temperature usually in liquid nitrogen (LN) at −196 °C. It ensures cost-effective and long-term safe storage of different types of plant materials (Engelmann 2004). Ex situ collections are at risk of being lost due to environmental or other natural disasters. Storage of plant germplasm in LN is more economical compared to plantations and maintenance of plant material in the field (Reed et al. 2004). USDA-ARS National Plant Germplasm system maintains collections of diverse plant genetic resources in cryotanks containing LN (Volk et al. 2017b; Wang et al. 2014). Shoot tips are used for cryopreservation and are treated with cryoprotectants before storage in LN. Recovery of plant material after long-term storage has not been extensively reported in citrus (Volk et al. 2008). Cryopreservation and successful recovery of plant material has been reported to be 20–70% in different mandarins, tangor and tangelos (Volk et al. 2017b). Cryopreservation technology needs to be worked out in mandarin species for long-term storage and recovery.

13.5 Environmental Components: Flowering, Breeding Behavior and Fruit Setting

Among different environmental components, temperature, photoperiod and water play major roles in regulating timing and intensity of flowering in mandarins. Hence, frequency of flowering varies in different regions having variable climatic conditions. The climatic factors also effect flower types, distribution on the tree, fruit set percentage and yield.

13.5.1 Floral Bud Induction and Differentiation

In citrus, vegetative growth stops in the winter season (rest period) in sub-tropical and dry tropical regions. Shoot growth ceases and root growth decreases when temperature falls below 12 °C and flower bud induction begins (Davenport 1990; Krajewski and Rabe 1995). In Satsuma, flower bud differentiation was observed in late December in Japan (Iwasaki et al. 1959). However, in early November, treatment of trees at a high temperature of 25 °C along with defoliation, initiated flower bud development before morphological differentiation (Nishikawa et al. 2007). Floral induction and bud development require different temperatures and seasons in Satsuma. Flowers were induced in trees grown at 15 °C for 45 days and there was no induction in trees grown at 25 °C (Nishikawa et al. 2007, 2009) indicating a critical role of low temperature in inducing floral buds.

Water stress and cold conditions are the main flower induction and regulating factors. Under subtropical conditions, cold plays a major role while in tropical conditions, water stress is the key factor. Flower bud induction requires several weeks below 25 °C (Inoue 1990) as reported by Nishikawa et al. (2007, 2009) in cv. Satsuma. Similarly, a drought period (4–5 weeks) during winter season is required to induce heavy bloom and flowering intensity is directly proportional to the intensity and duration of water stress (Southwick and Davenport 1986). Hence, one of the main reasons of low production in citrus particularly cv. Kinnow mandarin in Pakistan is intercropping of berseem, wheat and other related crops and heavy flood irrigation of fields during the winter season leading to less flowering and higher flower drop (authors' personal observations).

Water stress is used as a main tool in regulating and forcing bloom in citrus species in many leading citrus-producing countries. Generally, trees bloom 3–4 weeks after heavy irrigation suggesting application of heavy irrigation 3–4 weeks before the onset of spring. Under subtropical conditions, microscopic bud break starts during November–December, macroscopic bud differentiation occurs in December–January and flower bud visibility and anthesis starts in late January–early March. However, there may be considerable variation in these periods due to different climatic conditions in different regions and rapid fluctuations in temperatures and rainfall patterns. Off-season flowering has been commonly observed in different parts of the citrus

world at different times during the winter season. Plants normally have more leafless blooms compared with leafy blooms and later produce more fruit (Erner and Bravdo 1982). More leafless blooms are produced when low winter temperatures prevail for longer durations compared with prevalence of higher winter temperatures that lead to production of leafier blooms.

There are many other factors affecting flowering including nutrition, carbohydrates, hormones, water relations and temperature. Carbohydrate levels in roots have shown a relationship with flowering in mandarins displaying alternate bearing. In Murcott cv., excessive crop load leads to lower carbohydrate levels in roots, lower shoot and less bloom and trees may die, a condition known as *Murcott collapse*. This indicates that a minimum level of carbohydrates must be maintained in plants to sustain tree growth patterns. Similarly, slightly lower leaf N levels promote flowering in citrus and 2.5–2.7% leaf N should be maintained for moderate flowering and higher fruit yield. Low temperature and water stress also enhance leaf ammonia levels and flowering. Plant growth hormones like GA_3 has inhibitory effects on flowering. However, the physiological basis of flowering is poorly understood and needs more extensive studies.

13.5.2 Pollination, Fruit Setting and Drop

Most commercial mandarin cultivars do not need cross-pollination except cvs. Robinson tangerine and Orlando tangelo. Moderately higher temperatures (25–30 °C) enhance bee activity and growth rate of pollen grains through the style ensuring a higher rate of pollination and fruit setting. Early opening flowers have lower fruit set due to the prevalence of lower temperature and less bee activity leading to poor pollination. Higher temperatures (>40 °C) may lead to higher fruit drop. Fruit drop during May–June due to higher temperatures is termed *physiological drop* or *June drop*. This drop is severe when temperatures reach 35–40 °C associated with water stress. Together higher temperature and water stress may cause stomatal closure and a reduction in net CO_2 assimilation leading to more fruit drop.

13.6 Breeding Systems

13.6.1 Polyembryony, Genomics and Candidate Gene (s)

Asexual commercial propagation of fruit crops ensures clonal multiplication of heterozygous and hybrid plant material, thus avoiding the uncertainties of sexual reproduction. The Dutch scientist Antonie van Leeuwenhoek first described the formation of two plantlets from a single citrus seed, polyembryony, in 1719 (Aleza et al. 2010b; Batygina and Vinogradova 2007). The formation of nucellar embryos from

the nucellar tissue was named as *nucellar* or *adventive embryony* (Strasburger 1878). Polyembryony has two main types on the basis of cellular origin of embryogenesis including sporophytic and gametophytic polyembryony. Sporophytic polyembryony comprises development of embryos from nucellar tissue, integuments, endosperm and monozygotic cleavage (Batygina and Vinogradova 2007). However, sporophytic apomixis is still not very well studied. Different forms of polyembryony except the latter two are associated with apomixis (Savidan 2000) and citrus species exhibit both apomictic (polyembryony) and non-apomictic (monoembryony) behavior. However, the nature of apomixis in citrus is very stable. Apomixis enables fruit breeders to fix heterozygosity and other valuable traits (Bicknell and Koltunow 2004; Conner et al. 2015). Most citrus genotypes are apomictic in nature, except pummelo, citron, cv. Clementine and some mandarin hybrids (Aleza et al. 2011), due to the higher probability of apomictic lines to be selected and maintained during early selection and domestication process. In non-apomictic genotypes, seeds usually have a single zygotic embryo while in apomictic genotypes seeds have one zygotic and several nucellar embryos. Hence, non-apomictic 4x genotypes should be selected for interploid hybridization (4x × 2x) for triploid breeding production (Aleza et al. 2009).

Mandarins also exhibit higher frequency of nucellar embryony while some cultivars are monoembryonic e.g., cvs. King and Temple produce only zygotic seedlings. Cultivars of Clementine and some mandarin hybrids are non-apomictic in nature and do not produce nucellar embryos. These genotypes normally have only one zygotic embryo; however, a few more embryos may develop from two or more embryo sacs present in a single ovule or by fusion of a fertilized egg (Frost and Soost 1968). Frost (1938) reported production of morphologically similar individuals from hybrids which may be caused by cleavage of sexual embryos. Non-apomictic genotypes have shown consistent development of multiple small embryos by cleavage in partially-developed seeds of cvs Clementine and Iyokan mandarin (2x) when pollinated with cv. Kawano natsudaidae (4x) as reported by Oiyama and Kobayashi (1990) and in other triploid breeding programs (Aleza et al. 2010a). The additional zygotic embryos originated from cleavage of the original zygotic embryo when verified using isoenzymes (Oiyama and Kobayashi 1990). However, polyembryony may also originate from the existence of multiple functional embryo sacs in a single ovule (Bacchi 1943; Frost and Soost 1968). The other method of nucellar embryo production is in vitro culture of nucellus tissue. This technique has been widely used to produce pathogen-free plants in non-apomictic genotypes (Esan 1973) including Clementine varieties (Navarro et al. 1985). However, embryos developed from nucellus culture in vitro may have genetic variation and difference in their origin.

The hypothesis of Oiyama and Kobayashi (1990) was later confirmed by the findings of Aleza et al. (2010a) in non-apomictic citrus genotypes that the multiple embryos formed in the underdeveloped seeds in interploid crossing (2x × 4x) were genetically identical, sexual hybrids that have resulted from cleavage of the original zygotic embryo than the presence of multiple embryo sacs in a single ovule (Bacchi 1943; Frost and Soost 1968). Furthermore, the multiple embryo development in underdeveloped seeds is not affected by the embryo ploidy or sequence of the parental

genotypes. Rather, it is the outcome of the ploidy of the male parent and its access to the embryo or endosperm.

Parent-of-origin effect may also generate phenotypes depending upon direction of the cross. Such an effect is contributed by different genetic mechanisms including variable contributions of the mother parent to the endosperm affecting embryo: endosperm ploidy ratio, cytoplasmic inheritance, variable gene expression in gametes and parental alleles during the seed development (Dilkes and Comai 2004). All plants developed from the nucellus culture of cvs. Clementine and Fortune mandarins in vitro were found to be of zygotic origin than nucellus tissue indicating no embryogenic capacity in the nucellus cells/tissue of these varieties (Aleza et al. 2010a) and similar results could be found in other non-apomictic genotypes. The presence of the endogenous hormones like IAA, ABA and gibberellins could also regulate the potential of embryogenesis (Tisserat and Murashige 1977).

It is evident that there is no need to recover a greater number of embryos from the underdeveloped seeds in interploid crosses as they are more likely to have the same zygotic origin. Thus, the workload of breeders could be reduced and cost-effectiveness of the breeding programs could be increased. True-to-type and pathogen-free plants could not be developed using nucellus culture in vitro in non-apomictic citrus genotypes, rather, shoot tip grafting (STG) may be preferred (Navarro and Juárez 2007). The maternal or paternal parentage of the progeny could be confirmed later using suitable molecular markers.

13.6.1.1 Candidate Gene Controlling Polyembryony

The crossing of PE and ME cultivars revealed that one or two dominant loci are involved in controlling polyembryony and molecular markers linked to this trait has been identified (Kepiro and Roose 2010; Raga et al. 2012). The locus of the genomic region that has been mapped spans 380 kb (Nakano et al. 2012). Further fine mapping of polyembryony locus in 45 PE and 63 ME accessions in citrus including mandarins have revealed polyembryony locus as an 80 kb region on chromosome 4 containing 11 genes (Wang et al. 2017) embedded in a 380 kb region as reported previously (Nakano et al. 2012). The expression analysis of selected candidate genes revealed hyper expression of *Cg4g018970* in ovules of PE citrus cultivars and no expression in ME cultivars. The *Cg4g018970* encodes for RWP-RK domain, referred as *CitRWP*, containing a similar protein that regulates egg cell related genes in *Arabidopsis* (Koszegi et al. 2011). Four SNPs and insertion of miniature inverted repeat TE (MITE) were identified in the promotor region in chromosome four in the pummelo genome during sequence alignment of *CitRWP* of PE and ME citrus genotypes. The absence of MITE insertion in all the ME genotypes confirmed its association with the PE loci in all PE citrus genotypes (Wang et al. 2017). Hence, *CitRWP* has been identified as the key candidate gene that controls the polyembryony mechanism. Knockout studies could help to assess further contributions of this gene in regulating apomixis and related phenomenon in *Citrus* species.

13.6.2 Polyploids, Significance and Recent Developments

Polyploidization has been an important phenomenon during plant speciation and the angiosperm evolutionary process (Gallais 2003) and its exploitation has proved to be a very useful tool in citrus breeding programs (Cuenca et al. 2015b; Ollitrault et al. 2008a). Polyploids including triploids (2n = 3x = 27) and tetraploids (2n = 4x = 36) have been reported in citrus (Lee 1988). For a long time, chromosome doubling has been considered as the major source of polyploids and the role of meiotic restitution during the evolutional process has been nominal (Stebbins 1971). In contrast, Harlan and De Wet (1975) claimed that all polyploids arise from the meiotic restitution mechanism. The first polyploid was reported in the last century by Strasburger (1910); however, the genetic and genomic basis of the polyploidization and its role in the evolutionary history of citrus and other crops is still being investigated (De Storme and Geelen 2013).

13.6.2.1 Mechanisms of Polyploid Development and Role of Unreduced Gametes

Polyploids are formed by two major mechanisms in nature. First, by somatic doubling of the chromosomes known as *somatic polyploidization* where chromosomal restitution takes place during mitosis resulting in a cell having double the number of chromosomes (Carputo et al. 2003). Second, polyploids are developed by nuclear division restitution during meiosis leading to the formation of unreduced male and female gametes called *sexual polyploidization* (De Storme and Geelen 2013). Sexual polyploidization further involves three different mechanisms of meiotic restitution including *pre-meiotic*, *post-meiotic genome doubling* and *meiotic restitution*. The first two mechanisms are not frequently observed, compared with meiotic restitution which is commonly found and has been the main source of production of unreduced (2n) gametes. The production of unreduced gametes leading to spontaneous production of polyploids has been reported in many citrus species (Cuenca et al. 2015b; Esen and Soost 1971; Fatima et al. 2010) including mandarins (Fatima et al. 2015; Usman et al. 2006).

First division restitution (FDR) is produced by the occurrence of equational mitosis in the first mitotic division of chromosomes and non-sister chromatids will be included in the same gamete leading to unreduced gamete formation. Second division restitution (SDR) occurs by meiotic errors in the second meiotic division leading to the collection of sister chromatids in the same gamete (Cuenca et al. 2011; Park et al. 2007; Rouiss et al. 2017). These natural mechanisms are also the major source of diversity in gametic population resulting in diverse breeding progenies. Better understanding of these mechanisms could open new horizons to the application of this knowledge to citrus breeding programs.

Seedlessness is one of the major traits in mandarin breeding which can be obtained through interploid crossing which requires tetraploids (Aleza et al. 2012; Fatima et al.

2002; Grosser and Gmitter 2011; Usman et al. 2002). There are mainly two strategies for the production of triploids, either using sexual hybridization for polyploidization using 2n gametes or using tetraploids (doubled diploids) in interploid hybridization with diploids (Aleza et al. 2012). Production of unreduced gametes, a rare mechanism in citrus (about 2%), leads to the development of triploids in 2x × 2x crosses in cv. Clementine through the SDR mechanism (Aleza et al. 2010a; Cuenca et al. 2015b; Luro et al. 2004). However, Honsho et al. (2012, 2016) in mandarin cv. Nishiuchi Konatsu and Navarro et al. (2015) in 4x × 2x interploid hybridization in cvs. Clementine and sweet orange (CSO tangor), have revealed FDR as the major mechanism of 2n gamete formation and a relatively higher percentage of 2n gamete production in CSO tangor.

Cytogenetic studies have been handicapped in citrus due to the small size of the chromosomes (Barba-Gozalenz et al. 2005; Fatima et al. 2015). Molecular markers have been very useful in the identification and estimation of the parental heterozygosity restitution (PHR) by unreduced gamete production in the polyploid populations (Cuenca et al. 2011, 2015b; Luro et al. 2004). Molecular markers with low PHR (<50%) indicate the SDR-based origin of the polyploid progeny (Park et al. 2007). The centromeric position on the chromosome could be identified using half-tetrad analysis (HTA) which also helps in the estimation of both SDR and FDR (Tavoletti et al. 1996). Determination of marker centromere distance demonstrates the probability of 2n gamete production as homozygous or heterozygous. In case of a crossover event between centromere and the concerned locus, all SDR 2n gametes will be homozygous while FDR 2n gametes will be 50% homozygous and 50% heterozygous (Park et al. 2007). However, recent studies have revealed coexistence of both FDR and SDR mechanisms in the same genotype in citrus with SDR as the main mechanism of 2n gamete production in mandarins (Cuenca et al. 2015b; Honsho et al. 2016; Rouiss et al. 2017). Pollen size also correlates with its ploidy as the largest pollen grains are more likely to be FDR diploids compared with small-sized pollen.

Rouiss et al. (2017) reported production of tetraploid progenies from 4x × 2x sexual hybridization by unreduced male gametes of CSO tangor while previously such cases were reported as an outcome of selfing of tetraploid female parent (Aleza et al. 2012). Luro et al. (2004) reported the occurrence of few triploid plants from unreduced male gamete produced in cvs. Ananas, Hansen and King mandarins later confirmed by Cuenca et al. (2015b). Embryo endosperm ploidy ratio or genomic balance play critical roles in normal seed development (Johnston et al. 1980; Rouiss et al. 2017). During diploid × diploid hybridization 2/3 embryo endosperm ratio was more favorable compared with ¾ or other similar ratios. In tetraploid × diploid (4x × 2x) crosses the ploidy ratio was normally 4/6 (equivalent to 2/3 ratio) leading to normal seed development (Rouiss et al. 2017) compared with its reciprocal cross (2x × 4x). Hence, 4x × 2x crosses are more favorable for the determination of production of unreduced pollens (2n) leading to normal seed development having tetraploid embryos.

Citrus have been used as a model genus for studying meiotic behavior, the mechanism of unreduced gamete formation and ploidy manipulations (Aleza et al. 2009,

2016; Cuenca et al. 2015b; Rouiss et al. 2017). Recent advances in the genomics and cytogenetic studies have enhanced efficiency of citrus breeding programs. Understanding the production mechanism of the unreduced gametes to enhance their production frequency could further improve ploidy-based breeding programs. Both SDR and FDR gametes have advantages in polyploid breeding (Hutten et al. 1994). Production of FDR-2n gametes (heterozygous) will maintain parental heterozygosity followed by interploid hybridization with a doubled diploid (tetraploid) of the parent of interest. Production of SDR-2n gametes will help to create a large number of genetic recombinations leading to production of higher polymorphic progenies (Aleza et al. 2016; Rouiss et al. 2017) which may be used for interploid hybridization for triploid breeding program. These progenies will have more gametic diversity and higher heterosis due to more heterozygosity in the centromeric area.

13.6.2.2 Development of Polyploids Using Mitotic Inhibitors

Spontaneous polyploids produced through nucellar embryony (apomixis) are the result of simple somatic doubling in the nucellar cells (Aleza et al. 2011). Use of antimitotic chemicals including colchicine and oryzalin have produced tetraploid plants through somatic doubling in different citrus species including mandarins (Aleza et al. 2009; Fatima et al. 2015; Zhang et al. 2007). In citrus, tetraploidization has been routinely induced by colchicine treatment of vegetative buds in vivo (Wakana et al. 2005); however, this method may lead to several cytochimeras which need further screening and multiplication cycles. Colchicine treated calli developed from ovules could be used for embryogenesis in vitro and tetraploids have been developed in apomictic citrus genotypes (Zhang et al. 2007); however, this method involves a long juvenile phase and is time consuming.

Colchicine treatment and shoot-tip grafting (STG) techniques were developed for the production of disease-free plants in citrus (Navarro and Juárez 2007; Oiyama and Okudai 1986). To avoid cytochimeras and for complete tetraploidization, the initial bud size should be at least 0.5 mm. For successful tetraploidization, there is need to optimize colchicine dose and treatment time. The number of developed shoot tips are lower in colchicine-treated plants of mandarin cultivars compared with untreated control. In Clemenules cv. Clementine shoot tip immersion in colchicine solution (0.1%) effectively developed 37% shoots while increase in the dose or treatment time was found to be lethal. Hence, 0.1% concentration could be better for shoot-tip treatment in citrus. Micrografting after colchicine treatment produced 33% mixoploids (2x–4x). Direct colchicine application on the micrografted shoot tip was better compared with apical immersion of the shoot tip before grafting (Aleza et al. 2009). Oryzalin is relatively less toxic to colchicine and may be used for genotypes which are more susceptible to colchicine, although regeneration has been better on colchicine (Wu and Mooney 2002). Among the genotypes used, cvs. Clemenules and Fina were found less susceptible to colchicine compared with cv. Moncada. Flow cytometery could help in detection of stable tetraploid plants after screening and vegetative propagation of positive shoots. Triploids of all the three mandarin cultivars

were obtained in 4x × 2x crosses and about 3200 triploids were obtained in 3 years with an efficiency of 6.1 triploids per fruit (Aleza et al. 2009). This highly efficient method of producing triploids at a large scale in non-apomictic citrus genotypes could also be used in other fruit crops.

Among scion cultivars, tetraploids have been selected in mandarins including Anna, Kara, Tradivo di Ciaculli and Willowleaf, tangors (Murcott, Ortanique, Dweet and Nadorcott) and tangelos (Fairchild, Sunburst, Page, Orlando and Minneola) by IVIA in Spain (Aleza et al. 2011) and in Kinnow mandarin and Feutrell's Early by IHS, UAF, Pakistan (Fatima et al. 2015). The tetraploids are included as male parents in the breeding programs for the development of triploids. In 2x × 4x crosses, triploids obtained due to SDR-2n gametes will have higher frequency of having phenotypic traits of the parent producing 2n gamete compared with the parent in 2x × 2x crosses. However, triploids may have prolonged juvenile period and long thorns (Aleza et al. 2012).

13.6.2.3 Polyploid Rootstocks

Tetraploid rootstocks have higher tolerance to salt and water stress as compared to diploids due to modified abscisic acid synthesis (Allario et al. 2009; Saleh et al. 2008). Tetraploid rootstocks also induce dwarfism which is a highly desired trait in citrus orchards (Lee 1988). Development of autotetraploids or doubled diploids will be of high interest in rootstocks for better resistance against biotic and abiotic stress conditions without modifying the allelic composition. Tetraploid lines of cv. Cleopatra mandarin have been selected for their potential use in rootstock breeding and crop improvement program under abiotic stress (Aleza et al. 2011).

13.6.2.4 Temperature and Polyploidization

It has been postulated that low temperature may affect polyploidization in citrus and other crop species (Ramsey and Shemske 1998). Frequency of polyploidization has been more at higher elevations in the NH which may be attributed to their higher adaptability to low temperatures and extreme climates compared with diploids (Stebbins 1950) and higher heterozygosity (Johnson et al. 1965). The higher frequency of polyploidization in colder climates has also been favored by the presence of apomixis (Horandl 2006). Short vegetative periods in colder climates favor early seed development and maturity. The presence of apomixis ensures higher seed setting in the absence of pollinators and cold environment reduces pressure of predators and biotic stress. These conditions collectively favor a higher incidence of tetraploidization supplemented with gametophytic apomixis. Gene expression modifications reported in allopolyploids (Flagel and Wendel 2010; Wang et al. 2006) explain the reason for production of new phenotypes in allopolyploids with higher adaptability (Osborn et al. 2003). Major differences have been observed in the rate of tetraploidization under greenhouse and open-field conditions. The presence of one tetraploid 8 embryo

per 100 seeds was reported in cv. Tardivo de Ciaculli mandarin compared with 1 in 42 seeds under greenhouse conditions (Aleza et al. 2011); this discrepancy needs further investigation.

13.6.3 Haploids and Double Haploids in Breeding and Genomics

Haploidization followed by the development of double haploids (DH) through gametic embryogenesis and the application of antimitotic agents like colchicine, is a very useful and efficient tool. The phenomenon of DH shortens the breeding cycle by avoiding several generations of selfing to enhance homozygosity in classical breeding. Hybridization is handicapped in citrus by the prevalence of self-incompatibility, high heterozygosity and long juvenility (Fatima et al. 2015; Germanà 2011; Seguì-Simarro 2010). Recent advancements in the omics approaches, particularly structural genomics, have increased interest in development and utilization of haploids and DH.

13.6.3.1 Androgenesis

Haploids, double haploids, trihaploids and aneuploids have been obtained most commonly in citrus using anther culture or isolated pollen culture (Chiancone et al. 2015; Germanà 2003, 2007; Germanà et al. 2000, 2005). Isolated microspore culture has advantages over anther culture including avoidance of regeneration from somatic tissues of the anther wall. The haploid induction through the male gametophyte could further help in early genotype selection for desirable alleles available in pollen. Several factors are involved in inducing regeneration response in the immature gametes including donor genotype, growth condition and physiological status, gametic developmental stage, duration of pre-treatment shock to floral buds, media composition, physical growth conditions and interaction of these factors (Chiancone et al. 2015; Germanà 2011; Wang et al. 2000). In cv. Clementine the isolated microspore culture progress has been slow and it required 5 months for the development of callus tissue and embryogenesis, which continued for 11 months. The addition of meta-Topolin (mT) in replacement of BA and Zeatin showed embryo production in cv. Nules and the response was highly genotype dependent. The effect of mT upon embryogenesis could be attributed to its anti-senescence and growth stimulation activity as reported in polyamines which also improved callogenesis in anther culture in cv. Clementine (Chiancone et al. 2006, 2015). Despite several studies, there is still no single standard method available to obtain efficient plant regeneration from microspore culture. However, further investigation of the effect of anti-senescence substances in the induction of microspore embryogenesis could be quite helpful to enhance embryogenic efficiency in elite mandarin cultivars.

13.6.3.2 Gynogenesis and Induced Parthenogensis

Gynogenesis has also been used for the development of haploids and spontaneous DH in *Citrus* in interploid crosses (Germanà and Chiancone 2001; Toolapong et al. 1996). In vitro pollination of pistils of cv. Clementine with pollens of triploid cv. Oroblanco was used to develop haploid Clementine (Germanà and Chiancone 2001). Gynogenesis have also been induced using irradiated pollen grains and haploids have been reported in different citrus species. Haploid plants of cvs. Fortune, Ellendale tangor and Clementine were obtained using induced parthenogenesis by gamma rays of 150–900 Gy (Froelicher et al. 2007). However, there is need to enhance efficiency of this technology and to improve rate of plant recovery. Haploids were also obtained in cv. Clementine lines using irradiated pollens and recovered plants through embryo germination. The recovered plants bloomed after 4 years (Aleza et al. 2009). Spontaneous occurrence of a DH has also been reported. Seven haploids were recovered using irradiated pollens (250 Gy) and embryo culture in cv. Wilking (Jedidi et al. 2015). Normally haploids obtained through the different techniques discussed above show recovery of weak plantlets with low vigor and a high plant mortality rate during and after recovery, a subject which needs to be addressed.

13.6.3.3 Significance of Haploids

Development of haploids and DH offer several advantages as these could be used as reference material in genomics (Forster et al. 2007; Talón and Gmitter 2008), in physical and genetic mapping and their subsequent integration (Aleza et al. 2015; Chu et al. 2008; Hussain et al. 2007; Zhang et al. 2008). Such highly precise genetic information could be helpful in identification and labelling of candidate genes (Kunzel et al. 2000; Wang et al. 2001). In mutation breeding and genetic transformation ventures, haploids and DH are more desirable owing to production of homozygous lines in one step. These will also be better future candidates in whole genome sequencing (WGS) projects. Plant material having higher polymorphism adds to complications in WGS projects. Most of the commercial citrus cultivars are highly heterozygous; hence, the development of haploid and DH material could be the first priority. Among citrus, cv. Clementine was selected as a reference species due to its established protocol for the regeneration of haploids in vitro (Aleza et al. 2009), availability of a large number of SSRs and expressed sequence tags (ESTs), developed microarrays, BAC libraries and genetic maps (Luro et al. 2008; Martinez-Godoy et al. 2008; Ollitrault et al. 2008b; Terol et al. 2008). There is also need to establish in vitro haploid regeneration protocols for other commercially-important mandarin cultivars. These haploids could be further used for triploid breeding by somatic hybridization and for genomic studies.

13.6.4 Mutation Breeding

13.6.4.1 Spontaneous Mutations, Seedlessness and Parthenocarpy

Spontaneous mutations have played a vital role in recovery and development of new and seedless cultivars. These mutations occur frequently in mandarins like in other citrus species and expressed as limb sports, variants in nucellar seedlings or budded progeny. It is difficult to distinguish mutations with sexual seedlings. Several spontaneous mutants have been reported in cv. Satsuma mandarin including changes in rind color, fruit maturity time and seedlessness. Seedlessness has been one of the most desired characters in mandarins. Seedless cultivars usually have no seeds, aborted seeds or a few underdeveloped seeds. Fruit having less than five seeds is considered seedless (Varoquaux et al. 2000; Zhang et al. 2017). Seedlessness has been genotype dependent and in the absence of cross-compatible genotype pollination there may be no fruit set. Furthermore, the role of growth hormones is also documented in regulating fruit set in seedless cultivars. Seedless cultivars from the bud sports are reported in cvs. Satsuma and Clementine (Bono et al. 1996; Malidzan et al. 2004; Russo 2004). Ougan cv. mandarin seedless variant was recovered from a bud sport mutation in 1996 and retained all horticultural traits similar to the wild type parent. Seedless cultivars may have specific environmental requirements for the production of seeds and produce relatively low yields compared with their seedy parents (Bernier and Berna 2001). No significant differences were observed in fruit quality attributes in low seeded cv. Kinnow mandarin; however, the fruit was relatively small and lighter in weight compared with the seedy cultivar (Altaf et al. 2014). Seedless spontaneous mutant cv. Belelate, selected from cv. Owari satsuma, has been patented (Ano 2015). An overview of mandarin varieties showing compatibility, male sterility and parthenocarpic behavior is presented in Table 13.6.

Parthenocarpy and Self-incompatibility

Parthenocarpy has been a key requirement for the production of seedless fruits and may be coupled with self- and cross-incompatibility and male sterility to ensure development of seedlessness. In the case of self-incompatibility, isolation is required to avoid cross-pollinators. Most of the self-incompatible mandarin cultivars may show some parthenocarpy in the absence of pollinators or when cultivated in isolation, resulting in seedlessness (Chao et al. 2005). Alternatively, the self-incompatible cultivars may also develop seeded fruit if the given isolation is less than 9–10 km and foreign pollinators may contaminate. Self-incompatible cv. Clemenules planted at least 9 km away from Clementine fields in Valencia, Spain, resulted in the development of seedless fruit (Vardi et al. 2008). However, this approach is incompatible with current intensive citrus farming practices where growers are planting at higher densities.

Table 13.6 Mandarin and mandarin hybrids showing sexual compatibility, male sterility and parthenocarpy

Varieties	Compatibility		Male sterility		Parthenocarpy	References
	Self-comp.	Self-incomp.	CMS	GMS		
Satsuma	–	√	√	–	√	Yamamoto et al. (1992)
Encore (*Citrus nobilis* Andrews × *C. deliciosa* Ten.)	–	–	√	–	√	Yamamoto et al. (1997)
Ougan (*C. suavissima*)	–	√	–	–	√	Hu et al. (2007)
Clementine	–	√	–	√	√	Vardi et al. (2008)
Ellendale	–	√	–	√	√	
Edit	–	√	–	√	√	
Nova	–	√	–	√	√	
Michal	–	√	–	√	√	
Niva	√	–	–	–	√	
Shani	√	–	–	–	√	
Trovita	√	–	–	–	√	
Wilking	√	–	–	–	√	
Moncalina	–	–	–	√	√	Bermejo et al. (2015)
Kunenbo (*C. nobilis* Lour. var. kunep Tanaka)	–	√	–	–	√	Zhou et al. (2018)
Iyokan (*C. grandis* × *C. nobilis*) × *C. tangerina*	–	–	–	–	√	
Okitsu wase satsuma	–	√	√	–	√	
Kiyomi satsuma	–	√	√	–	√	

Male Sterility and Parthenocarpy

Breeders have developed cv. Satsuma mandarin which produces completely seedless fruit due to parthenocarpy and female sterility (Iwamasa 1976). The degree of male sterility could indicate the frequency of occurrence of seedlessness in different cultivars. Univalents are occasionally found during meiosis (Iwamasa 1976) and cytoplasmic male sterility (CMS) was found in cv. Satsuma mandarin (Yamamoto et al. 1997). A recessive gene is responsible for the occurrence of meiotic asynapsis, pollen and ovule abortion and parthenocarpy in inbred progeny of cv. Wilking mandarin (Spiegel Roy and Vardi 1981). Cytogenetic studies of natural seedless cultivars

revealed meiotic disruption due to chromosomal aberrations which have contributed to ovule abortion (Gmitter 1993). The analysis of segregating parthenocarpic populations in cv. Satsuma revealed that parthenocarpy is encoded by two dominant genes found in a heterozygous state (Vardi et al. 2008). A male sterility gene coding for fatty acyl coA reductase (lipid metabolism) has been identified in seedless cv. Ponkan mandarin hybrids with higher transcript levels compared with the seedy progenitors (Qiu et al. 2012).

Genetic Background of Seedlessness

In seedless and seedy fruit, different set of genes may be involved in controlling seedlessness; however, few genes may be in common in these genotypes. Venn diagram analysis depicted sharing of 48 genes with differences in transcriptional abundance (GDTA) at three different time points in seedless and seedy varieties of mandarin cv. Fallglo and 14 genes showed similar abundance ratios. The suppression of AtGA2ox8 homolog in citrus which is involved in gibberellin degradation indicated that seedless genotypes may have higher levels of endogenous GA leading to seedless fruit development (Zhang et al. 2017) as reported in cv. Fino Clementine. Furthermore, the transcript abundance of LOX2 gene homologue (lipoxygenases) was noted which was involved in the production of growth hormones including abscisic acid (ABA) and jasmonates. Balancing GA and ABA is known to regulate the fruit development in seedless mandarin varieties (Garciapapi and Garciamartinez 1984). Downregulation of UDP- glucosyltransferase and GA2ox8 and upregulation of PGP13 and LOX2 at early fruit development may affect the hormonal metabolism in the seedless varieties leading to parthenocarpic development (Zhang et al. 2017). Some transcription factors may also be involved in different citrus genotypes as ANAC102 was found downregulated in seedless cv. Ponkan during early fruit development (Fang et al. 2016). Genes coding for cysteine protease and aspartic protease were also involved in seedless fruit development. The gene expression changes in seedy and seedless varieties and transcriptional studies have revealed several interrelated processes including initiation of ovule abortion, degradation and loss of endogenous hormones and disrupted seed development leading to loss of ovules, thereby reducing fruit development (Zhang et al. 2017). These studies indicate a set of genes involved in seedless fruit development and open up new horizons for further extensive gene expression and function studies for better insights into the mechanism of seedlessness.

13.6.4.2 Induced Mutations

Induced mutations using physical mutagens like gamma rays and x-rays were reported in 1935 (Hensz 1971). In early growth stages, it is difficult to discern mutations due to higher frequency of nucellar embryony, spontaneous mutations and natural sterility. Budwood irradiation is commonly practiced in mandarins for induc-

ing seedlessness which is mainly caused by chromosomal aberrations (translocations and inversions) leading to higher abortion frequency of male and female gametes. Generally, vegetative buds are less tolerant (LD_{50} = 0.05–0.09 Gy) to gamma irradiation compared with seeds (LD_{50} = 0.01–0.15 Gy). The resulting buds are grafted on rootstocks in the field or cultured in vitro to develop multiple shoots and propagated. The seeds can also be either sown in vivo or cultured in vitro for further multiplications. Induced mutation is a very useful tool to develop new seedless cultivars; examples include Orri-seedless Orah and Morria-seedless Murcott. A few other irradiated mandarin cultivars exhibiting seedlessness include irradiated Or, King and Michal (Fig. 13.4; Goldenberg et al. 2014). Among physical mutagens, gamma irradiation has been most widely used to induce mutations in mandarins and develop seedlessness. An overview of mutation breeding in mandarins and other related species is provided in Table 13.7. Some newly released irradiated seedless mandarin varieties form the University of California breeding program include Tango a selection of Murcott, DaisySL, FairchildSL, KinnowSL, Encore IR6, Fremont IR2 and NovaSL. The mutants recently patented include N40 W-6-3, mutant of Snack tangerine (Grosser 2017), ODEM a mutant hybrid of Orah × Shani (Carmi et al. 2014) and US Early Pride a mutant of Fallglo (McCollum and Hearn 2013).

The effects of seedlessness and irradiation on fruit quality are still under investigation. In some cases, gamma irradiation has also reduced fruit size along with inducing seedlessness (Goldenberg et al. 2014). Hence, it is suggested that for induced mutation, priority may be given to already known low-seeded cultivars, having better fruit size and quality to have a minimal effect of irradiation on other fruit quality parameters. This is particularly important for mandarin varieties with small fruit size. However, due to the random nature of the induced mutations, chromosome damage may lead to undesirable traits and it may be necessary to screen large populations in the field involving considerable time, capital, manpower and space.

13.7 Hybridization Strategies

13.7.1 Conventional Hybridization

Mandarin breeding has relied on conventional hybridization strategies for long time, which is a time-consuming approach. Parent selection for breeding programs involves consideration of the following points. Zygotic cultivars should be preferred as seed parents while for rootstock breeding, at least one parent should possess nucellar embryony. The progeny of both ME parents should be ME. If ME parents are not available or unsuitable, slightly PE parents may be used. Selfing is a useful tool as most of the mandarin cultivars are highly heterozygous; however, it will lead to inbreeding depression and provide weak and inferior progeny. Furthermore, it may be handicapped by self-incompatibility in some cases. A few undesirable dominant characters include small fruit size, high number of seeds and pale fruit color devel-

Table 13.7 Mutation breeding in mandarins and mandarin hybrids including mutagens used and traits focused

Variety (Parent)	Mutagens	Trait (s) altered/studied	References
Kinnow mandarin	Colchicine	Morphology and plant growth	Khan et al. (1992)
Orri-seedless (Orah) Moria-seedless (Murcott)	Gamma rays	Seedlessness	Speigel Roy and Vardi (1992)
Clementines, Tangor, grapefruit	Colchicine	Tetraploidy	Wu and Mooney (2002)
Mandarins	Gamma irradiated pollen of cv. Meyer lemon	Seed setting for haploidy	Froelicher et al. (2007)
Tangor (mandarin × sweet orange)	Colchicine	Tetraploidy	Latado et al. (2007)
Kinnow mandarin	Gamma rays	Radiation sensitivity assessment	Altaf and Khan (2008)
Kinnow mandarin	Colchicine	Tetraploidy and In vitro regeneration	Usman et al. (2008)
Mandarin (*Citrus reticulata*) and pummelo (*C. grandis*)	Gamma rays	Seedleesness	Somsri et al. (2009)
Tangerine and pummelo	Gamma rays	Seedlessness	Somsri et al (2009)
Clementine	Gamma rays	Seedlessness	Majd et al. (2009)
Clementine hybrids	Colchicine	Tetraploidy	Aleza et al. (2009)
Ponkan Mandarin	Colchicine	Tetraploidy	Dutt et al. (2010)
Hongju 418 Hongju 420 Huegan 9-12-1 Zhongyu 7 Zhongyu 8	Gamma rays	Seedlessness	Latado et al. (2012)
Kedem (Rishon), Michal (Michal), Meravit (Merav), Vardit (Vered), Or (Ora), Mor (Murcott), Shani (Shani), King (King)	Gamma rays	Seedlessness, better fruit quality and color	Goldenberg et al. (2014)
Miyagawa (Satsuma)	Gamma rays	Seedlessness, antioxidant properties	Kim et al. (2012)
US Early Pride	Gamma rays	Seedlessness	McCollum and Hearn (2013)

(continued)

Table 13.7 (continued)

Variety (Parent)	Mutagens	Trait (s) altered/studied	References
ODEM (*C. deliciosa*)	Gamma rays	Seedlessness	Carmi et al. (2014)
Kinnow mandarin (*C. nobilis* Lour × *C. deliciosa* Tenora)	Gamma rays, ethyl methane sulfonate (EMS)	Leaf physio-biochemical parameters	Mallick et al. (2016)
N40W-6-3	Gamma rays	Seedlessness	Grosser (2017)

opment. Parent plants should also be selected for higher combining ability to transfer desired traits to the progeny. Cultivar Murcott, despite having several desired traits, is not chosen as a parent due to poor combining ability.

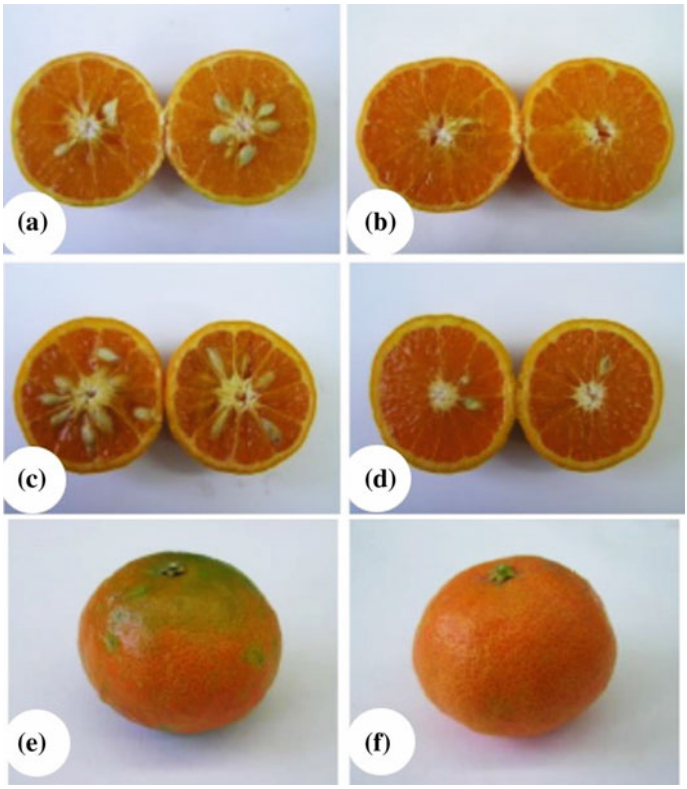

Fig. 13.4 Gamma irradiation induced mutagenesis for seedlessness in mandarin varieties. **a** Ora, **b** Irradiated Or, **c** King, **d** Irradiated King, **e** Michal, **f** Irradiated Michal (Goldenberg et al. 2014)

Conventional scion hybridization involves following steps

- Selection of ME parents for crossing having higher combining ability
- Sowing of hybrid seeds in the field and maintaining seedlings until maturity (6–10 years)
- Evaluation of trees for morphological traits, biotic and abiotic stress tolerance during juvenility
- Bud seedlings with desired traits on different rootstocks for evaluation of fruit yield and quality attributes (4–5 years)
- Release of budwood of desired cultivar (s) to nurserymen for field testing and plant multiplication

The above steps involve several years (20–30) to release a new cultivar. For example, crossing of parents of Robinson cv. tangerine (cvs. Clementine × Orlando) was done in 1942, while the variety was released in 1959 (17 years). Similarly, Kinnow cv. mandarin was bred in Riverside, California in 1915; the variety was released 1935 but took another 30–40 years to become a leading commercial mandarin cultivar in Pakistan (Khan and Kender 2007). Hence, conventional hybridization must be assisted with molecular tools to shorten the breeding cycle and release new improved varieties. An overview of spontaneous and crossed hybrids is provided in Table 13.8. Some newly-released mandarin hybrid cultivars from the University of California breeding program include Gold Nugget, Shasta Gold, Tahoe Gold, Yosemite Gold, Lee x Nova and Tango.

13.7.2 Somatic Hybridization

Success in a breeding program is mainly dependent upon the availability of genetic variation. Other than the variation available in the nuclear genome, cytoplasmic factors available in the organelle genome including chloroplast and mitochondria and interaction of the nuclear and cytoplasmic factors are also very important (Atienza et al. 2008; Del Bosco et al. 2017; Wang et al. 2010a, b). This interaction involves the control of several morphological, physiological and agronomic characters in citrus, including mandarins, which is still unexplored (Del Bosco et al. 2017; Satpute et al. 2015). Somatic hybridization using protoplast fusion has progressed well during the last three decades and considerable success has been achieved in citrus (Grosser and Gmitter 2011; Satpute et al. 2015; Wang et al. 2013). Somatic hybridization helps in resolving issues in conventional breeding including sexual incompatibility, male and female sterility, nucellar polyembryony and other problems (Grosser and Gmitter 2011; Grosser et al. 2000).

Combination of the entire parental genomes in symmetric fusion may also contain undesired nuclear encoded agronomic traits. The resulting somatic hybrids containing a hybrid tetraploid nucleus has not been of major use in citrus breeding programs due to sterility and morphological disorders with uncontrolled genomic instabilities (Grosser et al. 2000). The asymmetric somatic cybridization technique is based on the

Table 13.8 Overview of spontaneous and crossed hybrids in mandarins and mandarin hybrids

Hybrids		Parentage		Seedlessness
Spontaneous	Crossed	Female	Male	
Ellendale		Tangerine	Sweet orange	Seedy
Murcott		Tangerine	Sweet orange	Seedy
Temple		Tangerine	Sweet orange	Seedy
	Kinnow	King	Willowleaf	Seedy
	Fallglo	Bower	Temple	Seedy
	Osceola	Dancy	Clementine	Seedy
	Lee	Dancy	Clementine	Seedy
	Nova	Dancy	Clementine	Seedy
	Page	Minneola	Clementine	Seedy
	Sunburst	Robinson	Osceola	Seedy
	Kara	King tangor	Owari satsuma	Seedy
	Encore	King tangor	Willowleaf	Seedy
	Safor	Fortune (2n)	Kara (2n)	Seedless (3n)
	Albir	Fortune	Kara	Seedless (3n)
	Alborea	Fortune	–	Seedless (3n)
	C4-15-19	Sugar Belle tangelo	Nova + Succari (somatic hybrid)	Seedless (3n)
	Robinson	Dancy	Clementine	Seedless to seedy
	Minneola	Dancy mandarin	Duncan grapefruit	Seedless to seedy
	Orlando	Dancy mandarin	Duncan grapefruit	Seedless to seedy
	Ambersweet	(Clementine × Orlando)	Sweet orange 15-3	Seedless to seedy
	Dayse	Fortune	Fremont	Seedless
	UFGlow	(Clementine × Orlando)	Kishu	Seedless
	Bingo	LB7-11 (Clementine × Valencia)	KishuSL	Seedless
	GCM 305	Kara	–	Seedless
	Hadass	Ellendale	–	Seedless
	LB8-9	Clementine	Minneola	Seedless
	950	LB8-8	Fortune	Seedless

Table 13.9 Overview of somatic hybridization and cybridization in mandarin cultivars for seedlessness and crop improvement

Type of hybridization	Cultivar parents	Progeny/traits	References
Cybridization	Guoqing No. 1 (G1) satsuma + Hirado Buntan pummelo, Sunburst mandarin and Murcott Tangelo	Tetraploid (SH) and diploid (cybrids)	Guo et al. (2004)
Somatic hybridization and cybridization	Guoqing No. 1 (G1) satsuma + Page tangelo	Tetraploid (SH) and diploid (cybrids)	Cai et al. (2006)
Somatic hybrids	Yoshida navel orange + Okitsu satsuma	Tetraploids and diploids	An et al. (2008)
Somatic hybrids	Willowleaf mandarin SRA 133 + Eureka lemon	Allopolyploid, carotenoid gene expression behavior	Bassene et al. (2009)
Somatic hybrids	Guoqing No. 1 (G1) satsuma + Red Anliu and Taoye sweet oranges	Allotetraploids and diploids for scion improvement	Fu et al. (2011)
Cybridization	Guoqing No. 1 (G1) satsuma and Hirado Buntan pummelo	Genome constitution and proteome expression studies	Wang et al. (2010a, b)
Protoplast fusion	Mandarin/mandarin hybrid and sweet orange hybrid	Tetraploid (SH) and diploid (cybrids)	Soriano et al. (2012)
Somatic hybrids	Guoqing No. 1 (G1) satsuma and seedy sweet oranges cultivars	Transfer of CMS from Satsuma to orange cultivars, tetraploids and diploids developed	Xiao et al. (2014)
Cybridization	Chios and Clementine mandarins + Sanguinelli sweet orange	Tetraploid and diploid cybrids	Aleza et al. (2016)

unilateral chromosomal elimination leading to the development of morphologically-normal hybrids technically called *cybrids* which retain the diploid nucleus of the non-embryogenic parent (Guo et al. 2013). This type of somatic hybrids has the complete genome of one species and cytoplasmic genome of the other parent, known as a *non-nuclear cybrid* or it may have a combination of both the parental species. Some recent somatic hybridization and cybridization reports are presented in Table 13.9.

In citrus, protoplast events have always required the mitochondrial genome of the embryogenic parent (Cabasson et al. 2001) with few exceptions showing non-parental recombinant mitochondrial DNA genotypes in both somatic hybrids and

cybrids, suggesting a critical role of the mitochondrial genome in the regeneration of cybrids and somatic hybrids (Moreira et al. 2000a, b; Xiaodong et al. 2017). No specific patterns are reported in chloroplast genotypes of the regenerants and these could be found in both somatic hybrids and cybrids. Plant morphology has been quite variable in somatic hybrids and cybrids. Cybrids of cvs. Hazzara mandarin + rough lemon and Seminole tangelo + Lisbon lemon showed leaf morphology of the non-embryogenic parent (Moriguchi et al. 1996). On the other hand, somatic hybrids between cv. Ohta Ponkan mandarin + rough lemon had intermediate leaf morphology (Vardi et al. 1989).

In protoplast fusion of Cleopatra cv. mandarin (CLEO) and sour orange (SO), the cybrid nature of the genotype was confirmed by molecular analysis having the nuclear genome of leaf parent (SO), the mitochondrial genome of CLEO and the plastid genome of both parents (Del Bosco et al. 2017). In cybrids, coexistence of the plastid genome of both parents was also reported (Guo et al. 2007). Symmetric protoplast fusion in citrus at interspecific and intergeneric levels may successfully produce cybrids. Most such cybrids have the nuclear genome of the leaf parent and the mitochondrial and chloroplast genome of the embryogenic callus parent (Guo et al. 2013). Diversity in combinations of nuclear and cytoplasmic genomes in somatic hybrids and cybrids indicate the wide potential of protoplast fusion in creating genetic diversity and development of diverse germplasm for future breeding and biotechnology applications (Satpute et al. 2015). Cybridization between cvs. Dancy mandarin and grapefruit cvs. Ruby Red and Duncan showed enormous market potential due to better fruit quality attributes, including higher sugar, low acidity and better juice content (Satpute et al. 2015).

Cytoplasmic substitution has shown positive improvement in several breeding and agronomic traits including cytoplasmic male sterility CMS (Grosser and Gmitter 2005; Yamamoto et al. 1997), development of triploid seedless citrus or tetraploids for interploid hybridization (Grosser and Gmitter 2008, 2011), aroma (Abbate et al. 2012) fruit maturity and number of seeds (Grosser et al. 2000), disease tolerance (Tusa et al. 2000), metabolic traits in citrus (Wang et al. 2010a, b), rootstock improvement for disease resistance and soil adaptability (Grosser and Chandler 2003; Grosser et al. 2000) and Cleopatra cv. mandarin for improved polyphenol distribution in juice (Fig. 13.5, Del Bosco et al. 2017). Inclusion of the organelle genome in cybrids affects important breeding traits including gene expression behavior as reported in Eureka lemon cybrids having the mitochondrial genome from embryogenic Wilking mandarin (Fig . 13.6; Bassene et al. 2009, 2011). Mitochondrial gene modulation has also been observed under the foreign nuclear genomes (Zheng et al. 2012) suggesting the need for further investigations to underpin the mechanisms involved in interaction of organelle and nuclear genomes in diverse genetic backgrounds. Cybridization has emerged as a useful tool to transfer specific desired cytoplasmic traits in mandarins which is not possible through conventional breeding strategies.

Fig. 13.5 The new diploid cybrid (CLEO–SO), Sour cv. orange (SO) and Cleopatra cv. mandarin (CLEO) (Del Bosco et al. 2017)

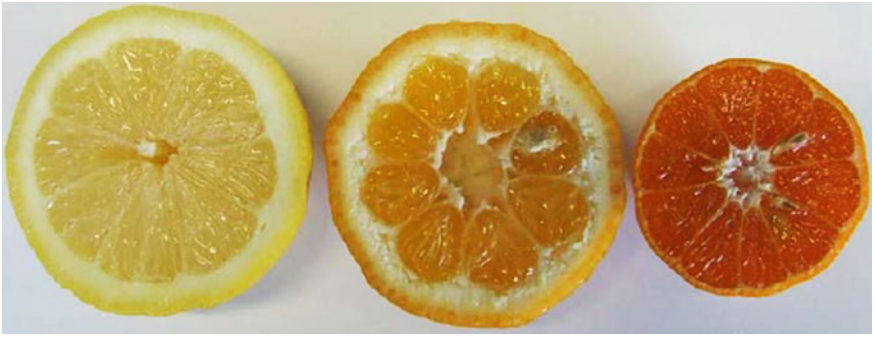

Fig. 13.6 Fruits of Eureka lemon (left), allotetraploid hybrid (middle) and Willowleaf mandarin (right) differing in their flesh color (Bassene et al. 2009)

13.8 Molecular Approaches in Crop Improvement

Fruit tree research has advanced less as compared to annual and herbaceous perennial crops due to problems like long juvenility, heterosis, self-incompatibility and polyploidy. Recent advances in the molecular techniques and the advent of next generation sequencing (NGS) approaches, shortening of breeding cycle and early detection of genetically diverse citrus material for different crop improvement programs, have opened new possibilities. The advanced molecular techniques involve omics including genomics, proteomics, trascriptomics including RNAseq, marker assisted selection (MAS), marker assisted breeding (MAB), genetic linkage mapping, use of bioinformatics databases and transgenics. Single cell omics has become a popular trend in biological sciences and transcriptomics of different traits and phenomenon including polyploidy, somatic hybridization, flesh and rind color have been investi-

gated in citrus (Allario et al. 2011; Bassene et al. 2010; Guo et al. 2015; Shiratake and Suzuki 2016; Yu et al. 2012). A combination or integrated omics performance on a single sample is known as *integrated omics* or *multiomics*. Supported by NGS and bioinformatics, key genes, proteins and metabolites could be identified and the output could be used for crop improvement programs.

13.8.1 Functional Genomics

The genome represents a complete haploid set of chromosomes including the set of genes in an organism. Advances in NGS have enhanced cost-effectiveness of sequencing and enabled whole genome sequencing in crops like citrus including *Citrus clementina* and *C. sinensis* (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/493/195/GCF_000493195.1_Citrus_clementina_v1.0). The available genome sequences of different cultivars and species could be compared for cultivar specific and species specific traits enabling identification of specific genes including the genes controlling flowering and regulating the alternate bearing mechanism in mandarins.

13.8.1.1 Flowering Genes and Floral Regulation

Many genes involved in the flowering process have been identified, mainly in annual herbaceous species like *Arabidopsis*. Recently, homologues of these genes, particularly Flowering Locus T*, FT,* have been identified as playing an important role in regulating flowering in woody perennial fruit crops as well (Hsu et al. 2006; Nishikawa et al. 2007, 2009). Overexpression of citrus *FT* homologue *CiFT* in satsuma mandarin corresponded with the period of flower induction (Nishikawa et al. 2007). Seasonal flowering also corresponded with higher expression of citrus LEAFY *CsLFY* and lower expression of citrus *TERMINAL FLOWER 1 CsTFL,* whereas transcription of *APETALA 1 CsAP1* (Pillitteri et al. 2004a, b) during flower bud induction and development remained unaltered (Nishikawa et al. 2007). The detailed gene expression analysis of satsuma mandarin revealed that *FT, TFL1, LFY* and *SEP* homologues were conserved while the expression of *AP1* and *FUL* remained low during flower induction and bud development, and environmental conditions, indicating no correlation of these genes with flowering (Nishikawa et al. 2009).

A recent study in Satsuma mandarin revealed a correlation of ABA with *CiFT3*-differentially expressed in shoots. Endogenous ABA was higher in shoots during flower inductive temperature (15 °C) and field conditions which in turn enhanced *FT* transcripts and flowering frequency. Carotenoid precursors act as substrate for the biosynthesis of ABA and were decreased with higher accumulation of ABA in leaves indicating that both ABA and carotenoid precursors are involved in flower induction in mandarin under low temperature conditions (Endo et al. 2017).

13.8.1.2 Fruit Load, Floral Regulation and Gene Expression

Citrus plants are evergreen and their floral buds do not show dormancy under tropical and subtropical conditions (Davenport 1990). Like other fruit crops, fruit load acts as a floral inhibitor in citrus as well, which is determined by factors such as cultivar, environmental conditions, fruit load per tree and harvest time (Martinez-Fuentes et al. 2010). The nutritional and hormonal status of the tree along with seasonal changes are involved in floral regulation (Yahata et al. 2004). In citrus, flowering is the outcome of a complex interaction of promoter and inhibitor genes including *CiFT*, *CsLFY*, *CsAP1*, SUPPRESSOR OF OVER EXPRESSION OF CONSTANS (SOC1; *CsSL1*), TERMINAL FLOWER 1 (*CsTFL*) (Endo et al. 2005; Pillitteri et al. 2004a, b; Tan and Swain 2007). The promotor gene *CiFT* plays a major role in flower inhibition due to crop load through a repressor system. In leaves, *LFY* and *AP1* are strongly regulated by the fruit load and are involved in floral identity, while *FLC* and *TFL1* are not correlated to fruit load. The floral identity genes are mainly expressed in floral meristems instead of leaves in citrus (Nishikawa et al. 2007; Yu et al. 2006) cv. Moncada mandarin (Munoz-Fambuena et al. 2012a).

Expression of *CiFT* in leaves regulates flowering and alternate bearing. Its expression is higher in buds from unloaded trees leading to higher flowering intensity in the spring season. After bud induction, the higher *CiFT* expression in both leaves and buds maintains a flux of FT protein to developing meristematic regions until floral morphogenesis is initiated (Giakountis and Coupland 2008; Munoz-Fambuena et al. 2012a). The expression of *FD* is higher in buds than leaves indicating its key role in transition from the vegetative to floral bud, and modulates the action of *FT* in the meristem (Jaeger et al. 2006; Munoz-Fambuena et al. 2012b).

The expression of inhibitor genes is different during *on* and *off* years. All the genes discussed above showed low expression during *off* year in leaves indicating floral repressor genes controlling the promoters. The expression of *FLC* was not different in leaves and buds and is associated with flower regulation through a vernalization pathway and its low expression may facilitate floral promoters (Michaels et al. 2005). Promotor genes like *FT* and *FD* contribute to floral morphogenesis (Notaguchi et al. 2008). The fruit load affects flowering and changes gene expression levels in both leaves and buds in alternate bearing (AB) citrus trees. Fruit load inhibits *CiFT* and *TSF* expression and the expression of floral identity genes *CsLFY* and *CsAP1* is reduced. The flowering inhibitor genes *FLC*, *CsTFL* and *TLF2* have lower expression in *off* years thus supporting the action of the promotor genes. Leaf defoliation reduces the activity of the promoters including *CiFT*, *CsAP1* and *TSF* during the inductive stage (Munoz-Fambuena et al. 2012b).

Alternate bearing is a cyclic behavior of trees to bear a heavy crop one year (*on* year) and extremely low or no yield in the next year (*off* year) which continues until certain management practices are followed to break the cycle. Alternate bearing is quite common in *Citrus* particularly in seed cultivars; however, some polyploids and interspecific hybrids also show AB. The fruit retention on the tree plays a major role in regulation of AB (Martinez-Fuentes et al. 2010). The alternate fruiting behavior also effects crop quality as during an *on* year fruit yield is more but the quality

is low, and in *off* year yield is less but fruit quality is again poor due to large-sized fruit which is highly problematic in commercial citrus orchards. Management practices also focus on flower regulation using different growth retardants including Cycocel (CCC), paclobutrazol (PBZ) etc. Retention of fruit after attaining proper weight has inhibitory effects on flowering as the time coincides with flower induction period (Martinez-Fuentes et al. 2010). Molecular studies have revealed that fruit affects flowering behavior by altering the gene expression behavior in leaves and meristem. The expression of flowering gene *CiFT* is inhibited in both leaves and the meristem while floral identity genes including *CsAP1* and *CsLFY* are significantly reduced (Martinez-Fuentes et al. 2010; Munoz-Fambuena et al. 2011). In Moncada and Satsuma cv. mandarins, *CiFT* expression starts in October–November when conditions are favorable for floral bud induction i.e., low temperature and less fruit load (Munoz-Fambuena et al. 2011; Nishikawa et al. 2007). PBZ has shown flower induction by enhancing *CiFT* gene expression when fruit load is less (inductive conditions) while under non-inductive conditions (heavy fruit load), *FT* repressor gene *FLC* expression is increased (Munoz-Fambuena et al. 2011). However, PBZ and GA_3 have shown no effect on *FLC* gene expression (Munoz-Fambuena et al. 2012b).

13.8.2 Genome Sequencing and Diversity Assessment for Domestication and Ancestry

Mandarins have basic genotypes that originated through interspecific and introgressive hybridization events with their wild ancestors. These domestication events have thousands of years of history of an undocumented domestication process. Wide variations have been observed mainly as somatic mutation, limb sports or variation among the apomictic seedling populations. It is believed that pummelo domestication mainly originated from mandarins and oranges. The pummelo (*C. maxima*) is indigenous to Southeast Asia while most of the mandarins are identified as *C. reticulata*, also native to the region. There is some controversy in the naming and classification of mandarins (Swingle and Reece 1967; Tanaka 1977). Wu et al. (2014) referred to the name *mandarin* as a commercial designation referring to small-sized, easy-peeling and sweet fruit and is most commonly used now for the recent mandarins and their hybrids including cvs. Clementine and W. Murcott (Nadorcott and Afourer). However, it may be taxonomically different from the traditional mandarins (lacking admixture with other ancestral *Citrus* species) including Willowleaf cv. a common Mediterranean variety and Ponkan cv. a widely grown Asian variety.

Development of the admixture structure of interspecific germplasm has proved essential for better utilization of citrus resources to develop innovative breeding programs. Plant evolution analysis has been modified using NGS technologies and is based on the whole genome variability analysis termed *phylogenomics*. The whole genome resequencing (WGS) technology is more expensive compared with geno-

typing by sequencing (GBS) (Elshire et al. 2011). The uncovering of the hypothesis of pummelo-mandarin species using genome sequencing and diversity analysis tools revealed interesting findings using a haploid derived through induced gynogenesis from Clementine cv. mandarin (Germanà et al. 2013). The traditional mandarin varieties are the outcome of interspecific introgression of pummelo (*C. maxima*) into the wild mandarins (*C. reticulata*). The extensive sharing of haplotype among wild and modern-day mandarins confirms their relationship which were previously thought to be unrelated (Wu et al. 2014). Similarly, identification of cv. Willowleaf and sweet orange alleles at each locus of cv. Clementine confirmed its hybrid origin as reported previously (Nicolosi et al. 2000; Ollitrault et al. 2012a; Wu et al. 2014). Likewise, the hybrid origin of cv. W. Murcott mandarin which was presumed to be a chance seedling of cv. Murcott tangor (sweet orange × unknown mandarin) has also been confirmed by Wu et al. (2014).

Surprisingly, nuclear and chloroplast genome sequencing and analysis of commercial and a wild mandarin named Mangshan from China revealed a variable chloroplast genome compared with *C. reticulata* and *C. maxima* with limited heterozygosity, indicating no admixture of pummelo and mandarin. Hence, Mangshan cv. mandarin nomenclature as *C. mangshnensis* by Liu et al. (1990) is supported and confirmed as a distinct species from *C. reticulata* (Wu et al. 2014). Furthermore, the conclusions of previous reports (Xu et al. 2013) about the ancestry of mandarins has been ruled out by Wu et al. (2014) who revealed a pummelo admixture in cvs. Huanlingmiao and Clementine mandarins. Willowleaf, Huanlingmiao and Ponkan cvs. mandarins are also related to each other. The admixture analysis further revealed that breeding of *C. reticulata* and *C. maxima* occurred early in the domestication process. Sour orange has also been confirmed as a hybrid of *C. maxima* and *C. reticulata*. In future, the identification of citrus somatic mutants with their genetic backgrounds will also be possible using advanced genomic tools for changes in the fruit color, aroma and flavor in modern mandarins and other citrus cultivars.

Recent NGS development advances have enabled simultaneous discovery and genotype assessment of SNPs and indels in different plant species including citrus (Davey et al. 2011). Among different methods, GBS is more advantageous in the preparation of libraries from small amounts of DNA (Sonah et al. 2013). Genotype calling and varietal diversity in 55 citrus accessions including mandarins was assessed using *ApeKI* restriction enzyme and Cornell GBS methodology (Elshire et al. 2011). The heterozygosity level varied as 0.135 with 5110 heterozygous markers for Cleopatra cv. mandarin, mandarin hybrids including tangors, tangelos and orangelos showed average heterozygosity of 0.359 (Oueslati et al. 2017). Overall, mandarins showed lower average heterozygosity (0.217) compared with oranges (0.490–0.544) and grapefruit (0.476). Out of 55 varieties, 95.76% of the polymorphism was found in 17 mandarins and pummelo accessions, and mandarin accessions were found to be more polymorphic compared with pummelo as reported with indels and SNPs (Curk et al. 2015; Garcia-Lor et al. 2012). All mandarin hybrids and other citrus species including *C. aurantium*, *C. sinensis* and *C. paradisi* found intermediate positions among mandarins and pummelos in overall genetic organization. The introgression of *C. maxima* was limited in cvs. Cleopatra and Sunki mandarins com-

pared with cvs. King, Satsuma and other mandarins. Furthermore, the pure mandarin species including cvs. Willowleaf, Ponkan and Huanglingmiao have also displayed introgression of *C. maxima* (Curk et al. 2015; Oueslati et al. 2017) which might be attributed to frequent natural interspecific hybridization and backcrosses of *C. maxima* on *C. reticulata* germplasm. Recent WGS based studies have further confirmed the parentage of cv. Wilking (King × Willowleaf) and cv. Kiyomi (Satsuma × sweet orange). It has confirmed that one grandparent of cv. Fallglo was cv. Clementine, cv. Ponkan as the parent of cvs. Dancy and Huanlingmiao (a somatic mutant of cv. Kishu) as the parent of Satsuma. Other cultivars have shown higher inbreeding and rounds of homozygosity including Dancy, Satsuma, Clementine, Fallglo, Kiyomi and BTJ mandarins. Furthermore, late admixture mandarin varieties including cvs. Clementine and Kiyomi were found to be direct progenitors of the sweet orange, while early admixture cv. Ponkan mandarin has more likeliness ($r \approx 0.36$) towards sweet orange. Cultivar Sun Chu Sha Kat, the pure mandarin, has also relatedness ($r \approx 0.23$) to sweet orange (Wu et al. 2018). However, due to the absence of pummelo haplotypes in mandarins, sweet orange may not be considered as a common ancestor of mandarins. Hence, it is postulated that the phylogenomic patterns discovered may drive the wide phenotypic diversity observed in citrus and its related germplasm. It could be concluded that using modern tools like molecular markers, NGS technologies, and admixture analysis could enhance our understanding of parentage and introgression patterns during the evolutionary process and enhance molecular breeding efficiency.

13.8.3 Molecular Markers and Applications

Development of genetic markers is one of the most important milestones in plant breeding. A gene or DNA sequence with its known locus and trait is generally termed a *molecular* or *genetic marker*. Some important molecular markers include amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), simple sequence repeats (SSRs), diversity arrays technology (DArT) and single nucleotide polymorphism (SNP) (Nadeem et al. 2017). DNA markers can be dominant or co-dominant, hybridization or PCR based, and can depend upon the mode of transmission as maternal or paternal, based on organelle or nuclear inheritance (Nadeem et al. 2017; Semagn et al. 2006). Molecular markers are being routinely used for a variety of objectives including evolutionary and phylogeny assessment, heterosis assessment, identification of haploid, double haploids, polyploids, cultivar genotyping, genetic diversity assessment, genetic and QTL mapping, structural mapping, genome wide association studies (GWAS), marker assisted selection (MAS) and genome editing (CRISPR). Reconsideration of citrus classification began after the discovery of DNA markers (Breto et al. 2001; Kepiro and Roose 2010; Luro et al. 1994; Tripolitsiotis et al. 2013; Uzun et al. 2009) and citrus phylogeny and genetic associations have been revealed gradually and new classifications have

been proposed. An overview of different markers used in mandarin genetic diversity assessment and other applications is presented in Table 13.10.

13.8.3.1 Genetic Diversity Assessment in Germplasm

Among a variety of molecular markers, SSRs, SNPs and indels have been most widely used for germplasm description because of their abundance, multiallelic nature, codominance and wider genetic coverage (Curk et al. 2014; Garcia-Lor et al. 2012; Ollitrault et al. 2012a). The size of germplasm collections could be reduced up to 15–20% by analyzing genetic diversity including SSRs (Djè et al. 2000), AFLP (van Treuren et al. 2006) and SNPs (McKhann et al. 2004). Mandarin core collections available at IVIA (Moncada, Spain) and INRA-CIRAD (Corsica, France) have been developed using molecular markers including SSR markers, indels and SNP markers (Garcia-Lor et al. 2012, 2013; Ollitrault et al. 2012b).

13.8.3.2 Identification of Zygotic and Nucellar Seedlings and Cultivars

Citrus nucellar embryony is a normal phenomenon in apomictic genotypes and identification of the nucellar and zygotic embryos is critical for breeders. Isozymes have been used previously for this purpose; however, now SSRs and SNPs are being widely used for the identification of nucellars and zygotics due to a higher level of available polymorphism in these markers (Curk et al. 2015). Mandarins have evolved through sexual hybridization during evolutionary processes; hence, cultivar and homonym identifications are possible through genetic markers, particularly SSRs as reported in cv. Sunki mandarin using the 07B05 SSR marker (Ben Yahmed et al. 2015). Molecular markers are highly efficient in identifying genotypes available in different genetic resource centers and to assess comparative phenotypic variation under contrasting climatic conditions.

13.8.3.3 Systematic Classification and Diversity Organization

Most studies using different genetic markers have revealed the structure and organization of three ancestral species including pummelos, mandarins and citron. Development of allele specific markers has enabled interspecific and intraspecific diversity assessment in mandarins and other groups and their putative parentage (Luro et al. 2012). The phylogeny of citrus genotypes can also be assessed using mitochondrial and plastid genome (Carbonell-Caballero et al. 2015; Curk et al. 2016; Yamamoto et al. 2013). The parentage of cv. Clementine and other important mandarin cultivars has been discovered using DNA markers (Shimizu et al. 2016).

Table 13.10 Applications of different molecular markers in genetic diversity assessment in mandarins and mandarin hybrids

Mandarin varieties	Markers	Primer series	No. of primers used	Optimal primers	No. bands/loci	No. of polymorphic bands/loci	Polymorphism (%)	References
Citrus reticulata hybrid Zhelong Zhoupigan	nSSR cpSSR	Geneworks	10 8	– –	– 60	– 42	– 1.428	Li et al. (2006)
4 varieties (Ellendale, Fortune, Clementine, tangerine)	STMS	Froelicher et al. (2007)	4	–	–	–	–	Froelicher et al. (2007)
Satsuma × tangerine (2x X 3x) hybrid	SSR	Kijas et al. (1997)	TAA15 TAA27	–	–	13 9	–	Tan and Swain (2007)
Satsuma+pummelo & sweet orange cybrids	SSR cpSSR	TAA1 TAA15 NTCP9	2	1 (TAA15) NTCP9	–	–	–	Cai et al. (2007)
C. reticulata spp.	SSR	Geneworks	7	–	52	5	94.5	Golein et al. (2011)
Mandarin × pummelo somatic hybrid	EST-SSR	–	4	–	–	–	–	Chen et al. (2008)
Ponkan	AFLP conversion into SCAR	EcoRI and MseI	72	13	–	19	18	JinPing et al. (2009)
15 mandarin & mandarin hybrids	SRAP	–	28	Em 1-16 Me 1-12	376	17	4.52	Uzun et al. (2009)
Interspecific tetraploid	SSR	Froelicher et al. (2008)	17	–	–	–	–	Kamiri et al. (2011)

(continued)

Table 13.10 (continued)

Mandarin varieties	Markers	Primer series	No. of primers used	Optimal primers	No. bands/loci	No. of polymorphic bands/loci	Polymorphism (%)	References
5 mandarin and mandarin hybrids	SSR SRAP CAPS-SNP	–	61 33 24	–	596 656 135	9.7 19.8 5.6	1.62 3.01 4.14	Amar et al. (2011)
Mandarin germplasm	SSR SRAP	–	26 21	14	14 187	2 148	2.7 77	Kacar et al. 2013
C. reticulata	ISSR	–	11	–	160	153	95.62	Pal et al. (2013)
165 mandarin genotypes out of 191 citrus genotypes	SSR indels SNPs cpDNA mtDNA	Garcia-Lor et al. (2012, 2013) Cheng et al. (2005), Froelicher et al. (2011)	50 24 67 8 4	10	529 74 134	– 3.1 12	60 19 27	Garcia-Lor et al. (2015)
Murcott mandarin	SSR SRAP	–	26 28	–	56 149	0 0	0 0	Polat et al. (2015)
Fortune × Murcott 116 F1 hybrids	SNPs	–	10	–	–	–	–	Yu et al. (2016)

13.8.3.4 Identification of Unreduced Gametes and Polyploids

Polyploidization frequency is higher in citrus as compared with other fruit crops due to the presence of nucellar embryony; the percentage may be increased further (10–20%) depending upon the genotype and prevalence of low temperature during blooming. In citrus, intervarietal and interspecific sexual hybrids and polyploids can be identified using SSR markers (Fatima et al. 2015; Luro et al. 2008). Genetic analysis using SSR and SNPs revealed >80% tetraploid hybrids produced from 2n pollen in 4x × 2x interploid hybridization having cv. CSO tangor as the male parent and helped to identify the origin of 2n pollen (Rouiss et al. 2017).

13.8.4 Bioinformatics

The bioinformatics of mandarins is still in its infancy; however, more genomic information is available compared with other citrus species, excluding *Citrus sinenesis*. The existing data banks present valuable information about different mandarin species including *C. reticulata*, *C. clementina* and *C. unshiu* (Table 13.11). The information in Table 13.10 also contributes to the development of such databases which provide information about the genome size, available markers, genomes and genome maps, QTLs, ESTs, genes and proteins which is being used for omic studies and other molecular-breeding programs. Genome sequencing and comparative analysis in different citrus species have enabled reinforcing the extent of genomic introgression during the process of domestication and to identify the ancestry of the important species including mandarins (Garcia-Lor et al. 2015; Wu et al. 2014). Flesh color, flavor and seedlessness are key traits for cultivar improvement. Several SNP markers associated with fruit traits were identified in F1 population of cvs. Fortune × Murcott and aligned with cv. Clementine genome for the construction of genetic maps. There were 48 QTLs identified, including QTLs for flavedo color and juice color space values, carotenoid biosynthetic pathway genes *pds1* and *ccd4* which could be further used for MAS (Yu et al. 2016). Bioinformatics databases help in prediction of miRNAs using cloning, deep sequencing and miRBase. Out of 9 miRNAs reserved for citrus, 5 miRNAs were contained in *C. clementina* and 4 in C. *reticulata.* Ponkan and clementine also shared similar genome sequence and the miRNAs discovered in Ponkan complement the number of miRNAs in *C. reticulata* (4–170), and enriched citrus miRNAs (Fang et al. 2014). The mandarins represent a highly diverse group and there is still much scope of genome sequencing in different mandarin species.

Table 13.11 Mandarin genomic databases and related information

Databases	Species (common name)	Genome size (Mb)	Available markers	Available genomes and (maps)	Available QTLs and (traits)	Web links	References
Citrus genome database	*Citrus reticulata* (tangerine)	~370	595	0 (3)	128 (36)	www.citrusgenomedb.org/search/featuremap/list?organism=Citrus%20reticulata	Yu et al. (2017)
	C. clementina (Clementine)	370	1728	1 (3)	341 (145)	www.citrusgenomedb.org/organism/Citrus/clementina	Aleza et al. (2009)
NCBI genome	*C. clementina* (Clementine)	295	–	–	–	www.ncbi.nlm.nih.gov/genome/11310	Aleza et al. (2009), Wu et al. (2014)
	C. unshiu (Satsuma)	360	–	2	–	www.ncbi.nlm.nih.gov/genome/46555	Shimizu et al. (2017)

13.8.5 Transgenics

Plant transformation has been widely used as a valuable tool for genetic modification and horticultural trait enhancement of economically-important citrus species. Transformation tools allow the incorporation of different desired traits into important cultivars without making major changes to their genetic constitution and integrity. Classical breeding has limited scope in citrus crop improvement as discussed in previous sections. Issues like long juvenility could be reduced by transformation and overexpression of genes inducing early flowering, thus shortening the breeding cycle or by direct transformation of mature tissue (Bohlenius et al. 2006; Cervera et al. 2008; Endo et al. 2005; Orbovic et al. 2015). In mandarins, cv. Murcott can be used as a female parent due to the higher number of zygotic embryos available and in the absence of pollinators it produces seedless fruit. However, in mandarin hybrids and cv. Murcott, *Agrobacterium* transformation is more difficult as compared with other citrus species. Furthermore, tissue regeneration in citrus is highly genotype dependent (Cervera et al. 1998, 2000). To date, chiefly mandarins and satsumas have been utilized for transformational studies using *Agrobacterium*-mediated transformation using ribonuclease, miraculin and calchone synthase genes (Al Bachchu et al. 2011; Khawali et al. 2006; Li et al. 2002; Wang et al. 2010a, b), as presented in Table 13.12. Out of nine transgenic lines of cv. W. Murcott developed by direct DNA uptake protoplast transformation system against citrus canker using green fluorescent protein (GFP) as a reporter gene and the *Xa21* (*Xanthomonas* resistance gene taken from rice), the WM-8 line was found consistently tolerant to canker (Omar et al. 2018). The promising transgenic lines are under field evaluation. It is further suggested that codon optimization of *Xa21* may be required to enhance gene

Table 13.12 Transformation in mandarin varieties using different methods and genes of interest for crop improvement

Varieties	Gene (s) inserted	Traits	Mode of transformation	References
Ponkan mandarin	Barnase suicide (ribonuclease)	Male sterility	*Agrobacterium*	Li et al. (2002)
Nagpur mandarin	GUS	–	*Agrobacterium*	Khawale et al. (2006)
Guoqing No. 4 satsuma	Chalcone synthase (CHS) and chalcone isomerase (CHI)	Flavonoid biosynthesis	pMD 18-T vector	Wang et al. (2010a, b)
Miyagawa wase satsuma	Miraculin	Sour to sweet taste	*Agrobacterium*	Al Bachchu et al. (2011)
W. Murcott mandarin	Green fluorescent protein (GFP) and *Xa21*	Citrus canker resistance	*Agrobacterium*	Omar et al. (2018)

expression for improved disease resistance. There is a need to develop efficient tissue culture regeneration systems following protoplast/GFP transformation system in emerging mandarin varieties and hybrids for better transformation efficiency and higher transgene expression.

13.9 Conclusions and Prospects

13.9.1 Overview of Current Status

The mandarins are a diverse group containing different species and a large number of varieties, cultivars and hybrids. The economic significance of mandarins is evident from its wide cultivation from the Mediterranean Region to New Zealand on about 8,000,000 ha furnishing about 25% of total world citrus production. Mandarins are consumed both as fresh fruit and as several processed products. Low mandarin productivity is due to its requirement of specific environmental conditions by a few leading cultivars including cold sensitivity, biotic stresses like citrus greening disease, CTV and canker. Classical breeding in mandarins is handicapped by factors like long juvenility, self-incompatibility and nucellar embryony. Genetic resources in the USA, China, Japan, Australia, Spain, France and Italy are rich in mandarin germplasm which needs further exploitation and exchange with other citrus growing regions in the world including Asian countries. The emerging concept of creating core collections and using molecular markers for genetic diversity assessment could be highly useful in making germplasm collection more cost-effective and diverse as compared to previous approaches. The identification of flowering genes *CiFT*, and

understanding the behavior of associated genes in flowering pathways under diverse climatic conditions has opened new horizons in mandarin breeding and enhanced the understanding of the flowering regulation and fruit setting phenomena. Genetic backgrounds of nucellar embryony are better understood and identification of nucellar or zygotic embryo frequency in the hybrids could eliminate development, maintenance and screening of large populations. Identification of the key candidate gene *CitRWP* controlling polyembryony will further enhance our capability to study gene expression patterns in different mandarin species under contrasting climatic conditions. The development of unreduced gamete formation and their role in polyploidization in mandarin species is now well understood. Tetraploids could be better used as female parents in the intetraploid crosses for higher production of 2n gametes and normal seed development with 4n embryos. Recently, development of haploids and double haploids in cv. Clementine and genome sequencing has enhanced our understanding of the mandarin genome and the significance of haploids in structural genomics. Induced mutations using gamma rays have been widely used in mandarins and have altered many economically-important traits. Omic studies and admixture analysis have revealed further insights into the ancestry and the domestication process of mandarins and highlighted their role as primary species compared with other citrus species. Among molecular markers, SSRs, indels and SNPs have been most useful in genetic diversity assessment and confirmation of parentage during hybridization. Bioinformatics tools are being enhanced by the upcoming genetic information and the databases are provided for further utilization of the available information. Transgenics is still under developmental phase and only a few genes have been transformed successfully. It is limited to specific varieties having higher in vitro regeneration potential.

13.9.2 Recommendations for Future Research

There is a need to enhance international germplasm exchange programs for extensive and wider utilization of the genetic resources for crop improvement and to spread diverse germplasm across different climatic conditions for better selection and hybridization approaches. Gene knockout and gene expression studies using *CitRWP* under diverse climatic conditions could help understanding its behavior across different mandarin and other citrus species and may lead to the development of ME mandarin cultivars. There is a need to develop tetraploid plant material in other elite mandarin varieties and hybrids for interploid crossing for seedlessness. Induced parthenogenesis is a useful tool for the development of haploids and will be utilized more efficiently for the development of haploids in other leading mandarin varieties for breeding purposes and for further genomic studies.

Mutation breeding should be more target oriented and efficient screening methods involving molecular markers, NGS technologies should be developed to avoid screening for several generations and to release new mutants with novel traits of interest. DNA markers associated with self-incompatibility, parthenocarpy, male sterility and

seedlessness could be used for directed breeding leading to identification and cloning of target genes of interest for development of transgenic seedless varieties. Efficient cell suspension and in vitro regeneration systems are required for elite mandarin cultivars to enhance transformation efficiency and for the development of overexpressing transgenic plants for early flowering, expression of gibberellin biosynthesis genes like GA20-oxidase for the development of seedless cultivars and against citrus greening and CTV. The emerging biotechnological strategies should be evaluated for potential contributions in the acceleration of the breeding efforts for the development of cultivars having better fruit quality, stress tolerance and early maturity.

Appendix 1

Leading research institutes and online resources involved in citrus breeding and biotechnology

Institutions	Country	Contact information and websites
Key Laboratory of Horticultural Plant Biology, Huazhong Agricultural University	China	http://www.hzau.edu.cn +86 27 87286965
National Citrus Engineering Research Center, Southwest University Chonquing		+86-23-68349601 http://www.sicas.cn
Citrus Research Institute, Chinese Academy of Agricultural Sciences Chongqing		http://www.caas.cn/en +861082106755
The French National Institute for Agricultural Research (INRA)	France	http://institut.inra.fr +33(0)1 42 75 90 00
French Agricultural Research Centre for International Development (CIRAD)		www.cirad.fr +33 1 53 70 20 00
USDA National Institute for Food and Agriculture (NIFA)	USA	www.citrusgenomedb.org
Citrus Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida		http://www.crec.ifas.ufl.edu +1 863-956-1151
Citrus Research Center and Agricultural Experiment Station, College of Natural and Agricultural Sciences, University of California, Riverside		http://cnas.ucr.edu +1 951 827-6555

Institutions	Country	Contact information and websites
Division of Citrus research, Institute of Fruit tree and Tea science NARO, Shimizu, Shizuoka	Japan	http://www.naro.affrc.go.jp +81-54-369-7100
Center of Citriculture, Instituto Valenciano de Investigaciones Agrarias – IVIA, Valencia	Spain	http://www.ivia.gva.es +34 963 42 40 00
Centro di Ricerca per l'Agrumicoltura e le colture mediterranee (Research Centre for Citrus and Mediterranean Crops), Catania	Italy	acm@crea.gov.it +39-095-7653111 http://sito.entecra.it/portale/cra_dati_istituto.php
Citrus and Subtropical Fruit Research Institute	South Africa	http://www.arc.agric.za/arc-itsc/Pages/Citrus.aspx
Indonesian Citrus and Subtropical Fruits Research Institute, Junrejo District, East Java	Indonesia	http://balitjestro.litbang.pertanian.go.id (+62341) 592683
Central Citrus Research Institute, Nagpur	India	http://www.ccringp.org.in 0712-2500813
Citrus Genetic Resources Bank, Jeju National University, Jeju 690-756,	Korea	http://www.knrrc.or.kr/english/rrc/rrc.jsp?category=75&order=1#36
Directorate of Horticulture, Ayub Agriculture Research Institute, Faisalabad	Pakistan	+92-489239212 http://www.agripunjab.gov.pk/research
Institute of Horticultural Sciences, University of Agriculture, Faisalabad		+92-41-9200186 directorihs@uaf.edu.pk
Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad		+92-41-9201087 directorcabb@uaf.edu.pk
Center for Advanced Studies in Agriculture and Food Security (USPCAS-AFS), University of Agriculture, Faisalabad		+92-41-2409462-4 uspcasafs@uaf.edu.pk

Appendix 2

Genetic resources

Cultivars	Important traits	Cultivation location
Miyagawa	Large fruit than Owari, early maturity, highly acidic	Japan
Nankan No 4	Balanced flavor	
Hayashi	Vigorous growth, high sugar content, most finest flavor	
Aoshima	Smooth rind, long term storage, late maturity	

Cultivars	Important traits	Cultivation location
Juman	High sugar level, prolonged storage	
Hassaku	Leaves are pummelo like, fruit resemble Marsh grapefruit	
Iyokan	Fruit have depressed area at stem end, sweet flavor without bitterness	
Natsudaidi	Dark green leaves, large grapefruit size fruit, seedy, late maturing, bitter and highly acidic flavor	
Kiyomi	Soft rind and tender texture, late maturity	
Seminole	Cup-shaped leaves, fruit borne inside canopy, deep reddish orange color, late maturity, high acid content	
Tankan	Large orange like leaves, medium-late maturing, rind have pebbly texture	South China, Japan
Okitsu	Early bearing, completely seedless, high sugar content than Miyagawa	Japan, Spain
Nules	Extended harvesting, multiple fruit setting	Spain
Esbal	Early bearing	
Fina	Small fruit, high juice content	
Guillermina	Reddish orange fruit color	
Hernandina	Late maturity, incomplete color development	
Arrufatina	Thorny, early bearing	
Clausellina	Early maturity than Owari	
Planellina	Early maturity than Clausellina, Juicy, thin rind and large fruit size	
Fortune	Bear fruit inside canopy, late maturing	
Fremont	Early maturity, reddish orange internal and external color, brittle rind	Spain, Turkey
Kara	Open habit, droop branches, rough rind, high acid content	Spain, Australia
Nova	Thorny, reddish orange rind, juicy fine flavor	Spain, Israel, USA
Wilking	Alternate bearer, firm fruits, seedless	Spain, Morocco
Pixie	Small and firm fruit, less juicy, mediocre quality	California, Arizona, USA
Fairchild	Thornless, dense foliage, early maturity, tight rind, tomato like aroma	
Robinson	Early maturing, thornless, cold hardy, flattish fruit	Florida, USA
Orlando tangelo	Cup shape leaf, early maturity, difficult to peel, cold hardy	
Temple	Cold sensitive, fairly thin rind, pulp color don't match with rind, spicy flavor, self-fertile	
Sunburst	Early maturing, smooth thin skin	USA

Cultivars	Important traits	Cultivation location
Minneola tangelo	Less cold resistant, large fruit, unique delicious and distinctive flavor	
Dancy	Thornless, frost susceptible, well balanced flavor	
Encore	Dark orange patches on fruit, distinctive rind oil aroma, highly seedy	USA, Japan
Honey Mandarin	Fruit borne on terminal, cold hardy, thin rind, higher juice and sugar content	USA, Brazil
Ellendale	Prone to crotch splitting, thin rind, long shelf life,	Australia, Argentina, Uruguay
Imperial	Early maturity	Australia
Malaquina	Rough rind texture, insipid flavor	Argentina
Malvasio	Seedy, late maturity	
Palazzelli	Clementine like flavor, late maturity, good storage	Italy
Sun Red	Small fruit size, the highest level of anthocyanins (800 ppm), seedy	
D2238	Fruit medium in size, pulp yellow to orange, low naringinin and furanocoumarins, TSS 10–12°Brix, seedless	
Miho	More vigorous than Okitsu	South Africa
Ortanique	Late maturity, tough segment walls, outstanding juice color	Jamaica
Kinnow	Late maturity, smooth rind, seedy, high TSS, fruit size large	Pakistan, India
Kinnow LS	Late maturity, smooth rind, low seeded, high TSS, fruit size large	Pakistan
Feutrell's Early	Early maturity, seedy, high TSS, good fruit quality, fruit size small to medium	

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Chapter 14
Strategies for Olive (*Olea europaea* L.) Breeding: Cultivated Genetic Resources and Crossbreeding

Luis Rallo, Diego Barranco, Concepción M. Díez, Pilar Rallo, María Paz Suárez, Carlos Trapero and Fernando Pliego-Alfaro

Abstract Olive cultivars represent an invaluable heritage of genetic variability selected over more than 5500 years of cultivation. This high diversity of local cultivars is a common feature in traditional olive-producing countries. Most cultivars are old and continue to be cultivated around areas where they have likely been selected. Crossbreeding in olives was only initiated in the second half of the twentieth century and currently represents the most promising strategy to provide farmers with new cultivars that are well adapted to the new high density olive plantations spreading

L. Rallo (✉) · D. Barranco · C. M. Díez · C. Trapero
Departamento de Agronomía, Edificio Celestino Mutis, Universidad de Córdoba, Campus Universitario de Rabanales, Campus de Excelencia CeiA3. Ctra. de Madrid km. 396, 14004 Córdoba, Spain
e-mail: lrallo@uco.es

D. Barranco
e-mail: dbarranco@uco.es

C. M. Díez
e-mail: cmdiez@uco.es

C. Trapero
e-mail: carlostrapero@uco.es

P. Rallo · M. P. Suárez
Departamento de Ciencias Agroforestales, Universidad de Sevilla, ETSIA, Carretera de Utrera, km 1, 41013 Seville, Spain
e-mail: prallo@us.es

M. P. Suárez
e-mail: maripaz@us.es

F. Pliego-Alfaro
Departamento de Biología Vegetal, Facultad de Ciencias, Instituto de Hortofruticultura Subtropical y Mediterránea (IHSM) "La Mayora" (UMA-CSIC), Campus de Teatinos s/n, 29071 Málaga, Spain
e-mail: ferpliego@uma.es

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in traditional and new olive-growing countries. This chapter focuses on cultivated genetic resources and crossbreeding strategies in olive. Exploration, cataloguing and authentication for the conservation and sustainability of true-to-type cultivars by morphological and DNA markers in the Network of Germplasm Banks promoted by the International Olive Council, is the most extensive and worldwide initiative to date. The strategies, methodologies and advances in crossbreeding programs worldwide are reviewed. Shortening the juvenile period, early selection and other strategies for the evaluation of valuable agronomical traits are integrated into the framework of alternative protocols that also provide information regarding the variability and heritability of these traits. In addition, the possibilities provided by new genomics tools to shorten the protracted crossbreeding process are also presented. Finally, new developments on in vitro culture and genetic transformation as well as the feasibility of using these tools in breeding programs are discussed.

Keywords *Olea europaea* · Clonal selection · Cryopreservation · Biotechnology Genomics · In vitro regeneration · Morphological descriptors · Molecular markers

14.1 Introduction

Olive (*Olea europaea* L.) is a Mediterranean tree species that was probably domesticated in the Middle East and in Central Mediterranean approximately 5500 years B.P. Since that time, the crop has expanded along both shores of the Mediterranean Sea. Olive growing was well-established in Roman times, as witnessed by the agricultural treatises of Pliny and Columela. Beginning in the fifteenth century, the transoceanic voyages of Christopher Columbus, Ferdinand Magellan and Juan Sebastián Elcano helped olives reach and spread throughout the New World. They are currently also grown in South Africa, China, Japan and Australia.

Empiric local selection within wild olives and crosses between the previous selected or introduced cultivars, and other local cultivars or wild olives, in all growing areas have yielded a huge number of local cultivars. This high diversity of local cultivars is therefore a common trait in traditional olive-producing countries. Most cultivars are old and are cultivated around areas where they were likely selected. In most cases, cultivars are self-rooted. Grafted trees are found only in the case of difficult-to-root cultivars or due to top grafting onto wild olives or onto other obsolete cultivars (Díez et al. 2015). Most cultivars are exclusively used for oil production. However, certain cultivars are used for table olives, and others may be used for both oil and table olives.

According to data published by the International Olive Council (IOC); (www.international.oliveoil.org), the world's olive-growing area reached approximately 11.4 million ha in 2015, which was mostly located (>96%) in the Mediterranean Basin. Seventy-eight percent of this area is rainfed, and 22% is irrigated. Nonbearing trees represent 13% of the total orchards. The average production and consumption per year for the periods 2012/2013 to 2016/2017 was approximately 2,760,000 and

2,951,700 mt of olive oil, respectively. For table olives, the average production and consumption for the same period were 2,632,100 and 2,542,300 mt, respectively. Production and consumption are currently balanced; the large price variations between years and seasons are usually due to the biennial bearing habit of olive crops.

Olives have traditionally been a long-lived rainfed crop that is well adapted to the Mediterranean climate, which is characterized by a low and irregular yield, a high demand for labor for harvesting, empirical technology and low investment. However, since World War II, olive growing has been changing in both Northern Mediterranean countries and in new olive-growing regions. Human migration from rural to urban areas has required more productive and mechanized orchards. The accumulated changes since that time have led to new olive plantations (Fig. 14.1). These new olive orchards bear earlier; however, their overall life span is shorter than in the past. Improved agricultural practices to avoid soil erosion and contamination of the environment are increasingly being employed. Annual cultural practices are being simplified, and pruning and harvesting have become more mechanized. Yield has increased through the implementation of irrigation and high-density plantations (Fig. 14.1). In many countries, traditional and local practices of selection and propagation of cultivars by farmers are being replaced by a few cultivars multiplied by nurseries. Finally, olive oil is now considered an important agricultural product for health, and its consumption has steadily increased. Extensive and comprehensive books on olive growing have been published in Italy (Fiorino 2003) and Spain (Barranco et al. 2017). An earlier edition of the latter book has been translated into English (Barranco et al. 2010).

During this time of change, the conservation and sustainable use of genetic resources and breeding have become necessary. For the first time in history, olive growers face an increasing risk of genetic erosion, and coordinated efforts to establish an International Network on Olive Germplasm Banks have been developed by the IOC since 1994 (Rallo et al. 2011). Additionally, the need for new improved cultivars has prompted the first olive-breeding programs in the world (Bellini et al. 2002a; Lavee 1990). Furthermore, recent advances in genomics and biotechnology (Rugini et al. 2016) will allow early genetic selection of progenies and the development of transformation protocols. This review focuses on the state of the art of the conservation of cultivated germplasm and breeding with a particular focus on crossbreeding and the pervasive concern of how to reduce the time needed to create and release new cultivars.

14.2 The Genetic Resources: First Strategy for Breeding

Exploration, conservation, evaluation and sustainable use of genetic resources have become a priority in most plant species of interest for agriculture and food. The diversity of cultivars in olive-growing countries represents a legacy of genetic variability from more than 5500 years of cultivation for the breeding and future growth of olives.

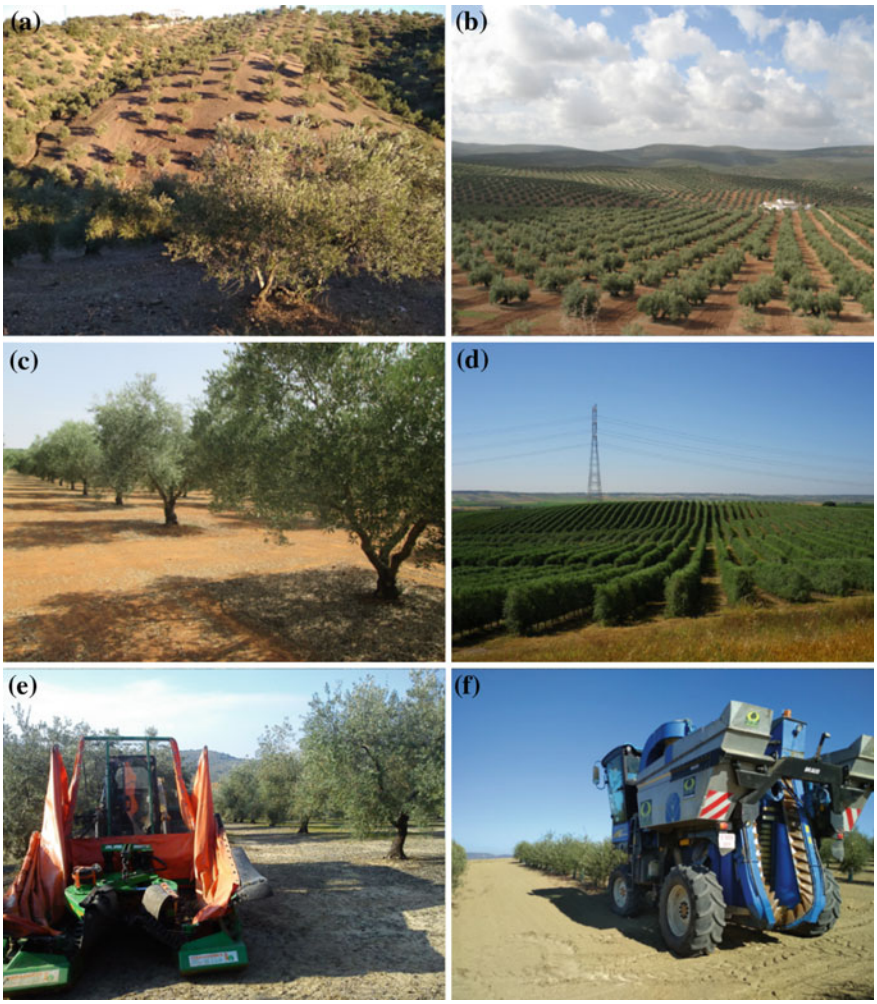

Fig. 14.1 Traditional and new olive plantations. **a** Traditional rainfed orchard on high-slope soil, **b** Traditional rainfed orchard on low-slope soil, **c** Intensive high density irrigated orchard, **d** Narrow hedgerow (super intensive) orchard, **e** Mechanical harvest in intensive orchard, **f** Mechanical harvest in super intensive orchard. Photos **a**, **c**, **e**, **f** by P. Valverde; **b** by D. Barranco; **d** by D. Cabello

A recent, extensive and updated review of olive genetic resources (Belaj et al. 2016) stated, as a first conclusion, that "*A better knowledge, management, and exploitation of cultivated, wild, and ancient trees are still needed, establishing common protocols for the molecular identification and for cultivar naming*,". This section will focus on the works exploring the conservation of true-to-type cultivars, which have been historically used as the first strategy for breeding. However, this critical aspect is still missing in olive cropping. Furthermore, true-to-type cultivars repre-

sent an urgent and compulsory requirement for olive growing in the world due to the global exchange of plant material. Within the requirement of trueness to type, we also review the works on *clonal selection* in olive.

For other aspects of the management of genetic resources, we refer the reader to the above-cited review (Belaj et al. 2016).

14.2.1 Exploring and Cataloguing Cultivars

To date, efforts to explore and catalogue true-to-type olive cultivars in different countries are incomplete. Until the beginning of the twentieth century, only a few scattered studies on olive cultivars had been published. At the VII Congreso Internacional de Olivicultura (olive growing) held in Seville in 1924, Coupin proposed a world catalogue of cultivars, their agronomic evaluation, and the need to breed new cultivars. However, catalogues of true-to-type cultivars are still pending in many countries; the common drawback of establishing synonyms and homonyms has not yet been solved in most cases. Furthermore, agronomic evaluation of cultivars is fragmentary and confusing because traits are assigned to varietal denominations (accessions) rather than to specific cultivars (Bartolini et al. 1998). As such, olive breeding is still in its infancy.

14.2.2 Olive World Germplasm Bank (OWGB) of Córdoba, Spain

The case of exploring and cataloguing true-to-type olive cultivars in Spain represent a long-lasting work on the exploration, cataloguing and conservation of true-to-type cultivars (Rallo et al. 2005). Initial works before the 1970s (Priego 1935) demonstrated incomplete sampling and a lack of representativeness of the characterized plant material, insufficient description methodologies and confusion in cultivar naming.

The Olive World Germplasm Bank of Cordoba (OWGB) was established in 1970 on the Alameda del Obispo Farm of INIA (National Institute for Agrarian Research), as a first attempt toward the conservation of olive cultivated genetic resources through an FAO-INIA project. Since the beginning, this collection has been cared for by INIA and the University of Córdoba (UCO). The collection was enlarged through prospecting surveys and cataloguing of cultivars in Andalucía (Barranco and Rallo 1984), Spain (Barranco et al. 2005a) and other international prospecting surveys and by exchanges with other germplasm banks (Caballero et al. 2006). The Alameda del Obispo collection was incorporated into the Andalusia Institute for Agricultural and Fishing Research (IFAPA in 2003 (Fig. 14.2a) and new exchanges with other banks have increased its accessions (Belaj et al. 2016). In 2011, a duplicate of true-to-type

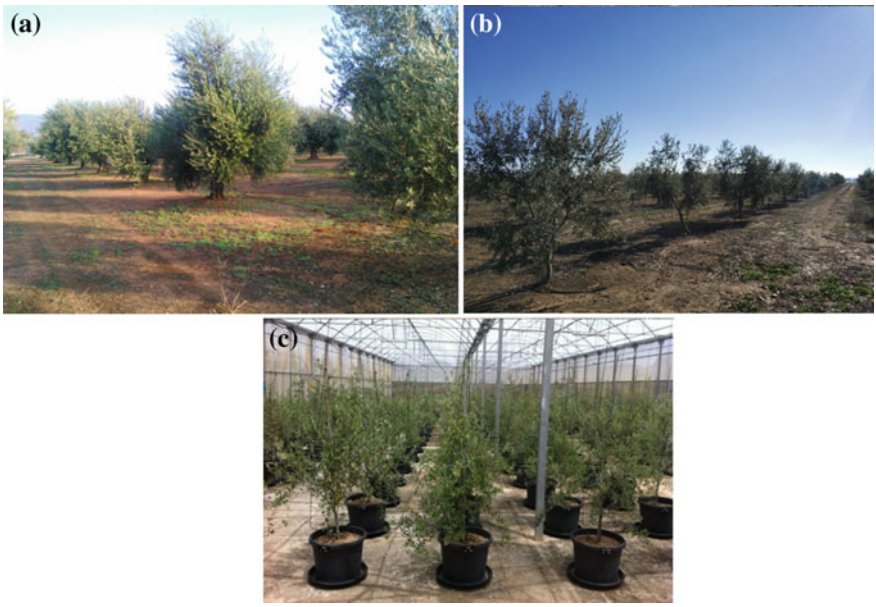

Fig. 14.2 The Olive World Germplasm Bank (OWGB) of Córdoba, Spain. **a** Collection in Alameda del Obispo Farm (IFAPA), **b** Collection in Rabanales Farm (UCO), **c** Isolated repository of true-to-type and pathogen-free cultivars (CAP-UCO-IFAPA). See text above. Photos: **a** by P. Valverde; **b**, **c** by P. Morello

cultivars of this collection was established in the UCO experimental Rabanales Farm in a soil free from *Verticillium dahliae* to guarantee the conservation of the cultivated genetic resources in the OWGB (Fig. 14.2b). In 2009, UCO, in cooperation with the CAP (Andalusia Agricultural and Fishing Administration) and IFAPA, established an isolated repository to conserve true-to-type and pathogen-free authenticated commercial accessions of the Alameda del Obispo collection (Fig. 14.2c). An agreement between the CAP, UCO and IFAPA for the management of the OWGB was signed in 2013. The OWGB was acknowledged by the International Olive Council (IOC) as an International Bank of Reference of its Network of Germplasm Banks in 2015.

14.2.3 Identification and Authentication

The identification of existing cultivars represents the first step in their cataloguing. Naming is the main difficulty in olive-cultivar identification because it has been historically based on common morphological traits (particularly of the fruit), toponyms or practical uses of varieties (Barranco and Rallo 1984; Barranco et al. 2005a). Until the 1980s, only morphological descriptors were used for identification purposes. The effect of environment on the expression of these traits was the main shortcom-

ing for the use of these characteristics. However, morphological descriptors have been a useful tool for naming true-to-type cultivars, synonyms, homonyms and inaccurate denominations for the most explored and sampled genotypes in Andalusia and Spain (Barranco and Rallo 1984; Barranco et al. 2005a), also establishing criteria for unambiguously naming genotypes carrying the same name (homonyms). Both catalogues represent the most complete morphological characterization of Spanish cultivars listing true-to-type cultivar names, homonyms, synonyms and inaccurate denominations. To distinguish among different genotypes carrying the same name, these authors proposed to add to the generic name of the cultivar the site of main diffusion (e.g. Manzanilla de Sevilla, Manzanilla de Jaén and Manzanilla Cacereña). True-to-type cultivars catalogued in this work were planted in the ex situ collection at Alameda del Obispo in Córdoba.

The use of molecular markers for genotyping olive cultivars started with isozymes in the 1980s (Pontikis et al. 1980; Trujillo et al. 1995). Molecular markers have provided a powerful tool to manage ex situ germplasm and distinguish between genotypes. The advent of DNA markers and their use for genotyping olives started in the mid-1990s with random amplified polymorphic DNA (RAPD) (Bogani et al. 1994; Fabbri et al. 1995).

Belaj et al. (2016) reviewed the use and DNA markers for molecular identification, the most used tool for studies of genetic variability worldwide. Their use has facilitated the management of the OWGB (CAP-UCO-IFAPA) (Table 14.1), evidencing duplications, synonyms and homonyms (Atienza et al. 2013; Belaj et al. 2003a, c; Fendri et al. 2010; Noormohammadi et al. 2007; Trujillo et al. 2014). The complementary use of SSRs and morphological characteristics of the endocarp have proved to be a powerful and discriminant method to authenticate accessions (true-to-type cultivars), duplications, homonyms, synonyms and inaccurate denominations in the OWGB of Córdoba (Trujillo et al. 2014). Comparison of endocarps between genotypes and true-to-type control samples allows the discrimination of molecular variants of a cultivar (i.e. genotypes with very high SSR similarity and undistinguished by endocarp morphological traits) and different cultivars (genotypes with a very high SSR similarity and very different endocarp characteristics) as shown in Fig. 14.3.

14.2.4 The International Olive Council (IOC) Network

Prospecting surveys in many countries and the exchanges of cultivars between countries have contributed to the very high number of conserved accessions in ex situ collections. Bartolini et al. (1998, 2008) reviewed for FAO the accessions conserved in approximately 100 regional and national collections in 53 countries, which include more than 4000 accessions. This inventory is indicative of the cultivated accessions conserved worldwide. However, the criteria for the choice of accessions are diverse, and their identification and authentication are still lacking in most collections. We currently do not know whether such accessions correspond to true-to-type cultivars

Table 14.1 Works on the exploration, cataloguing, conservation and authentication of olive cultivars jointly developed by the University of Córdoba and the Andalusia Institute for Agricultural and Fishing Research (IFAPA)

Years	Description	References
From 1971	*Exploration, cataloguing and conservation* Exploration (511 sampled trees in 83 localities), cataloguing by morphological descriptors (55), establishment of differentiated cultivars (156) and of synonyms and homonyms between the initial names (197) and conservation of Andalusia cultivars in the OWGB (CAP-UCO-IFAPA) of Córdoba	Barranco and Rallo (1984)
	Exploration in the rest of Spain (489 sampled trees in 196 localities) cataloguing by morphological descriptors (28), establishment of differentiated cultivars (262) and of synonyms and homonyms between the initial names (501) and conservation of Spain cultivars in the OWGB (CAP-UCO-IFAPA) of Córdoba	Barranco et al. (2005a)
	Exploration and genetic diversity of monumental olives in Andalusia and wild olives in Spain	Belaj et al. (2007, 2010, 2011), Diez et al. (2011, 2015)
From 1996	*Use of Molecular Markers for studies on genetic variability* Isozymes. First study on the genetic variability of the current accessions of the OWGB of Córdoba (CAP-UCO-IFAPA)	Trujillo et al. (1995)
	RAPDs. Further studies on the genetic variability of the current accessions of the OWGB of Córdoba (CAP-UCO-IFAPA)	Belaj et al. (2001, 2002, 2004b, c)
	RAPDs and AFLPs. Intra-cultivar variability in Manzanilla de Sevilla and Arbequina	Belaj et al. (2004a)
	SSRs Studies on the management of genetic variability in accessions of the OWGB of Córdoba (CAP-UCO-IFAPA)	Atienza et al. (2013), Belaj et al. (2012), Muñoz-Diez et al. (2012), Rallo et al. (2000a)
	Authentication 332 different cultivars identified from 823 trees representing 499 accessions from 21 countries of the OWGB of Córdoba (CAP-UCO-IFAPA) were characterized with 33 SSRs and 11 morphological characteristics of the endocarp. 200 cultivars were authenticated by comparison of SSRs and endocarp profiles with authentic control samples. 130 SSRs genotypes were considered molecular variants because they showed minimal molecular differences, but the same morphological profile, compared with 48 catalogued cultivars. 15 previously described and 37 new cases of synonyms as well as 26 previously described and seven new cases of homonyms were reported	Trujillo et al. (2014)
	Core collections SSRs, DARTs SNPs and morphological traits. Accurate definition of genetic variability and definition of core collections in the OWGB	Atienza et al. (2013), Belaj et al. (2012), Muñoz-Diez et al. (2012)

True-to-type cultivars are conserved in the Olive World Germplasm Bank (OWGB) of Córdoba

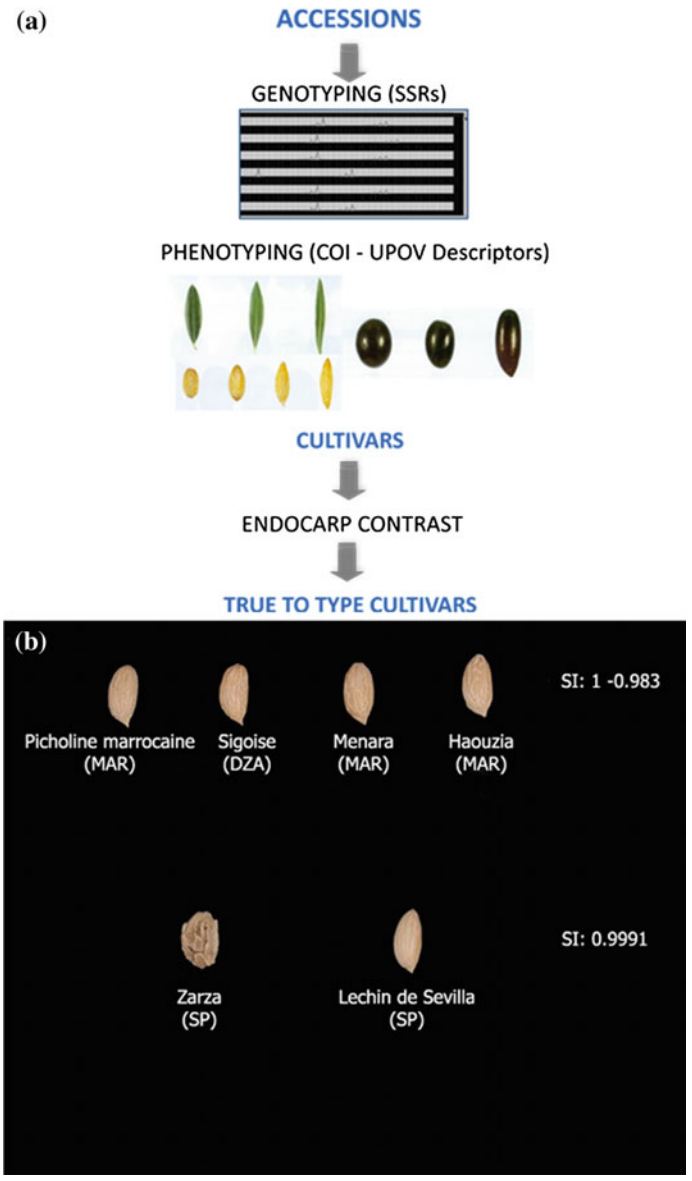

Fig. 14.3 Identification **(a)** and authentication **(b)** in the Olive World Germplasm Bank (OWGB) of Córdoba. Photo **b** from: Trujillo et al. (2014) used by permission of Springer

and both corresponding homonyms, synonyms and inaccurate denominations. Consequently, data on the phenotyping of many agronomic traits in these collections are not concordant for the same denominations, thus generating confusion. This finding

demonstrated a need to authenticate the accessions as a first step in any collection to avoid inconsistent phenotyping data in many publications based on the names of the accessions in any collection.

Since 1994, the IOC has promoted a network that currently includes National Germplasm Banks in 22 countries focusing on the exploration and conservation and cataloguing of local cultivars. The network conserves more than 1100 accessions, which were collected and have been partially characterized http://www.internationaloliveoil.org/resgen/index.html). The IOC published a first partial World Catalog of Cultivars (Barranco et al. 2000), an incentive publication of some additional national catalogues (Barranco et al. 2005a; Hosseini-Mazinani et al. 2013; Mendil and Sebai 2006; Moutier et al. 2004; Muzzalupo et al. 2010; Trigui et al. 2002, 2006).

The IOC has recognized three International Olive World Germplasm Banks in Córdoba, Spain, Marrakech, Morocco and Izmir, Turkey. These banks are enriched by the interchange of material with other banks, particularly those of the IOC Network. Currently, the OWGB of Córdoba conserves more than 1000 accessions from 25 Countries (Belaj and Barranco, pers comm), the OWGB of Marrakech conserves 591 accessions that have been partially identified (El Bakkali et al. 2013; Haouane et al. 2011) and the OWGB of Izmir has established 183 identified cultivars from 13 different countries to date (Gurbuz, pers comm).

Currently, the IOC aims to authenticate the accessions of all collections and to conserve them true-to-type and pathogen-free in all the acknowledged National Germplasm Bank of its network. Thus, the IOC has recently proposed a project to the UCO to cooperatively authenticate the accessions of the National Germplasm Banks by the morphological and SSR protocol proposed by Trujillo et al. (2014). This initiative will also guarantee the absence of devastating effects by diseases such as verticillium wilt (Jiménez-Díaz et al. 2012) and *Xylella fastidiosa* (Saponari et al. 2013). Thus, this project will ensure, over the short term, the conservation of true-to-type and pathogen-free of: (a) main commercial cultivars of the world in a first step. Afterwards other projects will progressively authenticated accessions in the IOC Networks.

14.2.5 Clonal Selection

Most of the traditional olive varieties grown today are the result of the selection of singular seedlings from wild, feral or cultivated trees with outstanding characteristics such as a larger fruit size, greater production, higher oil content or adaptation to certain climatic zones, among others, and the clonal propagation of those trees (Barranco and Rallo 1984). All current cultivars are in fact selected clones.

There is evidence that cultivars often contain somatic mutations. This phenomenon has been described in several fruit crops, such as grape, apple, peach, pear and plum (Badenes and Byrne 2012). Somatic mutations might not have phenotypical consequences but they might also become the basis for new varieties, as it has been

the case for citrus and grape, among others (Moore 2001; Pelsy et al. 2010; This et al. 2006).

Somatic mutations have also been reported in olives by using different molecular markers such as RAPD, AFLP and SSR (Charafi et al. 2008; Cipriani et al. 2002; Diez et al. 2011; Garcia-Diaz et al. 2003; Nikoloudakis et al. 2003; Trujillo et al. 2014). These mutations were responsible for subtle genetic differences among several genotypes and their closest cultivar (Diez et al. 2011; Trujillo et al. 2014). No morphological differences were observed among these genotypes and they were considered *molecular variants* of the standard genotype (Trujillo et al. 2014).

Variability within a cultivar due to somatic mutations should not be confused with a case of homonym; this is the existence of different cultivars sharing the same name. Clonal mutations happen at a very low rate and normally affect few loci (Heinze and Fussy 2008; Sarkar et al. 2017) leading to very close genetic similarity indexes (similarity index ~0.8 to 0.9) between the clones and the cultivar from which they derived. Because of this reason, the description of extensive genetic variation within a putative cultivar (Gemas et al. 2004; Martins-Lopes et al. 2009) should be treated with caution. In these cases, an accurate genetic, morphological and agronomical characterization of the accessions involved (Ben-Ari et al. 2014) should be required to corroborate the clonal origin of the observed variability.

Sampling surveys aimed to look for phenotypic diversity within olive cultivars showed that intra-cultivar phenotypic diversity seems to be an unusual phenomenon in olive. Since the 1970s, clonal selections within the main olive cultivars have been performed, such as cvs. Manzanilla de Sevilla, Picual, Arbequina and Arbosana in Spain (Garcia Berenguer 1988; Suarez et al. 1990; Tous et al. 1999); Chemlali de Sfax in Tunisia (Kamoun et al. 2002; Khlif and Trigui 1990); Picholine Marrocaine in Morocco (Boulouha 1986); Moraiolo, Canino, Biancolilla, Giarraffa Moresca, Cresuola, Tonda Iblea and Nera in Italy (Caruso et al. 2014; D'Hallewin et al. 1990); Souri in Israel (Ben-Ari et al. 2014; Lavee et al. 2008); and Cobrançosa, Santulhana, Verdeal Trasmontana, Azeitera, Blanqueta, Carraquenha, Redondil, Galega Vulgar and Macanilha Algarvia in Portugal (Fernandes Serrano 1990; Gomes et al. 2008; Leitao et al. 1999; Martins et al. 1998).

All these works were based on the selection of outstanding trees for some agronomic traits, usually the production or fruit size, within major cultivars in various locations. Comparison of these phenotypic selections in one comparative field trial with few replications did not provide a consistent genetic basis for selection.

To date these works have provided few new commercial selected clones. To our knowledge, only Arbequina IRTA i.18^{R} (Tous et al. 1999, 2005) selected from cv. Arbequina trees in Catalonia and Haouzia, Menara and Dhabia cvs. selected from cv. Picholine Marocaine trees in three distinct locations (Amal Hadiddou, pers comm) have been commercially propagated. However, Arbequina i.18^{R} did not show any significant difference, with the standard Arbequina in long-term experiments at 7 and 14 years after planting (De la Rosa et al. 2007; Diez et al. 2016). Additionally, Amal Hadiddou (pers comm) did not observe significant differences between Haouzia, Menara Dhabia and Picholine Marocaine cvs. in yield and oil content in 5-year crops in a comparative field trial in Marrakech. In both cases, only minor differences

for DNA markers leading to very close similarity indexes were detected. These differences were not associated with any morphological difference (Belaj et al. 2004a; Trujillo et al. 2014) (Fig. 14.3).

14.3 Crossbreeding

The first olive crossbreeding programs in the world were initiated between 1960 and 1971 in Israel (Lavee 1990) and Italy (Bellini et al. 2002a), respectively. Since the 1980s, new crossbreeding programs have been developed in different countries (Arias-Calderon et al. 2014; Bellini 1992; Dabbou et al. 2012; Lavee 2012, 2013; Lavee and Avidan 2011; Ozdemir et al. 2016; Rallo 1995; Rallo et al. 2008b; Trapero et al. 2013a; Zeinanloo et al. 2009). Most of the programs aim to obtain cultivars for olive oil, and only a few for table olives or both, with a special focus on early bearing, high yielding and adaptability to the new planting systems designed for mechanical harvesting (Rallo 2014a; Rallo et al. 2013). Recently, breeding programs are underway for resistance to verticillium wilt (Arias-Calderón et al. 2015a, b; Trapero et al. 2013a, b, 2015). Pre-breeding evaluation of cultivars and new genotypes from breeding programs for resistance to the bacteria *Xylella fastidiosa* are also under development (Landa B pers comm).

A previous revision of the olive-breeding protocol has been published (Rallo et al. 2011). Recently, Rugini et al. (2016) and Rugini and De Pace (2016) published two protocols focusing on biotechnological approaches.

14.3.1 Strategies for Crosses

The design of crosses requires previous knowledge of the genetics of the desired traits and information on pollen pistil compatibility. In their absence, the initial crosses of the current programs include progenitors with the desired phenotypic traits to incorporate or complement the other selected progenitors. In these programs, the progenitors are cultivars derived from the same or different regions of origin. Recently, crosses of cultivated olive (*Olea europaea* ssp. *europaea* var. *europaea*) with wild olive (*O. europaea* ssp. *europaea* var. *sylvestris*) and with other subspecies (*O. europaea* ssp. *cuspidata* and *O. europaea* ssp. *laperrini*) have been reported (Caceres et al. 2015; Klepo et al. 2014; Trapero et al. 2015), with the aim of enlarging the basis of genetic variability for olive breeding.

Olive is an allogamous species, in which self-incompatibility is the general rule with some cases of inter-incompatibility (Díaz et al. 2007; Koubouris et al. 2014; Rodriguez-Castillo et al. 2009; Seifi et al. 2011; Selak et al. 2014; Wu et al. 2002). The pollen tube of alien pollen grows faster than the pollen of the same genotype, triggering an earlier growth of both the seed and the fruit in cross-pollination than in self-pollination (Rallo et al. 1990). Therefore, emasculation it is usually not nec-

essary for performing crosses. However, there may be some problems related to contamination by undesirable alien pollen due to the fast and long distance airborne transport of pollen that may have contaminated the limbs before they were bagged (De la Rosa et al. 2004; Díaz et al. 2007; Rallo 2000b). Currently, paternity testing by different SSRs is being used in breeding programs (De Rosa et al. 2013; Díaz et al. 2007), representing a compulsory step for any genetic study.

14.3.2 Strategies to Shorten the Juvenile Phase

The time elapsed from seed germination to first flowering of a seedling is known as the *juvenile phase* (JP). This phase is usually very long in woody species and represents one of the major drawbacks for olive tree breeding by crossings since the JP in this species can reach up to 15–20 years (Bellini 1992; Fontanazza and Baldoni 1990). For example, in an olive crossbreeding program initiated in the early 1970s in Italy, many seedlings were still juveniles 25 years after planting (Bellini et al. 2002a). The cost of maintaining in the field a large number of trees that will take many years to flower is extremely high. Consequently, many efforts have been undertaken in different olive-breeding programs to shorten the JP to initiate evaluations of fruit traits in the progenies as soon as possible (Lavee et al. 1996).

The first strategies to shorten the JP consisted of forcing the growth of the seedlings to achieve a certain size in a short time, since the onset of first flowering in many woody species is reported to be related to the size of the plant (Hackett 1985). Santos-Antunes et al. (1999, 2005) established a forced-growth protocol in which seedlings are grown under continuous light and fertigation in the greenhouse for approximately 7–8 months, after which the plants are transplanted to field conditions. This protocol has been shown to be very efficient, obtaining approximately 50% of flowering seedlings by the third year (Fig. 14.4). Other management techniques to reduce JP have been studied regarding plant canopy height (Moreno-Alías et al. 2010a) or soil solarization (El Riachy et al. 2011), among other traits.

The relationship between seedling growth traits and the time of first flowering has been widely studied (De la Rosa et al. 2006; Pritsa et al. 2003; Rallo et al. 2008b; Santos-Antunes et al. 2005) to establish preselection criteria to cull seedlings with a long juvenile period in early stages, before transplantation to the field. Seedling vigor traits, mainly plant height and stem diameter, measured after the forced growth period in the greenhouse are strongly correlated to the earliness of first flowering and, thus, are currently used in breeding programs to preselect the seedlings to be planted. Threshold values based on a specific seedling height (approximately 100 cm; Moreno-Alías et al. 2010b) have been proposed, whereas Rallo et al. (2008b) suggest culling up to 35–40% of the shortest seedlings since growth performance may vary among progenies and years.

Differences in the earliness of first flowering among progenies have been reported, indicating a strong influence of the genitors on the length of the JP of the progenies (Bellini et al. 2002a; De la Rosa et al. 2006; Moral et al. 2013; Rallo et al. 2008b;

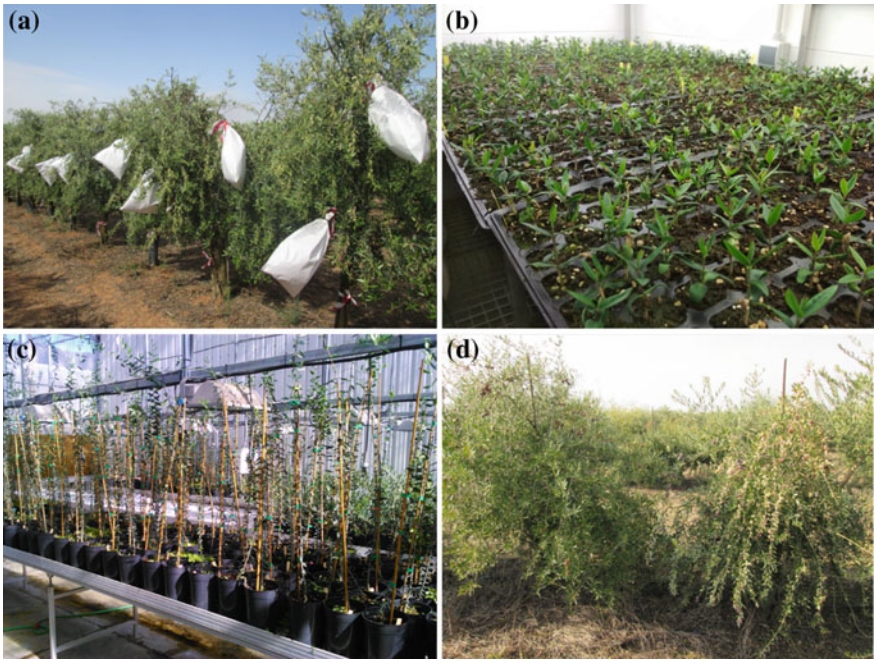

Fig. 14.4 Steps to shorten the juvenile period. **a** Cross, **b** Seed germination, **c** Forcing seedlings growth in greenhouse under continuous light, **d** Forcing growth in field until first flowering and fruiting. Photos by P. Valverde

Santos-Antunes et al. 2005; Suárez et al. 2011). Thus, parental selection of certain genitors is a good strategy to accelerate first flowering in progenies. Furthermore, the unproductive period of genotypes in the adult stage has been positively associated with the JP of the same genotypes (Leon et al. 2007). Therefore, early bearing is enhanced when selection for a short JP is performed, and similarly, the use of early-bearing parents will help to reduce the length of the JP of progenies.

The transition from the juvenile to the adult stage is not yet well understood in the olive tree. As described by Hackett (1985) and Hartmann et al. (2002), phase changes in woody species follow a cone-like pattern, known as the juvenility cone, during which the basal and inner parts of the adult seedling remain juvenile. In the case of the olive tree, the position of the first flower in seedlings has been studied as a marker of the end of the JP, and a juvenile cone has been confirmed in this species (Moreno-Alías et al. 2010a; Suárez et al. 2011). The attainment of a certain distance from the root to the meristem is required to flower, which was determined at approximately 200 cm for olive by Moreno-Alias et al. (2010a), although significantly different values among progenies were reported by Suarez et al. (2011). The latter authors also found correlations with the length of the juvenile period such that early flowering genotypes required a lower minimum distance to the ground to overcome juvenility.

In addition to the onset of flowering, other markers of the phase change from the juvenile to the adult stage have been studied, including proteins, morphological and anatomical traits and NIR (near-infrared) spectra of leaves, and rooting ability (Casanova et al. 2014; Garcia et al. 2000; Leon and Downey 2006; Moreno-Alías et al. 2009). Recently, much effort is being focused on the discovery of genes involved in the transition from juvenile to the adult stages and in flowering in olive species (Fernández-Ocaña et al. 2010; Garcia-Lopez et al. 2014; Haberman et al. 2017; Jiménez-Ruiz et al. 2015; Muñoz-Mérida et al. 2013; Sgamma et al. 2014). A better understanding of the complex genetic control of the juvenile-adult phase change will elucidate new avenues for the development of molecular tools for early transcriptomic selection of short JP seedlings.

14.3.3 Strategies for the Evaluation of Genotypes: Crossbreeding Steps

Crossbreeding in fruit trees is an extended process due to the length of the JP and the required multistep protocol to evaluate seedling progenies and their successive clonally-propagated selections (Hancock 2008). After crossbreeding pollination, seed germination and forced seedling growth to shorten the JP, selected genotypes are submitted to several selection steps for further evaluation. In each step, the number of genotypes is reduced and the number of replications per genotype and characters under evaluation increases up to the final release of new cultivars. The total number of evaluated genotypes and selection steps are usually determined by the available human and financial resources and facilities.

Figure 14.5 shows a general overview of the selection steps followed at the Spanish olive-breeding programs: UCO-IFAPA (University of Córdoba- Andalusia Institute for Agricultural and Fishing Research) and US (University of Sevilla).

Two alternative protocols are used. Two steps of evaluation in the first crosses (Rallo et al. 2016a; Fig. 14.5 blue arrow): (1) for progenies, and (2) a final step for selected genotypes. As an example, 15 of 748 genotypes were selected in the first step of evaluation, and one new cultivar (Sikitita/Chiquitita) was registered after a comparative trial, 15 years after plantation of the progenies (Rallo et al. 2008a). After some additional comparative trials, two selections will soon be registered. In the USA's table olive breeding program, 40 of 1800 genotypes were selected in the first step, 20 of which are currently in field trials for final evaluation (Rallo P, pers comm).

The second protocol proposes three steps (Leon et al. 2015; Figure 14.5 (red arrows): (1) initial progenies, (2) intermediate for preliminary selections and (3) final for advanced selection. Following this protocol, 108 of 1548 genotypes were selected in the first step, 14 of which remained after the intermediate step (15 years after plantation of the progenies). The final evaluation of the 14 advanced selections in comparative field trials ended approximately 20 years after planting of the seedlings.

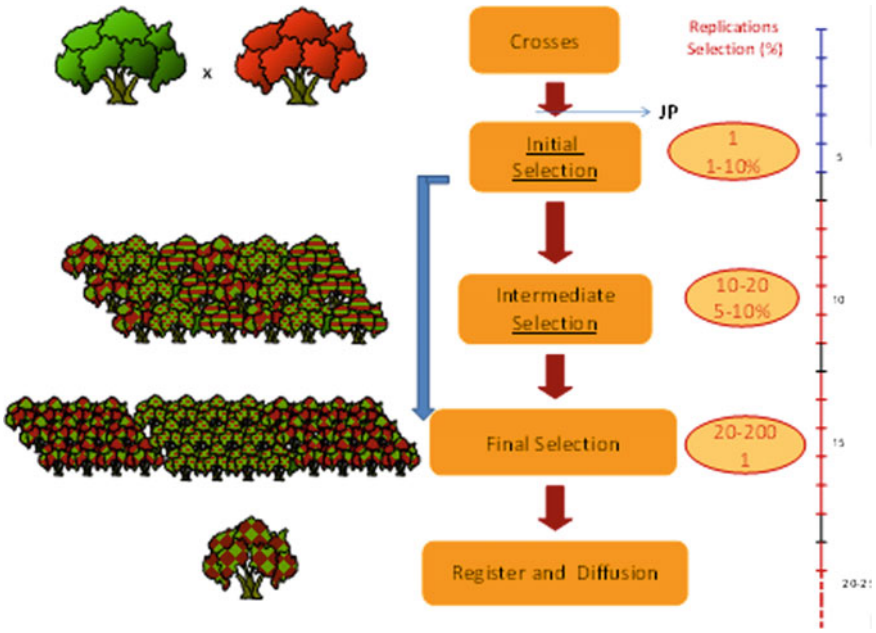

Fig. 14.5 Proposed breeding scheme in olive breeding programs in two (blue arrow) or three (red arrows) selection steps. JP: Early selection for the short juvenile phase

These data indicate that the choice between both protocols will depend on available human, financial and logistic resources. Cooperation with stakeholders may facilitate a two-step protocol and accelerate the breeding process (Fig. 14.7c).

14.3.3.1 Step 1: Initial (Evaluation of Progenies)

Initially, the breeder has to manage a large number of non-replicated genotypes per cross. Rigorous criteria and techniques for early or indirect selection according to the main objectives of the program must drastically reduce the initial population of genotypes in this first step to save time, labor and money. The earliness of bearing, oil content and composition and adaptation to new plantation systems are systematically evaluated in all progenies in all crosses of the UCO-IFAPA Breeding Program (Rallo et al. 2016a).

Early criteria for selection in the first period of seedling growth must be a general concern in any olive breeding program. Early selection tests have also been an excellent and general strategy to shorten the JP and to eliminate late flowering genotypes in any cross (see Sect. 14.3.2). Additionally, early selection for *Verticillium dahliae* resistance has be conducted in 2-month-old seedlings, eliminating more than 90% of them for later evaluation in field plantations in highly infested soils (Trapero

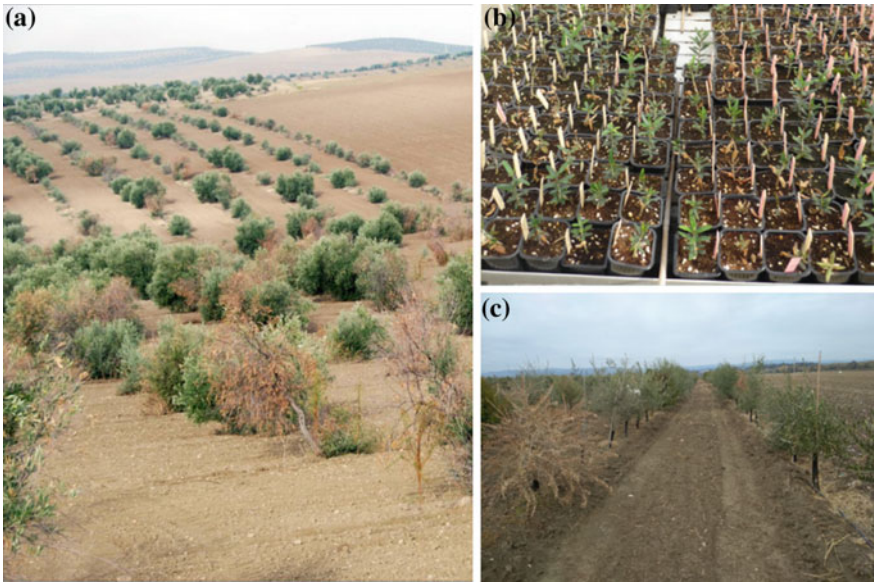

Fig. 14.6 Two-step selection for verticillium wilt resistance. **a** Young orchard devastated by this disease, **b** Evaluation of inoculated seedlings in greenhouse, **c** Evaluation of pre-selected seedlings in field. Photos:**a** by C. Trapero; **b**, **c** by P. Valverde

et al. 2013a) (Fig. 14.6). Currently, newly-developed genomic tools are providing some efficient tests for early genetic-based selection of relevant agronomic traits (see Sect. 14.4).

The evaluation of progenies usually persists for 5 years, i.e. the three first crops after overcoming the JP. Many traits have been evaluated in the initial step. Earliness of bearing (see Sect. 14.3.2), plant architecture, oil content and composition (see Sect. 14.3.6) of breeding oil cultivars are usually assessed in the initial step based on values correlated with those in subsequent steps and on moderate to high heritability values (Ben Sadok et al. 2013, 2015; Leon et al. 2015). Additionally, different fruit traits have also been accurately evaluated during this initial step for breeding table olives (see Sect. 14.3.6).

The simplification of recording and the use of suitable methods to evaluate small samples have been used in the initial step. Categorical data for the number of flower seedlings have demonstrated a fast, easy and reliable record for earliness of bearing that has been routinely adopted for seedling evaluation (Leon et al. 2015). Non-destructive and multicomponent trait evaluation by NIR spectrometry has allowed the evaluation of oil content, fruit traits, ripening index and oil composition in a fast and accurate manner (Bellincontro et al. 2013; Leon et al. 2003, 2004a; Morales-Sillero et al. 2011) in small samples, particularly in fruits, allowing an efficient way to evaluate the progeny variability in any segregating population for multiple traits.

The evaluation of progenies also represents a basic step for genetic and genomic studies that are undergoing active development (see below and Sect. 14.4).

14.3.3.2 Step 2. Intermediate (Evaluation of Preliminary Selections)

Vigor and yield are critical traits for final selection. Intermediate steps attempt to evaluate these traits with some replicates to first obtain and compare records for preliminary selections that cannot be accurately evaluated in the initial step. Additionally, the intermediate step may confirm the consistency of previously evaluated traits in the initial selection step.

Correlations between data recorded for seedling and intermediate steps indicate that selection for the earliness of bearing, fruit size and oil content can be efficiently performed at the seedling stage, whereas evaluation at the intermediate step would be necessary to select for yield and vigor. Similar conclusions were obtained from analyses of heritability estimated for these characteristics at the intermediate step of selection. Additionally, a high number of genotypes was retained in the intermediate step, allowing large variability for both previously evaluated and non-evaluated traits such as fatty acid composition, tocopherol and phytosterol contents and the phytosterol profile (Leon et al. 2015).

14.3.3.3 Step 3. Final (Evaluation of Advanced Selections)

Genotype × environment (G × E) evaluation is the main objective of the final evaluation of advanced selections before new cultivars are registered. In addition to vigor and yield, the most important traits in any new cultivar, other traits related to the breeding objectives must be re-evaluated at this step in several locations. Therefore, cooperation with growers in different sites provides information about the selection performance for different environments to other growers from those zones, thus promoting new potential registered cultivars. The UCO-IFAPA program has developed this strategy since the first crosses. Currently, more than a dozen stakeholders, including nurseries, participate in this step of evaluation.

Different field experiments corresponding to initial, intermediate and final evaluations are shown in Fig. 14.7.

14.3.3.4 The Components of Variability

Research on the genetics of agronomic traits in olive is in its infancy. Nevertheless, studies examining the components of genetic variability in cultivars and genotypes from crossbred progenies have been increasing in recent years. Several patterns have been observed in these studies.

A large variability among genotypes has been a general pattern for any evaluated trait in cultivars from germplasm banks and in genotypes from any crossbred progeny.

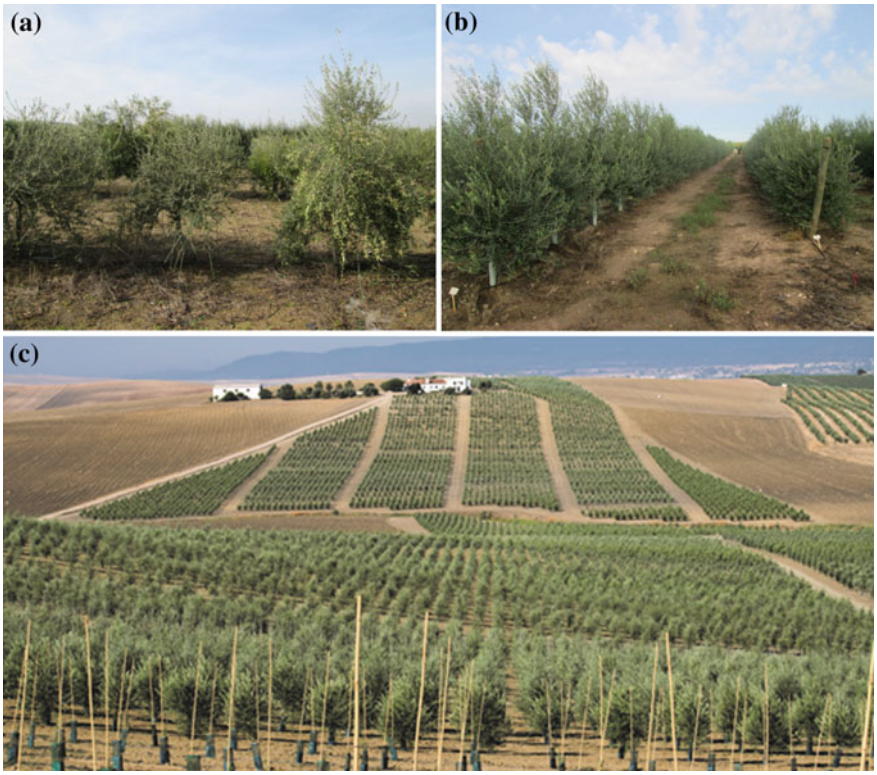

Fig. 14.7 Field evaluation steps for crossbreeding. **a** Initial evaluation of unreplicated genotypes from progenies, **b** Intermediate evaluation of preliminary soft selection, many genotypes with some replications, **c** Final selections of genotypes in a comparative precommercial field trial. Photos: **a**, **b** by P. Valverde; c courtesy of TODOLIVO

This is the case for all reported traits in this review (Sects. 14.3.2, 14.3.4–14.3.6). As a rule, the values of the descendants transgress the genitor values in the segregating progeny. In all analyzed models, the genetic factor within crosses is the main component of the variability of the evaluated traits, as expected due the extensive heterozygosity of this species. This is a favorable situation from a clonally-propagated species such as the olive.

Few studies have examined heritability in olive. Hassani and Tombesi (2008) measured the growth characteristics in the greenhouse of a full diallel cross among nine cultivars. The heritability of characters such as dry weight and internode length in progenies indicates high values, (0.75 and 0.97), respectively. Zeinanloo et al. (2009) designed one-way diallel crosses with six genotypes for nine quantitative characteristics. Broad sense heritability ranged from 0.31 to 0.86 and narrow-sense heritability from 0.17 to 0.28. Zard and Roghani cvs. were good combiners for fruit weight, percentage of dry matter, fruit length, fruit width, and percentage of oils.

Architectural traits have attracted attention due to the search for compact and early bearing trees for new high-density systems. Hammami et al. (2011, 2012) proposed five traits (main vertical axis, preferential distribution of lateral shoots, dominant length of lateral shoots, branch orientation and branch bending) as the most relevant descriptors for the olive seedling architecture based on the high capacity to indicate diversity, a strong influence of the parent genotype, a lack of correlation with each other, and a demonstrated value for agronomic performance. They also showed that cvs. Picual, Arbosana and Sikitita were promising cultivars for use as genitors because of their tendency to produce offspring with desirable growth habit traits.

Ben Sadok et al. (2013, 2015) studied the heritability of tree architecture and reproductive traits in a progeny of 120 trees from the Oliviere × Arbequina cross in two contrasting growing sites. After selection of a model, broad sense heritabilities were estimated. Despite strong environmental effects on most traits, no G × E interaction was found. Moreover, the internal structure of the trait covariation was similar in both sites. Ontogenetic growth variation, related to (i) the overall tree form and (ii) the growth and branching habit at growth unit scale was not altered by the environment. Finally, moderate to strong genetic control was identified for traits at the whole tree scale and at the internode scale. Among all studied traits, the maximal internode length exhibited the highest heritability ($H2 = 0.74$).

A detailed variance analysis of oil content components and fruit morphology traits in an olive progeny issued from the cross Oliviere × Arbequina (Leon et al. 2016) showed the lowest values of heritability for fruit moisture (0.40) and the highest values for oil content in fruit fresh weight (0.94). An estimation of heritability and environmental variation over varying numbers of years and tree replications revealed that the inclusion of more annual replications could be more effective than the addition of tree replications. Finally, this study provides a global view of adequate spatial and temporal replications required to accurately develop criteria for both conventional and marker-assisted selection on fruit traits from olive crossing progenies.

An evaluation of resistance to the defoliating pathotype of *Verticillium dahliae* Kleb was performed in 12 cultivars and 52 genotypes previously selected from a wider initial population based on their agronomic performance (Arias-Calderon et al. 2014) and 6017 genotypes derived from 48 crosses obtained by open pollination and crosses between olive cultivars, wild olive genotypes and other *Olea* species and *Olea europaea* subspecies (Trapero et al. 2015). In both studies, high genetic variability and wide segregation of resistance were observed. The estimates of heritability suggest that it is possible to breed for verticillium wilt resistance in the olive.

14.3.4 Strategies for Tolerance to Abiotic Stress Selection

Environmental adaptation is a cornerstone to successfully grow a crop in a specific region. Olive cultivars that are able to tolerate many abiotic limiting factors such as cold, heat, water stress or nutrient-deficient soils can be found due to uninten-

tional selection over thousands of years. However, these cultivars often lack other desired agronomic characteristics. Although the combination of both characteristics seems possible, the development of new cultivars with increased tolerance to abiotic stresses is not a major objective in current olive breeding programs worldwide, and research studying the genetic variability of tolerance to abiotic stresses is generally not extensive. Rootstock breeding also appears to be an opportunity to overcome some of these abiotic stresses.

Olive growth and productivity can be seriously limited by low temperatures, especially temperatures below −7 °C (Palliotti and Bongi 1996). Physiological responses related to cold damage in olive have been identified (Ruiz et al. 2006), and methods to quickly assess tolerance to damaging temperatures have also been established (Azzarello et al. 2009). This screening method, combined with the existence of genetic diversity regarding tolerance to cold conditions (Barranco et al. 2005b; Bartolozzi and Fontanazza 1999; Gómez-del-Campo and Barranco 2005), suggests that breeding for this trait should be feasible.

Chilling requirements in olive is an increasing limiting factor because of the expansion of olive plantations to new areas (Rapoport 2014), as well as the global temperature increase in established olive growing areas (Gabaldón-Leal et al. 2017). This trait is genetically dependent on field observations (Hartmann and Porlingis 1957; Zouari et al. 2017) and modeling (Garcia-Mozo et al. 2009). A method to determine the dormancy period has been recently developed (Ramos et al. 2018) and could accelerate the identification of previously initiated olive genotypes with low-chilling requirements (Cabello, unpublished data).

Olive is quite tolerant to drought stress in comparison to other tree species (Fernández and Moreno 1999). Some of the physiological factors that control drought tolerance are known (Sofo et al. 2004), and an important array of studies have reported genetic differences between olive cultivars in terms of tolerance to drought (Bacelar et al. 2009; Guerfel et al. 2007; Tugendhaft et al. 2016). Wild germplasm has also been identified as a possible source of tolerance to this abiotic stress (Besnard et al. 2012). Prospects for the breeding of this trait in the future are positive due to the development of a technique using chlorophyll fluorescence to screen for this factor (Faraloni et al. 2011) and the identification of a gene that enhances drought tolerance (Chiappetta et al. 2015). Tolerant genotypes may be used as rootstocks to alleviate this problem.

Chlorosis due to iron deficiency can result in important yield losses. This condition is common in plants growing in calcareous soils. Although olive species are considered quite tolerant in comparison to other plant species (De la Guardia and Alcántara 2002), they are grown in calcareous soils throughout the world and are therefore affected by iron chlorosis. Olive cultivars present notable genetic differences in their tolerance to this abiotic stress (Alcántara et al. 2003). As in other species, tolerance can be achieved by the use of rootstocks. A few other studies have reported genetic variability in the tolerance to other abiotic factors such as salinity (Marin et al. 1995) or heat stress (Mancuso and Azzarello 2002), although sources of tolerance have not been deeply investigated.

14.3.5 Strategies for Resistance to Biotic Agents

Diseases and pests have always been limiting factors for olive growing. Olive genotypes with a higher level of resistance to major pests and diseases have been selected intentionally and unintentionally for a long time, and therefore sources of resistance to a range of pests and diseases are available, as shown in Table 14.2. However, their use in breeding programs is limited due to other agronomic and adaptive traits that normally comprise the main breeding objectives (Leon et al. 2007). Breeding for disease or pest resistance aims to combine the best agronomic and adaptation traits with resistance to the desired disease or pest in a single genotype. It is a slow process due to the time necessary for plant generation evaluations, as well as the lack of knowledge about the mechanisms and genetics underlying the resistant expression (Johnson and Jellis 1992).

Verticillium wilt of olive is a vascular wilt caused by the soil-borne fungus *Verticillium dahliae*. It has recently become the main disease in many olive-producing countries and is threatening olive production in many areas (Jiménez-Díaz et al. 2012; López-Escudero and Mercado-Blanco 2011). Factors such as the inefficacy of chemical compounds to control the disease make the use of plant material that is resistant to verticillium wilt especially important. However, most of the olive cultivars evaluated to date have been identified as susceptible or extremely susceptible to this disease in the field (Trapero et al. 2013b) and under controlled conditions (López-Escudero and Mercado-Blanco 2011). For many years, efforts to develop new olive material that is resistant to *V. dahliae* have been quite limited (Hartmann et al. 1971; Wilhelm and Taylor 1965). However, due to recent increases in the importance of the disease in the last 10 years, verticillium-wilt resistance has been incorporated into breeding programs, sometimes as a major objective. Mass screening methodologies (Trapero et al. 2013a) have allowed the evaluation of large numbers of genotypes generated by open pollination and targeted crosses in an attempt to combine high levels of resistance and positive agronomic traits (Arias-Calderón et al. 2015b; Colella et al. 2008; Trapero et al. 2015). According to these studies, complete resistance to verticillium wilt in olive is unlikely to exist, although high levels of resistance have been identified in olive cultivars, wild populations and related *Olea* species (Table 14.2). New varieties with higher level of resistance are likely to be generated in the upcoming years, once they have been assessed for a number of years in fields infested by the pathogen. The selection of undomesticated material that is resistant to disease for use as rootstock should first be evaluated for efficacy for controlling the disease. Verticillium wilt resistance is believed to be highly polygenic (Leyva-Pérez et al. 2017) and molecular tools to select for this trait are currently not available.

The main foliar diseases attacking olive are peacock spot (caused by *Venturia oleginea*), anthracnose (*Colletotrichum* spp.), and cercospora leaf spot (*Pseudocercospora cladosporioides*). These pathogens cause tree defoliation, premature fruit drop and fruit rot, which can be devastating under favorable weather conditions (Viruega et al. 2011). Although traditionally managed by cultural and chemical methods, genetic resistance has been demonstrated to be highly effective, especially when

Table 14.2 Sources of resistance available against the major diseases and pests that affect olive and their current situation in breeding programs

Disease/Pest	Sources of resistance	Level of resistance	Breeding stage	References
Verticillium wilt	Cultivars	Moderate/high	Advanced phases	Arias-Calderón et al. (2015a), Trapero et al. (2015), Wilhelm and Taylor (1965)
	Wild genotypes	High	Rootstock (early phase)	Colella et al. (2008), Jiménez-Fernández et al. (2016)
	Related species	High	Rootstock (early phase)	Arias-Calderón et al. (2015b), Trapero et al. (2015)
Defoliating diseases	Cultivars	Moderate/high	Released/ advanced phase	Lavee et al. (1999), Moral et al. (2015)
	Wild genotypes	High		Xavier (2015)
Olive knot	Cultivars		Not applied	Penyalver et al. (2006), Young et al. (2004)
Xylella fastidiosa	Cultivars	Moderate/unknown	Not applied	Frisullo et al. (2014), Luvisi et al. (2017)
Olive fly	Cultivars	Moderate	Not applied	Garantonakis et al. (2017), Gonçalves et al. (2012), Iannotta et al. (2007), Malheiro et al. (2015)

disease pressure is high. The identification of sources of resistance has been based on both field assessment (Moral and Trapero 2009) and artificial inoculation methods (López-Doncel et al. 1999). Genotypes generated from crossings between varieties with different levels of resistance have been selected in several breeding programs (Ciccarese et al. 2002; Moral et al. 2015; Rhouma et al. 2013) and one cultivar has been released with a reported high level of resistance to peacock spot generated by the self-pollination of a moderately resistant cultivar (Lavee et al. 1999). The inheritance of resistance to these diseases is likely to be mostly polygenically controlled according to the cited studies.

Olive knot is a disease characterized by the formation of cankers on the olive trunk, branches and shoots. It is caused by the bacteria *Pseudomonas savastanoi* pv.

savastanoi. Although it is not a major objective in olive breeding programs, susceptible genotypes are usually identified in the field and culled. By using inoculation methods under controlled conditions, (Marcelo et al. 1999; Penyalver et al. 2006) identified a high level of resistance among olive cultivars (Young et al. 2004) that could be useful for breeding.

Xylella fastidiosa is a bacterium that infects and colonizes the xylem of a broad range of plant species and causes leaf scorch and *quick decline syndrome* in the olive. This disease was of little importance to olive until the recent outbreak in southern Italy (Saponari et al. 2013). Currently, the level of resistance of different cultivars is largely unknown, apart from recent field observations and studies using a small number of cultivars (Frisullo et al. 2014; Luvisi et al. 2017), which suggest a potential variability in resistance to the pathogen. *Xylella fastidiosa* is currently a major concern in Europe as it is likely to spread to other growing regions (Stokstad 2015). Therefore, breeding efforts are likely to arise in the near future.

The olive fly (*Bactrocera oleae*) is the major pest affecting olive worldwide, causing severe damage to fruits and reducing their quantity and quality (Daane and Johnson 2010). Differences in the resistance level of a number of olive cultivars have been reported in several studies using preference or oviposition experiments (Garantonakis et al. 2017; Gonçalves et al. 2012; Iannotta et al. 2007; Malheiro et al. 2015). Fruit traits such as size, maturity date and hardness usually explain a great proportion of the level of resistance, although Grasso et al. (2017) have shown that resistance to olive fly can also be induced. To date, resistance to this pest has not been targeted as a major objective by any olive breeding program.

14.3.6 Strategies for Quality

Quality is becoming a prime target for plant breeders since consumer demand is moving toward food products with improved sensorial, nutritional and nutraceutical value. Table olives and virgin olive oil, the two main products obtained from olive fruits, are staple foods of the Mediterranean diet, and the benefits of their consumption in human health have been extensively documented. Additionally, their extraordinary organoleptic properties are also responsible for their increasing demand worldwide (IOC 2017).

Olive quality is a wide and complex concept involving many traits that may be important for table olives, olive oil or both (Rallo et al. 2011, 2017). For instance, fruit appearance is essential for table olives, which encompass traits such as fruit size, shape, symmetry, color or absence of bruising, whereas oil content components and fatty acid profiles are particularly important for olive oil. Recently, a special emphasis has been placed on both products with respect to minor bioactive compounds such as tocopherols, phenolics, squalene, sterols or triterpenic acids, among others. Table 14.3 lists quality traits that have been evaluated in olive-breeding programs based on fresh unprocessed fruits, oil or processed table olives. High levels of variability have been observed for most of these traits, with cases of transgressed seg-

Table 14.3 Examples of quality traits evaluated in olive breeding programs

Product	Traits	References
Fresh olives	*Fruit attributes* Fruit weight, width and length Stone weight, width and length Fruit shape and circularity Flesh/stone ratio Fruit bruising Skin and flesh color Flesh texture Flesh detachment *Fruit compounds* Oil content and moisture Fatty acids profile Phenolic compounds Pigments Sugars Sterols Squalene Tocopherols	Arias-Calderon et al. (2014), Avidan et al. (2012), Bellini (1993), Bellini et al. (2002a, b), De la Rosa et al. (2013, 2014, 2016), Ersoy et al. (2008), Fourati et al. (2002a), Jimenez et al. (2011), Klepo et al. (2014), Lavee and Avidan (2011), Leon et al. (2004b, 2015, 2016), Ozdemir and Kurultay (2015), Ozdemir et al. (2016), Padula et al. (2008), Rallo et al. (2008b, 2012), Rjiba et al. (2010), Roca et al. (2011), Velasco et al. (2014), Zeinanloo et al. (2009)
Olive oil	*Oil composition* Fatty acids profile Omega3 fatty acid Phenolic compounds Pigments Sterols Tocopherols Volatile compounds *Legal quality parameters* Free acidity Peroxide value K232, 270 Oxidative stability	Baccouri et al. (2007), Bellini et al. (2002c, 2004), Dabbou et al. (2012), De la Rosa et al. (2013), El Riachy et al. (2012a, b, c), Fourati et al. (2002a, b), Garcia-Gonzalez et al. (2010), Hernandez et al. (2017), Klepo et al. (2014), Leon et al. (2008, 2011, 2015), Ozdemir et al. (2016), Perez et al. (2014, 2016), Ripa et al. (2008), Rjiba et al. (2010), Roca et al. (2011), Sanchez de Medina et al. (2015a, b, c), Velasco et al. (2014)
Table olives	Bruising Oil and water content Salt content pH, acidity Phenolic compounds Triterpenic acids Sensory attributes	Jimenez et al. (2011), Medina et al. (2012), Ozdemir and Kurultay (2015), Sorrentino et al. (2016)

regation and significant differences among crosses and/or genotypes within crosses (Arias-Calderon et al. 2014; El Riachy et al. 2012a; Lavee and Avidan 2011; Medina et al. 2012; Perez et al. 2014, 2016).

Breeding for quality in olive is more complex than in other fruit species since the olive drupe may not be consumed directly but may need to undergo industrial processing to obtain table olives or olive oil. Although some of the abovementioned

quality traits may be evaluated in fresh unprocessed fruits, most should be assessed in the final elaborated products. This procedure hampers olive breeder work, especially in the early stages of evaluation, because processing a large number of genotypes is time-consuming and expensive. Furthermore, the low yield of genotypes in this stage limits the amount of fruit required for processing. In this regard, the strategy applied to correlate traits in fresh fruit with quality traits of the final product, for indirect selection based solely on fresh olive evaluation, has been studied. High correlations have been found between fruit flesh and extracted oils for the main fatty acids, tocopherols, sterols and squalene (De la Rosa et al. 2016; Velasco et al. 2014), and between oleuropein content in fresh fruit and different quality traits of Spanish-style green olives and black olives (Rallo et al. 2018 P, pers comm).

The complex methodologies needed to assess many of the abovementioned quality traits, along with the large number of genotypes to be screened, have encouraged olive breeders to explore the use of alternative fast and non-destructive methods, such as NIR spectrometry, to evaluate both olive oil and table olive quality traits (Giovenzana et al. 2015; Leon et al. 2003, 2012; Mailer 2004; Morales-Sillero et al. 2011). Good calibration models have been obtained for fruit weight, diameter and volume, flesh texture, oil content, moisture content, chlorophyll pigment, oleic and linoleic fatty acids.

The recent detection of loci associated with interesting quality traits (fruit weight, flesh/stone ratio, oil traits, fatty acid composition, among others) via classical QTL mapping approaches (Atienza et al. 2014; Ben Sadok et al. 2013; Hernandez et al. 2017) or through genome-wide association studies (GWAS) (Kaya et al. 2016) will soon enable marker-assisted breeding for olive quality, allowing very early selection of outstanding genotypes at the juvenile seedling stage.

14.4 Genomic Tools for Olive Breeding Programs

Despite the remarkable recent development of olive breeding programs, breeders lack the capacity to generate cultivars quickly in response to new growing systems, evolving consumer preferences and crises. Olive breeding is still based on classic methods using directed crosses between suitable cultivars followed by selection within the progenies, and finally the cloning of outstanding individuals. Non-conventional breeding approaches, such as protoplast technology or the selection of mutants from induced in vivo mutagenesis, have also been applied to olive with variable levels of success (Rugini et al. 2016). However, to our knowledge only cv. Briscola (Roselli and Donini 1982) obtained by gamma rays mutation of cv. Ascolana Tenera was released for its ornamental value. Thus, this section will be focused on genetic and genomic approaches that could accelerate classical breeding methods in the near future.

The olive tree is an extremely heterozygous species with high genetic variability. Thus, seedlings from breeding programs have been shown to undergo segregation for all the agronomical characters evaluated to date (Rallo 2014a, b), even the frequent

presence of individuals showing transgressive segregation (seedlings with trait values exceeding the values of their genitors) (de la Rosa et al. 2016; Leon et al. 2004b; Trapero et al. 2015). Given this remarkable variability, breeding new olive cultivars with improved characteristics requires the germination and evaluation of thousands of seedlings. For example, more than 10,000 genotypes have been evaluated to obtain a new cultivar, two promising advanced selections and more than 30 preselections with high production, early bearing, and adaptation to mechanical harvesting and high-density at the UCO-IFAPA breeding program (Rallo et al. 2016a). In addition, olive-breeding cycles are extremely long (>12 years), mostly due to the length of the JP of long-lived perennials (van Nocker and Gardiner 2014). From an agronomic perspective, JP is the period between seed germination and first flowering, which are influenced by both environmental and genetic factors. As mentioned in Sect. 14.3.2, the length of the JP is inversely correlated with plant vigor. This association has led to the culling of approximately 40% of plants with a predictably long JP a few months after their germination (De la Rosa et al. 2006; Rallo et al. 2008b). Thus, the height of seedlings is currently the most effective early selection marker for the short juvenile phase in olive (Rallo 2014a, b). New biotechnological tools that are able to produce precocious flowering of juvenile plants via the action of the viral vector might also help to accelerate the breeding process of perennial crops in the near future (Haberman et al. 2017; Velázquez et al. 2016). Therefore, understanding the mechanisms controlling the JP transition in perennials and the detection of additional early selection markers related to other agronomic traits are important requirements to advance olive breeding.

The main use of genomics in breeding is marker-assisted selection (MAS) for traits controlled by major genes or quantitative trait loci (QTLs). By MAS, genetic markers that either are known to cause a phenotype or are strongly linked to the causal genetic variant can be genotyped at the seedling stage, allowing a prediction of the phenotype of the adult plant. The construction of a high-density linkage map is a necessary prior step to localize QTLs and genes controlling agronomic traits. Several olive linkage maps have been developed using the following molecular markers and cultivar progenies: RAPD, AFLP, RFLP and SSR—Leccino × Dolce Agogia (De la Rosa et al. 2003); RAPD, SSR and SCAR—Frantoio × Kalamata (Wu et al. 2004); AFLP and SSR Picholine Marocaine × Picholine du Languedoc (Aabidine et al. 2010); AFLP, ISSR and SSR Oliviere × Arbequina (Aabidine et al. 2010); DArT and SSR—Picual × Arbequina (Domínguez-García et al. 2012); SNP—F2 from the selfing of Koroneiki (Marchese et al. 2016); and SNP, CAP and SSR—Gemlik × Edincik Su (İpek et al. 2017). These mapping approaches, combined with the phenotypical characterization of genitors and progenies, have allowed the location of QTLs related to flowering and fruiting traits (Ben Sadok et al. 2013), oil content, moisture, ratio pulp to stone, fruit weight and trunk diameter (Atienza et al. 2014) (see also Sect. 14.3.4).

The development of new high-throughput sequencing techniques (also known as next-generation sequencing) has resulted in an increase in studies focused on olive transcriptomics. These studies have led to the identification of expressed sequence tags (ESTs) (Alagna et al. 2009), the assembly and annotation of the olive transcrip-

tome (Muñoz-Mérida et al. 2013), the assessment of differential expression patterns among tissues and plant treatments (Bazakos et al. 2012, 2015; Carmona et al. 2015; Guerra et al. 2015; Jiménez-Ruiz et al. 2015) and the generation of saturated linkage maps (İpek et al. 2017; Marchese et al. 2016). Many of these studies have ultimately pursued the identification of candidate genes for agronomic traits such as the length of the juvenile period (Fernández-Ocaña et al. 2010; Jiménez-Ruiz et al. 2015).

Despite these diverse and numerous initiatives, the development of early markers to increase the efficiency of olive-breeding programs is still a pending task. The application of MAS to complex traits, such as yield or biennial bearing behavior, is not straightforward. Difficulties in manipulating these traits are derived from their genetic complexity, principally the number of genes involved, interactions between genes (epistasis) and environment-dependent expression of genes.

A recent publication concerning the reference genome of the cv. Farga (Cruz et al. 2016) and a wild olive (Unver et al. 2017) provides a landmark that can steer the generalization of genomic tools for the characterization of genetic resources and the selection of candidate genotypes. According to Cruz et al. (2016) and Unver et al. (2017), the olive tree, with $2n = 46$ and a genome size ~1.3 to 1.4 Gb, has approximately 50,000 protein-coding genes and an evolutionary history marked by polyploidization events. The existence of these reference genomes along with the decreasing cost of sequencing will: (a) increase the number of sequenced cultivars, allowing the possibility of performing genome-wide association mapping and, ultimately, developing early markers to accelerate the breeding process; (b) allow an accurate evaluation of the germplasm variability for the classification of cultivars into gene pools to design crossbreeding strategies (Rugini et al. 2016) shed light on the controversial domestication history of olive (Besnard et al. 2013; Díez et al. 2015) by applying methodologies that have been previously used for annual plants with reference genomes (Gaut et al. 2015).

14.5 Biotechnology Applications

Olive is a species difficult to manipulate in vitro; however, up to date, significant progress has been made in the understanding of its culture requirements under controlled conditions. In this section, the state of the art of micropropagation of important cultivars as well as key factors involved in adventitious regeneration, genetic transformation and in vitro storage of juvenile and adult tissues are reviewed, with indications regarding the possibilities of using these technologies as breeding tools.

14.5.1 Micropropagation

In olive micropropagation, explants of juvenile origin generally show high morphogenetic capacity (Cañas et al. 1992); however, working with this type of material is

not of interest since agronomical traits are not known. Attempts to establish in vitro explants of adult origin has encountered several problems such as explant oxidation and necrosis, difficulties of disinfection, poor growth as well as a strong influence of the genotype (Lambardi et al. 2013; Rugini and Baldoni 2005). The obtainment of responsive material has required the use of explants from specific sources: (a) new growth obtained after severe pruning of field-grown trees (Peixe et al. 2007; Roussos and Pontikis 2002) shoots from rooted cuttings grown in the greenhouse (Sghir et al. 2005) suckers sprouting at the tree base (Rugini and Fontanazza 1981; Vidoy-Mercado et al. 2012), (d) new growth obtained after forcing ovules (Rama and Pontikis 1990) or hardwood cuttings (Vidoy-Mercado et al. 2012) to sprout in a controlled environment and (e) shoots from in vivo (Garcia-Férriz et al. 2002) or in vitro grafts (Revilla et al. 1996).

Explants used for culture establishment are generally nodal sections with lateral buds forced to elongate in vitro and subsequently propagated by segmentation of elongated shoots (Lambardi et al. 2013). This method is considered very reliable in terms of genetic stability of the obtained material (George 1993). Regarding nutritional requirements, Rugini (1984) developed the OM formulation, which, in comparison to the widely used MS (Murashige and Skoog 1962), contains higher levels of Ca, Mg, S, Cu and Zn, lower NH_4 as well as an additional supplement of reduced nitrogen in the form of glutamine. The OM formulation has been widely used in olive culture (Brhadda et al. 2003b; Chaari et al. 2002; Lambardi et al. 2013; Sghir et al. 2005; Vidoy-Mercado et al. 2012), although others mineral nutrient mixtures developed for woody plants have also shown to be adequate for olive; e.g. WPM (Lloyd and McCown 1980) in cvs. Chondrodia and Chalkidikis (Grigoriadou et al. 2002) and cv. Kalamon (Dimassi-Theriou 1994) or a modified DKW (Driver and Kuniyuki 1984) in cvs. Koroneiki (Roussos and Pontikis 2002) and Arbequina (Vidoy-Mercado et al. 2012). In terms of carbon source, either sucrose (Grigoriadou et al. 2002; Rugini 1984; Sghir et al. 2005) or mannitol (Lambardi et al. 2013; Peixe et al. 2007; Roussos and Pontikis 2002) have been used; however, in a comparative study with cv. Maurino, Leva et al. (2013) showed that mannitol promoted shoot sprouting and growth more than sucrose.

Shoot elongation and proliferation require the presence of a cytokinin in the culture media, zeatin or zeatin riboside (4.6–13.6 μM) being the most widely used (Grigoriadou et al. 2002; Lambardi et al. 2013; Roussos and Pontikis 2002; Rugini 1984; Sghir et al. 2005); however, due to the high cost of these hormones, attempts have been made to replace them with other cytokinins; along this line, Peixe et al. (2007) in cv. Galega Vulgar obtained good results with benzyladenine and a coconut milk supplement, while Peyvandi et al. (2009a) used 2-isopenteniladenine (2iP) in cv. Rowghani. In some cases, a GA_3 supplement has been used for shoot elongation either in combination with zeatin (Grigoriadou et al. 2002; Vidoy-Mercado et al. 2012) or as a separate treatment prior to rooting (Lambardi et al. 2013).

Rooting of olive microcuttings is generally carried out in two phases, e.g. Rugini (1984) recommended a 2-week exposure to 5.4 μM naphthaleneacetic acid (NAA) followed by transfer to another medium devoid of auxin but supplemented with zeatin and activated charcoal; however, in most cases, shorter exposures to auxin in

liquid medium with subsequent transfer to either basal solid medium (Peixe et al. 2007; Sghir et al. 2005) or directly to the acclimatization substrate (peat moss/coco fiber/perlite, at different ratios, or jiffy pots) (García-Férriz et al. 2002; Peyvandi et al. 2009a) are preferred. In several cases, incubation in darkness and/or incorporation of putrescine to root induction medium have given positive results (Grigoriadou et al. 2002; Rugini et al. 1993). In addition, Peyvandi et al. (2009a) indicated that shoots which had proliferated in the presence of mannitol, rooted better than those previously multiplied in the presence of sucrose did. Acclimatization, a critical phase of the micropropagation process, is generally carried out on mist benches within a greenhouse with controlled light and temperature. Cozza et al. (1997) showed that survival of micropropagated plants of cv. Nocellara Etnea was linked to a higher level of vascular differentiation in comparison to those of Nocellara Belice. After hardening, Rugini (1984) recommends the use of GA_3 sprays to speed up regrowth of acclimatized plantlets.

Genetic stability of micropropagated olive plants has been evaluated through RAPDs analysis and conflicting results have been obtained, e.g. while García-Férriz et al. (2002), in cvs. Arbequina, Picual and Empeltre and Leva and Petruccelli (2012) in cv. Maurino, observed similar banding patterns in micropropagated material and the corresponding mother plants, Peyvandi et al. (2009b) and Farahani et al. (2011a, b) obtained opposite results in several Iranian cultivars; in addition and, according to these authors, observed variations increased with the number of subcultures. Regarding field performance, while Leva (2009) found no differences between plants micropropagated through axillary buds and self-rooted controls of cv. Maurino in terms of vegetative and reproductive growth patterns, Briccoli-Bati et al. (2006) obtained different results depending on genotype, e.g. while plants of cv. Nocellara Etnea showed higher production and lower average fruit size than control-grafted plants, micropropagated material of cv. Carolea showed very low production throughout the 8-year evaluation period, leading these authors to indicate that further research is needed to elucidate whether epigenetic variations could have occurred during the long time the plants had spent in culture.

14.5.2 Organogenesis

Early studies on adventitious shoot formation in olive were carried out using juvenile explants; e.g. Cañas and Benbadis (1988) evaluated the in vitro organogenic capacity of basal and apical sections of cotyledons, observing a better response in the former. Shoot regeneration required a 3-week culture period on OMc medium ((OM formulation with BN macroelements (Bourgin and Nitsch 1967) and 1 g/l casein hydrolysate)) supplemented with 25 μM indole-3-butyric acid (IBA), followed by subsequent transfer to basal medium supplemented with the cytokinin 2iP. Petioles from apical and basal nodes of adult micropropagated shoots of cvs. Moraiolo and Dolce Agogia were used by Mencuccini and Rugini (1993) obtaining better response in apical nodes of Moraiolo. No response was observed when using petioles derived

from adult plants grown either in the field or in the greenhouse. A hormonal supplement containing either 10 μM 2iP and 2.2-μM benzyladenine or 5–40 μM thidiazuron (TDZ) was recommended (Mencuccini and Rugini 1993). Interestingly, when regeneration took place in the presence of TDZ, MS formulation at full strength was required, although in cvs. Canino and Moraiolo adventitious shoots could also be obtained in ½ MS supplemented with 30 μM TDZ and 0.54 μM NAA (Rugini and Caricato 1995).

14.5.3 Somatic Embryogenesis

Somatic embryogenesis in olive was firstly observed when using immature zygotic embryos for culture establishment (Rugini 1988). Embryogenic callus could be induced in the absence of growth regulators although a cytokinin supplement was beneficial (Table 14.4). Orinos and Mitrakos (1991) in wild, as well as Mitrakos et al. (1992) and Cerezo et al. (2011) in domestic olives, recommended the use of isolated radicles from mature embryos to induce the process (Fig. 14.8a). In these cases, a medium with high auxin/cytokinin ratio was required to induce formation of embryogenic callus (Fig. 14.8b) followed by transfer to a medium without growth regulators or a very low auxin concentration, to enhance embryo differentiation (Fig. 14.8c). Similar requirements were found when using cotyledon (Brhadda et al. 2003a; Leva et al. 1995; Trabelsi et al. 2003) or root segments, from in vitro germinated seedlings (Rugini 1995; Rugini et al. 1995; Shibli et al. 2001), as explants (Table 14.4). Wounding the root surface and placing the explants in horizontal position enhanced callus formation (Rugini 1995).

Somatic embryogenesis has also been observed in adult material. Rugini and Caricato (1995) used petioles from shoots of adventitious origin of cvs. Canino and Moraiolo and cultured them in a medium with 2iP (0.5 μM), BA (0.44 μM), IBA (0.25 μM) and 0.42 mM cefotaxime. Resulting embryogenic structures could be maintained proliferating in the dark under the same conditions or in hormone-free medium supplemented with 0.1% activated charcoal; in the last case, proliferation of secondary embryos took place directly from epidermal or subepidermal cells of primary embryos (Benelli et al. 2001). In the Moroccan cv. Dahbia, Mazri et al. (2013) were able to induce embryogenesis following a 4-day exposure of leaf sections from in vitro grown shoots to liquid medium supplemented with 30 μM TDZ and 0.54 μM NAA followed by transfer to solid hormone-free medium for 8 weeks and final culture in the hormonal combination recommended by Rugini and Caricato (1995) (Table 14.4).

Mineral requirements for somatic embryogenesis seem to depend on explant used and genotype; e.g. for radicles, the OMc formulation (Mitrakos et al. 1992; Orinos and Mitrakos 1991) has been used, while in the case of roots from seedlings, MS was preferred (Rugini 1995; Rugini et al. 1995; Shibli et al. 2001) (Table 14.4). For cotyledons, adult leaf fragments or petioles, either OMc (Rugini and Caricato 1995;

Table 14.4 Somatic embryogenesis in olive

Cultivar	Explant	Induction/Callus proliferation	Embryo development	Conversion	References
Frantoio/Moraiolo/Dolce Agogia	75-day old embryos	½ MS + (0.5–2.5) μM BA ± 0.5 μM NAA	½ MS basal medium	OM + (0.5–2.5) μM zeatin	Rugini (1988, 1995), Rugini et al. (2005)
Wild olive	Mature embryo radicle/cotyledon	OMc + 2.5 μM 2iP + 25 μM IBA	OMc + 0.5 μM IBA	½ MS basal medium	Orinos and Mitrakos (1991)
Koroneiki	Mature embryo radicle	OMc + 2.5 μM 2iP + 25 μM IBA	OMc ± 2.5 μM IBA	-	Mitrakos et al. (1992)
San Agostino	Roots of seedlings	MS + 25 μM NAA + 0.5 μM BA	1/2MS + 0.5 μM NAA + 0.5 μM BA	¼ MS + 2.5 μM zeatin	Rugini (1995), Rugini et al. (1995)
Frangivento/Picholine/Frantoio	Immature cotyledons	SH + 2.5 μM NAA ± 0.5 μM 2iP	SH basal medium	SH basal medium	Leva et al. (1995)
Canino/Moraiolo	Leaflets/petioles from adult shoots of adventitious origin	OMc + 0.2 μM 2iP + 0.44 μM BA + 0.25 μM IBA	Basal liquid OMc or solid OMc + 0.1% AC	OMc + 1.3 μM zeatin	Benelli et al. (2001), Rugini and Caricato (1995), Rugini et al. (2005)
Nabali	Roots of seedlings	MS + 5.0 μM 2,4D + 0.5 μM Kin + 5.0 μM NAA	MS + 10 μM 2iP	MS basal medium	Shibli et al. (2001)
Picholine Maroccaine	Cotyledons	MS + 2.3 μM zeatin + 10.7 μM NAA	MS + 2.3 μM zeatin	MS + 2.3 μM zeatin	Brhadda et al. (2003a)
Chetoui/Chemlali/Arbequina	Cotyledons	OMc + 25 μM IBA + 2.5 μM 2iP	OMc basal medium	OM + 4.6 μM zeatin	Trabelsi et al. (2003)
Chondrolia Chalkidikis	Immature cotyledons Mature cotyledons	½ MS + 4.44 μM BA + 2.26 μM 2,4 D ½ MS + 4.44 μM BA	½ MS + 4.44 μM BA + 2.26 μM 2,4D ½ MS+4.44 μM	– –	Pritsa and Voyiatzis (2004)
Wild olive	Adult leaf/Petiole	MS + 12.25 μM IBA + 4.56 μM zeatin	MS basal	–	Capelo et al. (2010)
Picual	Mature embryo radicle	OMc + 2.5 μM 2iP + 25 μM IBA/OMc + 0.5 μM IBA/ ECO + 0.2 μM 2iP + 0.44 μM BA + 0.25 μM IBA	ECO+0.1% AC	1/3 MS macros +10 g/l sucrose	Cerezo et al. (2011)
Dahbia	Adult leaves	½ MS + 30 μM TDZ + 0.5 μM NAA/1/2MS basal/ ECO + 0.2μM2iP + 0.44 μM BA + 0.25 μM IBA	–	–	Mazri et al. (2013)

AC (activated charcoal); BA (6-benzylaminopurine); 2,4-D (2-4 dichlorophenoxyacetic acid); ECO (¼ OM macroelements, ¼ MS microelements, ½ OM vitamins and 550 mg/l glutamine); IBA (indole-3-butyric acid); 2iP (6-dimethylaminopurine); MS (Murashige and Skoog 1962); NAA (naphthaleneacetic acid); OM (Olive medium, Rugini 1984); OMc (OM in which macroelements were replaced by those of Bourgin and Nitsch (1967) with addition of 1 g/l casein hydrolysate and omitting glutamine); SH (Schenk and Hildebrand 1972); TDZ (thidiazuron)

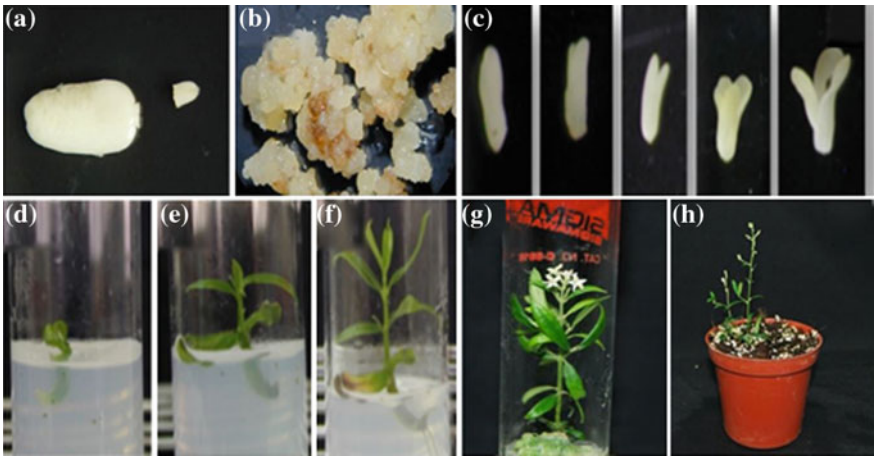

Fig. 14.8 Olive regeneration and transformation via somatic embryogenesis. **a** Radicle from mature embryo used for initiation of cultures, **b** Embryogenic callus, **c** Somatic embryos at torpedo and cotyledonary stages, **d–f** Germination of somatic embryo, **g**, **h** Transgenic plants, obtained after inoculation of globular somatic embryos with *Agrobacterium tumefaciens*, overexpressing the *Mt*FT gene developed flowers in vitro (**g**) or after the acclimatization phase (**h**). Photos by S. Cerezo

Trabelsi et al. 2003), MS (Brhadda et al. 2003a; Capelo et al. 2010) or ½ MS (Mazri et al. 2013; Pritsa and Voyiatris 2004) have shown to be adequate (Table 14.4).

In a comparative study between the basal formulations OMc (Sect. 14.5.2) and ECO (¼ OM macroelements, ¼ MS microelements and 550 mg/l glutamine), Cerezo et al. (2011) observed similar callus proliferation rates, although embryogenic structures grown in ECO formulation, of lower mineral strength, showed a higher capacity to form mature embryos. The same authors also found that culture of embryogenic callus for 4 weeks in liquid medium followed by sieving though a 3 × 3 mm mesh, could help to synchronize cultures prior to undergo embryo maturation in the presence of semipermeable cellulose acetate membranes. Embryos under this treatment showed a much lower water potential and germinated at 37.8% rate in a modified MS basal medium with ½ macroelements and 10 g/l sucrose (Fig. 14.8d–f) (Cerezo et al. 2011).

14.5.4 Somaclonal Variation

It is widely known that phenotypic variations can appear in plants regenerated in vitro, with the chances to occur being higher when adventive regeneration protocols, including a callus phase, are used (Bairu et al. 2011; Larkin and Scowcroft 1981). In olive, Cañas and Benbadis (1988) found that plants regenerated from cotyledon fragments showed morphological alterations, such as dichotomy or presence of double leaves.

In attempts to evaluate the effect of time in culture on regeneration capacity of olive embryogenic cells, Bradai et al. (2016a) studied the in vitro behavior of several embryogenic lines which had been kept in culture for 2 and 8 years. A strong genotypic effect was found, although embryos of aged lines showed a general decrease in maturation capacity and a reduced germination rate in comparison to younger ones; however, plants could be regenerated from material of all ages. Phenotypic evaluation of plants showed several alterations in vegetative traits (plagiotropic growth, fasciated stem and the appearance of 3 axillary shoots per whorl), phyllotaxy (4 leaves per verticil), leaf morphology (double leaves) and reproductive traits (flowers with 5–7 petals and 3–5 stamens). Again, genotype had a strong influence although, as expected, the frequency of variant phenotypes (19.78 vs. 4.0) and the percentage of plants showing these anomalies (37.36 vs. 10.0) were higher in aged than in younger lines (Bradai et al. 2016b). In plants obtained via somatic embryogenesis from immature cotyledons of cv. Frangivento, Leva (2009) characterized two types of somaclones: columnar and dwarf, differing in plant height, canopy projection, canopy volume and reproductive traits.

14.5.5 Genetic Transformation

Genetic transformation has been attempted in olive through biolistics and via *Agrobacterium tumefaciens*. Using the PDS-1000/He system to compare the efficiency of two promoters, Lambardi et al. (1999) found that the sunflower ubiquitin yielded higher *Gus* gene expression than the cauliflower 35S, in torpedo stage somatic embryos of cv. Canino, although opposite results were obtained with embryos at more advanced stages. Pérez-Barranco et al. (2009) using globular embryos derived from a mature embryo of cv. Picual reported similar results. These authors established a 6 cm target distance and a 900-psi bombardment pressure as optimum conditions for transformation. Gus expression could be observed 12 weeks after bombardment; however, as reported by Lambardi et al. (1999), no transgenic plants could be regenerated. Pérez-Barranco et al. (2009) also evaluated the response of olive cells to different antibiotics, pointing out the relatively high tolerance to kanamycin and paromomycin in solid medium while in liquid, antibiotics impaired cell growth at much lower concentrations.

Rugini et al. (2000) first attempted genetic transformation via *Agrobacterium tumefaciens* in somatic embryos of cv. Canino. In this protocol, after an initial 48 h exposure to the bacterial suspension, somatic embryos were transferred for 30 days to a medium deprived of antibiotics for recovery and subsequently exposed to 0.21 mM kanamycin in the dark; afterwards, embryogenic material was cultured in liquid medium in the light and only those embryos that turned green were selected. Finally, embryos were returned to the dark for secondary embryo production; these embryos were then induced to germinate in liquid medium deprived of antibiotics and supplemented with 1.3 μM zeatin. A different approach was undertaken by Torreblanca et al. (2010), using the highly virulent strain AGL1 for inoculation of globular stage

embryos, with continuous exposure to the antibiotic paromomycin 2 days after the co-culture phase. To avoid selection of chimeric tissues, a 3-week culture in liquid medium in the presence of antibiotic was used. For transgenic plant recovery the protocol of Cerezo et al. (2011), previously described (Sect. 14.5.3), was used.

Transformation via *Agrobacterium tumefaciens* has been used in attempts to improve agronomic traits as well as to undertake functional genomic studies. In an attempt to modify growth habit, somatic embryos of cv. Canino were transformed with rol ABC genes from *A. rhizogenes*; resulting transgenic plants showed hairy root phenotype, a long juvenile phase and maintained vegetative growth until late autumn (Rugini et al. 2008). To enhance stress tolerance, Rugini et al. (2000) were able to transform somatic embryos of the same genotype with the osmotin gene. Obtained transgenic plants have shown an outstanding resistance to water stress as well as enhanced tolerance to peacock spot (*Spilocaea oleagina*) although they are particularly attractive to the cribate weevil, *Otiorhynchus cribricollis* (Rugini et al. 2016). In order to elucidate the role of flowering locus T (FT) gene in olive, somatic embryos derived from a mature embryo of cv. Picual were transformed with FT-homologue from *Medicago truncatula*, following the protocol of Torreblanca et al. (2010). Some of the obtained transgenic lines flowered either during the in vitro phase (Fig. 14.8g) or following acclimatization (Fig. 14.8h); inflorescences were formed all year around although they were more abundant in spring. These plants also showed profuse axillary branching and reduced size (Haberman et al. 2017).

14.5.6 In Vitro Storage

14.5.6.1 Low-Temperature Conservation

Initial attempts to preserve olives under low temperature regimes were carried out by Micheli et al. (1998) through encapsulation of apical and lateral buds of cv. Moraiolo in sodium alginate beads; buds could be stored up to 45 days at 4 °C, although those of apical origin responded better in terms of sprouting. A few years later, Micheli et al. (2007) showed that 3–4 mm long nodal sections with lateral buds could be kept for 30 days at room temperature, in alginate nutrient solution kept in plastic cuvettes, suggesting that this could be an useful methodology to exchange material between countries. The encapsulation method has also been used by Cabello Moreno et al. (2013) using nodal sections of cv. Arbequina; these authors encapsulated buds at 4 °C over a 4-week period, indicating that pretreatments with growth regulators such as abscissic acid are beneficial for the conservation process.

Shoot cultures of cvs. Leccino and Frantoio, were maintained at 4 °C under darkness in OM basal medium for 8 months with a regrowth capacity above 80% (Lambardi et al. 2002), while in the case of cv. Arbequina maintained in RP medium (Roussos and Pontikis 2002) at 8 °C under light, shoots could be kept viable for 12 months (Imbroda et al. 2014).

14.5.6.2 Cryopreservation

Attempts at long-term conservation of buds have been carried out using the apical dome with 1–2 pairs of leaf primordia as explants. Martinez et al. (1999) obtained a 30% bud recovery in cv. Arbequina while Lambardi et al. (2002) reported a 15% in cv. Frantoio; however, in both cases, regrowth and further development or cryopreserved material occurred at a very low rate. Subsequent attempts to improve regrowth after cryopreservation through the inclusion of hormones in post-thaw medium, allowed Lynch et al. (2007) to maintain growth up to 10 weeks. Histological examinations showed the occurrence of damage in subapical cells, which could explain the failure in shoot recovery.

Somatic embryos seem to be a more suitable material for cryopreservation than buds. Shibli and AI-Juboory (2000) were able to successfully cryopreserve somatic embryos derived from seedling roots, via the encapsulation-dehydration or the encapsulation-vitrification methods; in both cases, inclusion of adequate dehydration steps (drying beads up to 21.1% moisture in the first case or keeping them for 3 h in plant vitrification solution) was critical for the process. In addition, exposure of embryogenic callus to 30 °C for 1 day allowed achievement of 58% survival rate when using encapsulation-dehydration or 68% in the case of encapsulation-vitrification protocol. Moreover, plants derived from cryopreserved cultures showed no morphological differences with controls. The importance of a correct dehydration step has also been emphasized by Sanchez-Romero et al. (2009) when using the droplet vitrification method after a 60 min dehydration in PVS2 (Sakai et al. 2008). In addition, Bradai and Sánchez-Romero (2017) indicated that preculturing embryogenic structures in a medium with high sucrose concentration was beneficial for culture recovery. Following regeneration, no morphological differences were observed between control plants and those derived from cryopreserved embryos. A different approach, including a 3-day sucrose pretreatment followed by incubation in a cry protectant mixture, prior to freezing at controlled rate (0.5 °C/min until −35 °C) and plunging into LN, allowed Lynch et al. (2011) to get noticeable callus regrowth and subsequent embryo recovery. Biochemical analysis indicated that applied pretreatments could enhance glutathione reductase, proline and sugar levels in the tissue, preparing them to withstand freezing.

14.6 Conclusions and Prospects

The choice of plant material is the first decision of the olive grower at the time of establishing a new plantation. A high diversity of local cultivars is a common trait in traditional olive-producing countries. Most olive cultivars are old, and they are grown around areas in which they were likely selected. In most cases, cultivars are self-rooted. Grafted trees are found only in the case of difficult-to-root cultivars or due to top grafting onto wild olives or onto other obsolete cultivars. In new olive-producing countries, imported cultivars represent most of the olive orchards. The

intensification of olive plantations after World War II in producing countries is based on a few selected national or foreign cultivars. This change has heightened interest in the conservation of olive genetic resources and in breeding olives.

The exploration, cataloguing and conservation of true-to-type traditional olive cultivars are the first steps for the sustainable use of genetic resources in any olive-growing country. Currently, ex situ collections are common facilities for conserving cultivars in germplasm banks. In all banks, confusion between names of accessions and cultivars remain an unresolved issue. An International Network with 22 National Germplasm Banks coordinated by the International Olive Council (IOC) with more than 1300 accessions is attempting to identify and to authenticate them. Morphologic characterization and DNA markers, particularly SSRs, have been shown to be powerful and discriminant methods to authenticate accessions (true-to-type cultivars), duplications, homonyms, synonyms and incorrect denominations. The identification and authentication of the IOC Network accessions is on course. The publication of a World Catalogue of True-to-Type Olive Cultivars will aid in solving the confusion in olive varietal denominations and developing International Plant Certification Protocols that will guarantee true-to-type and pathogen- and pest-free nursery plants in a time of global exchange of plant material. Other strategies, such as agronomic evaluation in banks and comparative trials, are in their infancy. The exploration and evaluation of wild and ancient olives is also under development to expand the genetic diversity to confront new breeding challenges such as climate change and devastating pests and diseases, as well as for studies related to the origin and domestication of the olive, among others.

New plantations systems have increased the need for new specific cultivars that have not been previously considered by empirical local breeders, i.e. the traditional farmer and breeder. The new systems require a high investment and early return, adaptation to mechanical harvesting, high yield, high oil content, resistance or tolerance to biotic and abiotic stresses, and quality of the olive oil and table olives, among others. In the last sixty years, crossbreeding has progressively developed in various olive growing countries. The breeding programs have demonstrated the following issues:

(1) A consistent increase in trained qualified breeders and publications.
(2) Evaluation and selection of parents for crosses will improve breeding efficiency.
(3) Reduction of the juvenile period (JP) to 2–3 years after planting accelerates the breeding process.
(4) Implementation of early selection methods of evaluation.
(5) Genetic studies demonstrates transgressed segregation of any trait in any crossbred progeny, consistency among genotype values data during the different steps of evaluation for most evaluated traits, and gain in the evaluation of a smaller number of well-selected crossbred progenies rather than many crossbred progenies with a smaller size.
(6) Moderate and high values of heritability for relevant agronomic, olive oil, and table olives traits is observed

(7) Progressive and fast development of genomic tools, such as MAS and GWAS, in addition to a recent publication concerning the first sequenced genome will accelerate the process of crossbreeding.
(8) Cooperation with farmers or other stakeholders during the final step of evaluation is critical for a sound G × E evaluation and for visibility among farmers of the new material advancements.
(8) New materials from public breeding programs for olive oil and table olive cultivars and rootstock will be progressively released in the next decade, providing farmers with cultivars for new mechanized plantation systems and/or resistance or tolerance to verticillium wilt and other diseases.
(10) New joint public-private consortia will develop new breeding programs.

Finally, registration represents the birth of a new cultivar. Marketing strategies appear as a major factor for its diffusion. This last step will determine whether any bred cultivar is well adapted to different environments in which it is dispersing, thus providing information about the real value of this innovation. A further requirement is the need for certification of nursery plants to ensure that newly-bred cultivars are true to type and free from pests and pathogens to demonstrate the potential performance of the new cultivar.

Regarding the possibilities of using in vitro regeneration and transformation technologies in breeding programs, micropropagation protocols are currently available for different olive cultivars; hence, although the genotype has strong influence on in vitro behavior, the technology could be useful for rapid multiplication of new releases as well as for international exchange of material. Regeneration via somatic embryogenesis is well established for juvenile material and very promising results are being obtained for explants of adult origin. Optimizing this pathway would make it feasible the use of cryopreservation for long-term in vitro storage as well as to undertake transformation of selected genotypes with gene coding for important agronomical traits. In any case, it appears that the interest of private companies for genetic manipulation of commercial cultivars, will depend on changes of consumer's acceptance of transgenic products.

Acknowledgments We dedicated this review to the collective effort of all researchers working on olive genetic resources and breeding in different Andalusia institutions since the establishment of the Olive Work Germplasm Bank of Córdoba in 1970, a landmark on olive breeding. We also acknowledge Isabel Trujillo for critical reading of the Section on Genetic Resources and to the graduate students Diego Cabello, Hristofor Miho, Pablo Morello and Pedro Valverde for their photographs and support in preparing the manuscript. Authors also recognize the plant breeding group of IFAPA-Alameda del Obispo, the biotechnology groups at IHSM (UMA-CSIC), Plant Biology Department, Malaga University, and IFAPA-Churriana, for their contributions to part of the research reviewed. Finally, we acknowledge the Spanish funding research agencies for their economic support for more than 40 years. Project P11-AGR-7992 is currently supporting part of the research indicated in Sect. 14.5.

Appendix 1

Research institutes and online resources

Country	Institution	Specialization and research activities	Contact information and website
Albania[a]	Centre of Agricultural Technology Transfer. Centre of Agricultural Technology Transfer Shamogjin, Komuna Novosele, Vlorë. Phone.: 00355 33 404144/145 Fax: 00355 33 404144/145	Genetic resources	Ms. Aulona Veizi aulona10@gmail.com qttbvlore@yahoo.com
Algeria[a]	ITAF. Tessala El Merdja - Birtouta -Alger. Phone: +213 023 58 38 60/61/66 Fax: +213 023 58 38 64/65	Genetic resources	M. Mahmoud Mendil mbmendil@gmail.com Itafv.dg@gmail.com webmaster@itafv.dz
Argentina	Laboratory of Genetic and Health Quality/ Faculty of Agricultural Sciences/UNC. Faculty of Agricultural Sciences National University of Cuyo. Almirante Brown 500. Chacras de Coria - Luján de Cuyo. CPA M5528AHB - Mendoza - Argentina. Phone: (+54 261) 413-5010	Genetic resources Breeding Genomics	L.E. Torres itorres@agro.unc.edu.ar http://www.fca.uncu.edu.ar/
Argentina	IBAM/ CONICET/ INTA. Faculty of Agricultural Sciences National University of Cuyo. Almirante Brown 500. Chacras de Coria - Luján de Cuyo. CPA M5528AHB - Mendoza - Argentina. Phone: (+54 261) 413-5010	Genetic resources Breeding Abiotic stresses Biotic stresses. Oil quality Genomics	R. W. Masuelli masuelli@fca.uncu.edu.ar https://inta.gob.ar/mendoza
Argentina[a]	EEA/CONICET/ INTA. Agricultural Experiment Station San Juan, Calle 11 y Vidart (5427) Villa Aberastain San Juan. Phone: (0264) 492 1079, (0264) 492 1191	Genetic resources Abiotic stresses	Dra. Mariela Torres mtorres@sanjuan.inta.gov.ar https://inta.gob.ar

Country	Institution	Specialization and research activities	Contact information and website
Australia	WWAI/ NSW DPI. Wagga Wagga Agr Inst, EH Graham Ctr Agr Innovat, Wagga Wagga, NSW 2650, Australia. Phone: (02) 6938 1999 International: +61 2 6938 1999 Fax: (02) 6938 1809	Genetic resources	www.dpi.nsw.gov.au
Australia	UNE. Univ New England, Sch Environm & Rural Sci, Armidale, NSW, 2351 Phone: +61 2 6773 2323 Fax: +61 2 6773 2769	Genetic resources Breeding	msedgle2@une.edu.au ers@une.edu.au
Belgium	Ghent University. Univ Ghent, Dept Plant Biotechnol & Bioinformat, B-9052 Ghent, Belgium Phone. +32 9 331 38 00 Fax +32 9 331 38 09	Genomics	marc.vanmontagu@ugent.be
Croatia	Faculty of Agriculture/ University of Zagreb. University of Zagreb, Faculty of Agriculture, Svetošimunska cesta 25, 10000 Zagreb, Croatia, Phone: +385 (0)1 2393 777 Fax: +385 (0)1 2315 300	Genetic resources Breeding Genomics	Z. Satovic zsatovic@agr.hr http://www.agr.unizg.hr/en
Croatia[a]	Institute for Adriatic Crops and Karst Reclamation. Inst Adriat Crops & Karst Reclamat, Put Duilova 11, Split 21000, Croatia. Phone: +385.21.43.44.44 Fax: +385.21.31.65.84	Genetic resources Biotic stresses	Mr. Slavko Perica Slavko.Perica@krs.hr info@krs.hr
Cyprus[a]	Agricultural Research Institute // Officer Olive Technology Laboratory. Agricultural Research Institute. P.O.Box 22016, 1516 Nicosia, Cyprus. Phone: ++357 22 403100 Fax: ++357 22 316770	Genetic resources	Ms. Dora Chimonidou dari@arinet.ari.gov.cy info@ari.gov.cy
France	AGAP/INRA/ Montpellier SupAgro. Montpellier SupAgro, 2 place Pierre Viala, 34060 MONTPELLIER Cedex 02. Phone: +33 (0)4 99 61 22 00 Fax: +33 (0)4 99 61 29 00	Genetic resources Breeding	L. Essalouh laila.essalouh@supagro.inra.fr https://www.supagro.fr

Country	Institution	Specialization and research activities	Contact information and website
France[a]	UMR-AGAP. Avenue Agropolis, 34398 Montpellier Cedex 5, France. Phone: +33 4 67 61 58 00	Genetic resources Breeding Oil Quality Genomics	B. Khadari khadari@supagro.fr https://umr-agap.cirad.fr
France	Université de Toulouse III/ EDB - UMR 5174. 118, route de Narbonne Bât. 4R131062 TOULOUSE cedex 9 Phone (+33) 05 61 55 73 84 Fax: (+33) 05 61 55 73 27	Evolution	G. Besnard guillaume.besnard@univ-tlse.fr http://www.edb.ups-tlse.fr/
Greece	Laboratory of Pomology/ Department of Crop Science/ Agricultural University of Athens. Agricultural University of Athens. Iera Odos 75, Athina 118 55, Greece. Phone.: +30 21 0529 4900 Fax. +30210-5294081.	Genetic resources Breeding	M. Hagidimitriou marianna@aua.gr http://www2.aua.gr
Greece	Institute of Viticulture, Floriculture and Vegetable Crops (I.V.F.V.H)/ NAGREF. Institute of Viticulture, Floriculture and Vegetable Corps of Herakleion // I.V.F.V.H // PO Box 2229 // 71003 Herakleion Phone: 2810 302 300// 245 851, 240 986. Fax 2810 245 873	Genetic resources Breeding Abiotic stresses Biotic Stresses Oil Quality	A.G. Doulis grandreas.doulis@nagref-her.gr http://www.nagref-her
Greece[a]	Olive Cultivation and Post Harvest Physiology Laboratory/ Institute for Olive Tree and Subtropical plants of Chania/NAGREF. Institute for Olive Tree and Subtropical plants of ChaniaLeof. Soudas 131, Chania 731 34, Greece. Phone:+30 2821 083472	Genetic resources Breeding Abiotic stresses Biotic Stresses Oil Quality	Dr. G.C. Koubouris koubouris@nagref-cha.gr Info@nagref-her.gr
Greece	Aristotle Univ. Thessaloniki. Aristotle Univ Thessaloniki, Dept Hort, Lab Biol Hort Plants, Thessaloniki 54124, Hellas, Greece	Biotic Stresses Breeding Oil Quality	Maria Tsimidou tsimidou@chem.auth.gr info@agro.auth.gr

Country	Institution	Specialization and research activities	Contact information and website
Iran	National Institute of Genetic Engineering and Biotechnology. Shahrak-e Pajoohesh, km 15, Tehran - Karaj Highway, Tehran, Iran P.O. Box: 14965/161. Phone: +98 21 44787301-9. Fax: +98 21 44787399	Genetic Resources Breeding Genomics	M. Hosseini-Mazinani hosseini@nigeb.ac.ir nigeb_manager@nigeb.ac.ir
Iran	Horticulture Department/Gorgan University of Agricultural Sciences. Gorgan University of Agricultural Sciences and Natural Resources. Gorgan, 49138-15739, Iran. Phone: +98-171-2220320. Fax: + 98-171-2220640	Genetic Resources Breeding Genomics	M. Sharifani mmsharif2@gmail.com International@gau.ac.ir
Iran	SPII/HD. SPII, Hort Dept, Mahdasht Rd,POB 31359-33181, Karaj, Iran	Breeding	A. Zeinanloo info@abrii.ac.ir
Israel	The Robert H. Smith Faculty of Agriculture, Food and Environment The Hebrew University of Jerusalem. Hebrew Univ Jerusalem, Fac Agr, Inst Plant Sci, IL-76100 Rehovot, Israel. P.O Box 12, Rehovot 76100	Genetic resources Breeding Abiotic stresses Biotic stresses. Oil Quality Genomics	A. Samach alon.samach@mail.huji.ac.il http://departments.agri.huji.ac.il
Israel[a]	ARO/ Volcani Center[a]. Agricultural Research Organization - the Volcani Center, 68 HaMaccabim Road, P.O.B 15159 Rishon LeZion 7505101, Israel Phone: +972-3-9683226 Fax: +972-3-9665327	Genetic Resources	Dr. Giora Ben Ari giora@agri.huji.ac.il http://www.agri.gov.il
Italy	IVALSA. National Research Council of Italy, Trees and Timber Institute Follonica (Grosseto) via Aurelia, 49 58022 - Follonica (GR) Phone. +39 056 652356	Genetic Resources Breeding Genomics	C. Cantini cantini@ivalsa.cnr.it http://www.ivalsa.cnr.it

Country	Institution	Specialization and research activities	Contact information and website
Italy	CNR/Institute of plant genetics. Institute of plant genetics. Via Madonna Alta, 130-06128 Perugia (PG) - Umbria Phone: +39 0755014862 Fax: 0755014869	Genetic resources Breeding Genomics	L. Baldoni luciana.baldoni@ibbr.cnr.it http://www.ibbr.cnr.it
Italy	DEMETRA/ University of Palermo. University of Palermo. Piazza Marina, 61// 90133 - PALERMO. Phone: +39 091 238 93011	Genetic Resources Abiotic stresses Biotic stresses	T. Caruso f tiziano.caruso@unipa.it http://www.unipa.it
Italy	Management Department of Agricultural and Forestry Systems/ University of the Mediterranean Studies of Reggio Calabria. Mediterranea University of Reggio Calabria. Salita Melissari 89124 Reggio Calabria. Tel.: +39 0965 169 1207 Fax: +39 0965 332201	Genetic Resources Breeding	R. Mafrica rocco.mafrica@unirc.it http://www.unirc.it
Italy	UNIFI/DISPAA. Universita degli studi fii Firenze. Dipartimento di Scienze delle Produzioni Agroalimentari e dell'Ambiente Piazzale delle Cascine, 18 - 50144 Firenze Phone: +39 055275-5700	Genetic resources Breeding Abiotic stresses	http://dispaa.unifi.it
Italy	UNITU. Universita della Tuscia, DAFNE, Via San Camillo de Lellis Snc, I-01100 Phone +39 0761357581/554; Fax +39 0761357558/434	Biotechnology Genomics	Eddo Rugini rugini@unitus.it dafne@pec.unitus.it
Italy	CNR/ IVALSA. Sesto Fiorentino (Firenze) via Madonna del Piano, 10 50019 - Sesto Fiorentino (FI) Phone: +39 055 52251	Genetic Resources Genomics	M. Centrito centrito@ivalsa.cnr.it info@ivalsa.cnr.it

Country	Institution	Specialization and research activities	Contact information and website
Italy	UNIBA/DISAAT. Universita di Bari, Dipartimento di Scienze Agro Ambiental e Territoriali (Di.S.A.A.T.) Amendola 165-A, I-70126 Bari, Italy	Genetic resources Biotic stresses	Franco Nigro franco.nigro@uniba.it info@agr.uniba.it
Italy	ENTECRA OLI. CRA OLI, I-06049 Spoleto, PG, Italy. Centro di ricerca per l'olivicoltura e l'industria olearia (Rende) Via Nursina 2 06049 - *SPOLETO*. Phonel: +39 0743-49743 Fax: +39 0743-43634	Genetic resources Biotic stresses	Adolfo Rosati rosati@entecra.it info@entecra.it
Italy	UNIPG/DSAAA. Univ Perugia, Dipartimento Sci Agr Alimentari & Ambientali, Via Borgo 20 Giugno 74, I-06121 Perugia, Italy	Oil quality	Maurizio Servili maurizio.servili@unipg.iti nfo@unipg.it
Italy[a]	CRA-OLI Research center for olive growing and oil industry. Centro di ricerca per l'olivicoltura e l'industria olearia – Sede Scientifica di Città S.Angelo (OLI.PE) Viale Petruzzi 75 65013 - CITTA' SANT'ANGELO	Genetic resources Breeding Abiotic stresses Biotic stresses Oil quality	Enzo Perri enzo.perri@crea.gov.it http://sito.entecra.it
Jordan[a]	NCARE. National Center for Agricultural Research and Extension PO Box 639, Baq'a 19381, Jordania Phone: +962 (6) 4725071 Fax: +962 (6) 4726099	Genetic Resources Breeding Abiotic stresses Biotic stresses Oil quality	Dr. Salam Ayoub salamayoub@hotmail.com http://www.ncare.gov.jo/
Lebanon	Lebanese University/ Faculty of Agricultural Sciences. Faculty of Agricultural Sciences. PO Box 90775, Horst Tabet, Beirut - Lebanon Phone: 484130/01 484131/01 484132/01 Fax: 510870/01 510867/01	Genetic resources Breeding Genomics	L. Chalak lamis.chalak@gmail.com https://www.ul.edu.lb

Country	Institution	Specialization and research activities	Contact information and website
Lebanon[a]	LARI. Lebanese Agr Res Inst, Lab Olive Oil, Tal Amara, Bekaa, Lebanon	Genetic resources Oil quality	Milad El Riachy mraichy@lari.gov.lb info@lari.gov.lb
Montenegro[a]	Biotechnical Faculty/Centre For Subtropical Cultures. Biotechnical Faculty. Centre for Subtropical Cultures Bar-University of Montenegro. Ul. Bjelisi bb 85000 Bar// Montenegro Phone: (382) 69516165	Breeding Genomics	B. Lazovic biljanal@t-com.me http://www.ucg.ac.me
Morocco[a]	INRA/ URAP. Centre Régional de la Recherche Agronomique de Marrakech Unité de Recherche sur l'Amélioration des Plantes et de la qualité B.P. 533 Menara MARRAKECH Maroc Phone: +212 524447882/ +212 524435175/ +212 524432627 Fax: +212 524446380	Genetic Resources Breeding	Sikaoui Lhassane sikaouilhassane@yahoo.fr http://www.inra.org.ma
Portugal[a]	INIAV. UEI de Biotecnologia e Recursos Geneticos. Polo de Elvas Estrada de Gil Vaz, Apartado 67351-901 Elvas – Portugal Phone: (+ 351) 268 637 740	Genetic resources Breeding Oil quality Table olives quality Genomics	António M Cordeiro antonio.cordeiro@iniav.pt polo.elvas@iniav.pt
Slovenia[a]	Experimental center for olive growing. Agriculture and Forestry Institute Nova Gorica.Ulica 15. maja 17, 6000 Koper Tel: ++386 (0)5 631 32 32/ ++386 (0)41 815 302	Genetic resources	Ms. Vesel Viljanka viljanka.vesel@siol.net www.kmetijskizavod-ng.si
Spain[a]	IFAPA Centro Alameda del Obispo. Centro Alameda del Obispo Avda. Menéndez Pidal s/n 14004- Córdoba Phone. +34 957016000	Genetic resources Breeding Abiotic stresses Biotic stresses. Oil quality Genomics	Raul De la Rosa raul.rosa@juntadeandalucia.es cordoba.ifapa@juntadeandalucia.es

Country	Institution	Specialization and research activities	Contact information and website
Spain	IMIDRA. Finca El Encin, Autovía del Noreste A-2, Km. 38.200, 28805// Alcalá de Henares, Madrid Phone: +34 918 87 94 00	Genetic resources Breeding Oil quality Genomics	B. E. Sastre blanca.esther.sastre@madrid.org www.madrid.org/imidra/
Spain	Instituto de la Grasa/ CSIC. Instituto de la Grasa, CSIC. Ctra. de Utrera, km. 1. Campus Universitario Pablo de Olavide - Edificio 46. 41013 - SEVILLA (España) Phone:(+34) 95 461 1550 Fax:(+34) 95 461 6790	Genetic resources Breeding Oil quality Table olives quality Genomics	J. M. Martínez-Rivas mrivas@cica.es www.ig.csic.es
Spain	Plant Physiology/ Faculty of Science/ University of Extremadura. University of Extremadura. Avda. de Elvas, s/n. 06006 Badajoz Phone:+34 924 289 300 Fax.: +34 924 272 983	Genetic resources Abiotic stresses Biotic stresses. Oil quality Genomics	M. C. Gomez-Jimenez mcgomez@unex.es https://www.unex.es
Spain	IAS-CSIC. Instituto de Agricultura Sostenible Avenida Menéndez Pidal s/n Campus Alameda del Obispo 14004 Córdoba (España) Phone: +34 957 49 92 00 Fax:+34 957 49 92 52	Genetic resources Breeding Abiotic stresses Biotic stresses.	H. F. Rapoport hrapoport@ias.csic.es http://www.ias.csic.es/
Spain[a]	Department of Agronomy/ University of Córdoba[a]. Universidad de Córdoba Departamento Agronomía Campus Univ. de Rabanales Ctra. Madrid-Cádiz Km. 396 14071-Córdoba Phone +34 957218433/ 34/ 35 Fax +34 957218438	Genetic resources Breeding Abiotic stresses Biotic stresses. Oil quality Genomics	Diego Barranco ag1banad@uco.es infoetsiam@uco.es
Spain	IRTA Mas de Bover. Instituto de Investigación y Tecnología Agroalimentarias. Ctra. de Reus El Morell Km 4,5 Phone: 977 32 84 24 Fax: 977 34 40 55	Genetic resources Breeding Abiotic stresses Biotic stresses. Oil quality Genomics	Agusti Romero agusti.romero@irta.cat http://www.irta.cat

Country	Institution	Specialization and research activities	Contact information and website
Spain	CSIC/EEZ. Plant Reproductive Biology Laboratory, Estacion Experimental del Zaidin (CSIC), Profesor Albareda 1, 18008 Granada, Spain. Phone +34 958572757 Fax +34958572753	Abiotic stresses Genomics	Juan D. Alche juandedios.alche@eez.csic.es info @csic.eez.es
Spain	US/ETSIA. Departamento Agroforestal. ETSIA. Universidad de Sevilla. Ctra. Utrera km 1. 41013 Sevilla. Phone +34 954486455 Fax +34 954486436	Breeding Quality table olives	Pilar Rallo prallo@us.es agroforestal@us.es
Spain	CNAG CRG. Barcelona Inst Sci & Technol, Ctr Genom Regulat, CNAG CRG, Baldiri i Reixac 4, Barcelona 08028, Spain Phone +34 93 316 01 00 Fax +34 93 316 00 99	Genomics	Toni Gabaldon toni.gabaldon@crg.eu. info@crg.esi
Spain	UJA/DBE. Univ Jaen, Dept Biol Expt, Campus Lagunillas S-N, Edif B-3, Jaen 23071, Spain. Phone:+34 953 212527	Genomics Oil Quality	Francisco Luque fjluque@ujaen.es info@ujaen.es
Spain	CSIC/IRNAS. CSIC, IRNAS, Irrigat & Crop Ecophysiol Grp, Ave Reina Mercedes 10, Seville 41012, Spain. Phone: +34 95 462 47 11	Abiotic Stresses	Enrique Fernandez Luque jeferse@irnase.csic.es www.irnas.csic.es
Spain	IHSM/UMA-CSIC, Inst Hortofruticultura Subtrop & Mediterranea, Univ Malaga-CSIC, Dept. Biol Vegetal, Fac Ciencias, E-29071 Malaga, Spain. Phone: +34 952131947	Biotechnology	F. Pliego, ferpliego@uma.es
Tunisia	IRESA. Univ Sousse, IRESA, High Agron Inst, B.P. n° 47 -4042 Chott Meriem *Sousse*, *Tunisie Phone*: (+216) 73 327 544/ (+216) 73 327.592. Fax: (+216) 73 327	Genetic resources	Ibtisissem Laribi ibtissem.laaribi@yahoo.fr isa.chott@iresa.agrinet.tn

Country	Institution	Specialization and research activities	Contact information and website
Tunisia[a]	Institut de l'Olivier Sfax, Route de l'Aéroport, B.P. 1087 3000 Sfax Tél: (+216) 74 241 240/ 74 241 589 Fax: (+216) 74 241 033	Genetic Resources Breeding Biotic Stresses	Monji Msallem msallemonji@yahoo.fr http://www.iosfax.agrinet.tn/
Turkey	Department of Bioengineering/Ege University. Ege University Faculty of Engineering Department of Bioengineering. Erzene Quarter, Ege Unv. No:180, 35040 Bornova/İzmir Phine: 0 232 311 58 11 Fax: 0 232 311 58 80	Genetic Resources and Breeding Genomics	B. Tanyolaç bahattin.tanyolac@ege.edu.tr http://biyomuhendislik.ege.edu.tr info@ege.edu.tr
Turkey	Bornova Olive Research Station. Olive Culture Research Station Üniversite Cd. No: 43 35100 Bornova/ İzmir Phone: +90 (232) 462-7073	Genetic Resources	Dr. Unal Kaya unal.kaya@gthb.gov.tr posta@zae.gov.tr
Turkey[a]	GFAR. Olive Research Institute - İzmir Universite Cd. No:43 35100 BORNOVA IZMIR Phone: +90 232 462 70 73 Fax: +90 232 435 70 42	Genetic Resources	Melek Gurbuz melekgurbuz11@gmail.com izmirzae@tarim.gov.tr
Uruguay	INIA/ National Agricultural Research Institute. National Agricultural Research Institute. Andes 1365 - piso 12 CP 11100 Montevideo, Uruguay Phone: + 598 2902 0550 Fax: + 598 2902 3666	Genetic Resources and Breeding Abiotic stresses Biotic stresses	P. Conde pconde@inia.org.uy inia@inia.org.uy
USA	National Clonal Germplasm Repository/USDA. Nat'l Clonal Germplasm Rep - Tree Fruit & Nut Crops & Grapes. Davis, CA One Shields Ave, UC Davis Davis, CA 95616 Phone: (530)752-7009 Fax: 530-752-5974	Genetic Resources Breeding Genomics	J. E. Preece John.Preece@ars.usda.gov https://www.ars.usda.gov

[a]IOC Network Germplasm Bank

Appendix 2

Genetic resources: Main native cultivars

Country	Cultivar	Use	Fruit weight (g)	Oil content
Albania	Kalinjot	Oil, Table	3.5	High
Algeria	Azeradj	Oil, Table	4	Medium
	Chemlal de Kabylie	Oil	2	Low
	Sigoise	Table, Oil	3	Medium
Argentina	Arauco	Table, Oil	8	Medium
Chile	Azapa	Table, Oil	8	Medium
Croatia	Lastovka	Oil	3	High
	Oblica	Table	5	Medium
Cyprus	Ladoelia	Oil	2.5	High
Egypt	AggeiziShami	Table	8	Very low
	Touffahi	Table	12	Very low
France	Aglandau	Oil, Table	2.5	Medium
	Bouteillan	Oil	5	High
	Grosanne	Table, Oil	2.5	Low
	Lucques	Table	3	Low
	Picholine du Languedoc	Table, Oil	3	Medium
	Salonenque	Table, Oil	3	Medium
	Tanche	Oil, Table	2.5	High
Greece	Adramitini	Oil	2.5	High
	Amigdaloia	Table, Oil	8	Medium
	Chalkidikis	Table	8	Medium
	Kalamata	Table	3.5	Medium
	Konservolia	Table	4	Medium
	Koroneiki	Oil	1	Very high
	Mastoidis	Oil, Table	2.6	Very high
	Megaritiki	Oil	2	High
	Valanolia	Oil	2.5	Medium
Iran	Fishomi	Table		Medium
	Mari	Table, Oil	3.5	High
	Roghani	Oil	4	High
	Zard	Oil, Table	4.5	Medium

Country	Cultivar	Use	Fruit weight (g)	Oil content
Israel	Barnea	Oil, Table	3	Medium
	Nabali	Oil, Table	3	High
	Souri	Oil, Table	3	High
Italy	Ascolana	Table	7	Low
	Bosana	Oil	3	High
	Cellina di Nardo	Oil	2	Low
	Coratina	Oil	5	Medium
	Frantoio	Oil	3	High
	Leccino	Oil	2.5	Medium
	Moraiolo	Oil	2	High
	OgliarolaBarese	Oil	2	High
	Pendolino	Oil	2	Low
	Taggiasca	Oil	2	High
Jordan	Nabali Baladi	Table, Oil	3	High
	Rasei	Oil	3	Medium
Lebanon	Baladi	Oil, Table	3	High
	Souri	Oil, Table	3	High
Libya	Endory	Oil	1	High
	Hammudi	Oil	2.2	High
Malta	Bidni	Oil, Table	2	High
Montenegro	Zutica	Oil	3.2	High
Morocco	Picholine Marocaine	Oil, Table	3.5	Medium
Palestine	Nabali	Oil, Table	3.1	High
	Souri	Oil, Table	3	High
Perú	Criolla	Table	8	High
Portugal	Cobrançosa	Oil	3	Medium
	GalegaVulgar	Oil, Table	2.5	Medium
Slovenia	Buga	Oil		
Spain	Arbequina	Oil	1.9	High
	Arbosana	Oil	2	Medium
	Cornicabra	Oil	3	High
	Empeltre	Oil	2.7	Medium
	Farga	Oil	2.4	Medium

Country	Cultivar	Use	Fruit weight (g)	Oil content
	Gordal Sevillana	Table	12.5	Low
	Hojiblanca	Oil, Table	4.8	Medium
	Lechin de Sevilla	Oil	3	Medium
	Manzanilla de Sevilla	Table	4.6	Medium
	Picual	Oil	3.2	High
Syria	Doebli	Oil, Table	4.5	High
	Sorani	Oil, Table	3	High
	Zaity	Oil	2.5	Very high
	Kaissy	Table	5	Low
Tunisia	Chemlali Sfax	Oil	1	Very high
	Chetoui	Oil, Table	2.5	Medium
	Oueslati	Oil, Table	2	High
Turkey	Ayvalik	Oil	3.6	High
	Domat	Table	5.3	Medium
	Gemlik	Oil, Table	3.7	High
	Memecik	Oil, Table	4.8	Medium
USA	Mission	Oil, Table	3	Medium

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Chapter 15
Pomegranate (*Punica Granatum* L.) Breeding

Doron Holland and Irit Bar-Ya'akov

Abstract Pomegranate (*Punica granatum* L; Punicaceae), is a unique fruit tree with showy edible and healthful fruit which symbolizes fertility and abundance. Breeding of deciduous fruit trees was based until recently on a combination of intuition and science. The development of high throughput sequencing and mapping techniques changed the equation and enable efficient introduction of new scientific tools in breeding. Pomegranate breeding is expected to benefit much as the pomegranate tree is most suitable for genetic analysis. Pomegranate has a short juvenile period, high number of progeny formation by a single genetic cross and is a diploid plant. Indeed, in a relatively short period of time, several transcriptomes from different pomegranate tissues including leaves, fruits and flowers were assembled, the genome was determined and the phylogenetic structure of several collections in different geographical locations was resolved. In parallel with the dramatic development in pomegranate genomics, increased economic interest in the fruit and its products resulted in the establishment of several breeding centers in countries such as Spain, Israel, Turkey Iran, the USA, China and India. The next challenge is now to integrate the data from the scientific achievements into the practice of pomegranate breeding. As genetic engineering is still not efficient for most plants, it is expected that in the short term breeding of new pomegranate cultivars will be based on classical breeding or on marker-assisted selection (MAS). Despite the prominent progress in pomegranate genomics, functional analysis and identification of genes involved in important agricultural traits were achieved in only very few cases in pomegranate. Finding genetic markers or important genes for fruit quality, content of healthful compounds and resistance to pests and diseases could be among the achievable challenges.

D. Holland (✉) · I. Bar-Ya'akov
Unit of Deciduous Fruit Tree Sciences, Newe Ya'ar Research Center, Agricultural Research Organization, P.O.Box 1021, 30095 Ramat Yishay, Israel
e-mail: vhhollan@agri.gov.il

I. Bar-Ya'akov
e-mail: iritby@agri.gov.il

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15.1 Introduction

Pomegranate (*Punica granatum* L.; Punicaceae) is a fruit tree highly praised in many human cultures since ancient times. The tree and its fruit are symbols of beauty and fertility and serve as a nutritional and healthful pharmaceutical source. Pomegranate is highly adaptive to a wide range of climates and soil conditions and is grown in many different geographical regions. Despite its ethnobotanical importance, pomegranate culture is considered as a minor crop. During the last decade a prominent increase in the consumption of this fruit in the western world has occurred following the expanding awareness to the healthful benefits of the pomegranate fruit.

The pomegranate is a well-known fruit tree with deep heritage in human culture and history. In the last decade novel methods have been developed for its cultivation and for the exploitation of its nutritional and medical benefits. Much of the progress in utilizing the full spectrum of the pomegranate tree depends on the accumulation of pharmacological and genetic data. Such data were absent for many years and inhibited rapid progress in pomegranate research and horticulture. In the last few years a dramatic development in our knowledge of pomegranate origin, variability, genetic inheritance of important traits, the pomegranate genome, the expression of the gene encoding parts of the genome (transcriptome), the metabolome and pharmacological aspects of its metabolite activity was achieved. Data assembly of this recently-produced vast knowledge makes the pomegranate for the first time a favorite and useful plant system for academic and applicative research. In this review we focus on and outline the important aspects that are relevant for pomegranate breeding. This chapter is aimed to enlighten pomegranate improvement since ancient times and throughout the world, emphasizing modern biotechnology. These new methods have led to reveal the pomegranate genome and transcriptome and to trace through mapping and clustering, genes and genetic markers related to important agricultural or health beneficial traits, for marker assistant breeding.

15.1.1 Botanical Classification

Pomegranate belongs to the order Myrtales, and to the Punicaceae or Lythraceae family dependent on taxonomists (Rana et al. 2010). The genus *Punica*, first described by Linnaeus in 1753, contains only two species: *P. granatum* L. and *P. protopunica* Balf. f. *Punica granatum* is the only species cultivated while *P. protopunica* is a wild species endemic to Socotra Island, Yemen. Some taxonomists classify the dwarf *P. granatum* var. *nana* as a distinct species, namely *P. nana* L. (Holland et al. 2009; Rana et al. 2010).

Based on xylem anatomy *Punica protopunica* has been suggested as the ancestral type species of the genus (Shilkina 1973). This is strengthened by the different chromosome number as *P. protopunica* has n = 7 versus n = 8 in *P. granatum*. However, not all botanists accept *P. protopunica* as the ancestor species (Zukovskij 1950).

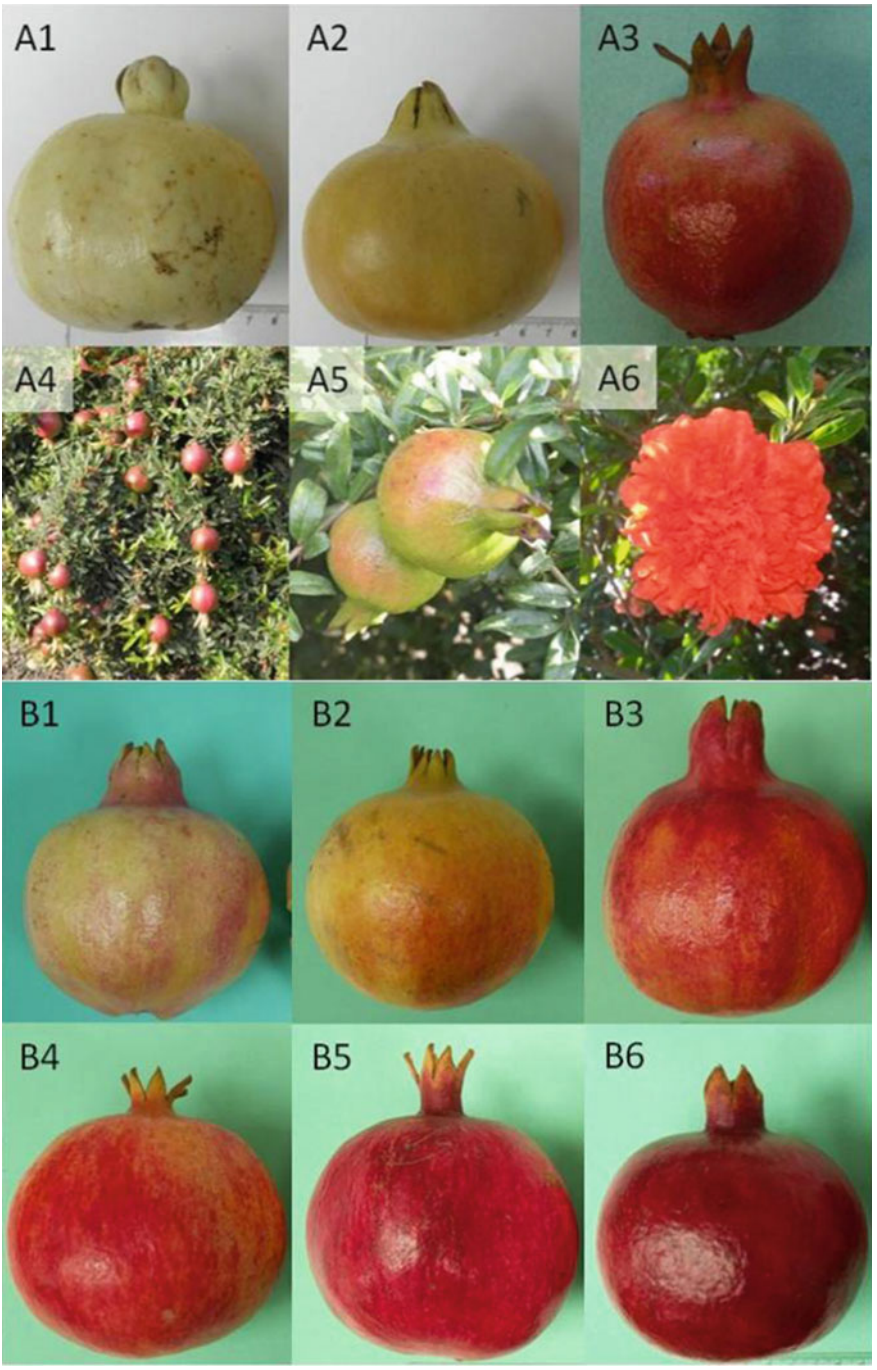
Wild *Punica granatum* are growing today throughout Central Asia extending from Iran and Turkmenistan to Northern India and are considered native to these regions. However, there is insufficient evidence that clearly defines what differentiates true wild from cultivated or cultural escapes. Botanists generally agree that Iran and its surrounding geographical regions are the origin of pomegranate, as earlier determined by de Candolle (Holland et al. 2009).

Recent single nucleotide polymorphism (SNP) analysis of the Israeli germplasm pomegranate collection, that holds accessions from Asia, Europe and North America, classified the pomegranate accessions into two major groups of kinship: one includes ornamental, inedible pomegranate accessions such as *Punica granatum* var. *nana*, as well as edible accessions from India, China and Iran and the other includes domesticated pomegranate accessions from the Mediterranean region, Central Asia and California (Fig. 15.1). One branch spreads from the putative origin of the pomegranate species towards the Far East, while the other spreads towards the West (Ophir et al. 2014). These findings support the alleged assumption of the pomegranate place of origin.

15.1.2 Genome Size and Chromosome Number

Punica granatum is a diploid species, but the exact chromosome number is in disagreement among botanists. Both cytological and genomic methods were used to determine the chromosome number. Some authors report a chromosome number of n = 8 while others report a chromosome number of 9 (2n = 16 or 18) (Darlington and Janaki Ammal 1945; Masoud et al. 2005; Nath and Randhawa 1959; Raman et al. 1971; Qin et al. 2017; Sheidai and Noormohammadi 2005; Teixeira da Silva et al. 2013; Yasui 1936). Rana et al. (2010) suggest that the chromosome number of wild pomegranates in India is 2n = 16 (2010). Interestingly, the chromosome number for *P. protopunica* is 2n = 14 (Teixeira da Silva et al. 2013).

The Plant DNA C-values Database of the Kew Royal Botanic Gardens informs that the diploid pomegranate genome size is 704 Mbp (Bennett and Leitch 2012). Recently, the first genome sequence of pomegranate was published by Qin et al. (2017). Based on the genome sequence, these authors concluded that the pomegranate genome size is 657 Mbp as compared to 714 Mbp estimated by flow cytometry. They conclude that whole-genome duplication occurred before the divergence of pomegranate and *Eucalyptus*. It is worth mentioning that Qin et al. (2017) report on 9 pseudo-chromosomes. The genome of pomegranate is similar in size to that of fruit trees of the Rosaceae family such as peach *Prunus persica* L. (Verde et al. 2013).


A1
A2
A3
A4
A5
A6
B1
B2
B3
B4
B5
B6

◀**Fig. 15.1** SNP analysis of the Israeli germplasm pomegranate collection classified the pomegranate accessions into two major groups of kinship: **a** Includes accessions such as A1. P.G.164-165, A2. P.G.167-168, A3. P.G.279-290, A4. P.G.149-150, A5. P.G. 261-272, A6. P.G.239-250 from India, China and Iran. **b** Includes domesticated pomegranate accessions such as B1. P.G.204-215, B2. P.G.106-107, B3. P.G.132-133, B4. P.G.100-101, B5. P.G.105-106, B6. P.G.116-117 from the Mediterranean region, Central Asia and California

15.1.3 Domestication and Traditional Breeding

Pomegranate was among the first fruit crops to be domesticated (Janick 2011). It is estimated that pomegranate domestication initiated somewhere in the Neolithic Era (Levin 2006; Still 2006), at first in the Transcaucasian-Caspian and northern Turkey regions (Harlan 1992; Zohary and Spiegel-Roy 1975). This region generally corresponds nowadays to Georgia, Armenia, Azerbaijan, Iran and Turkey, the regions that are believed to be the origin place of pomegranate.

Domestication is actually a process of selection and improvement of wild inferior varieties and consequent generation of varieties that excel over the former ones. Over many generations people selected local plants due to their desired qualities (larger fruit or seeds, better taste, higher yield etc.). In the case of pomegranate, which is easily propagated by cuttings, such selections resulted mainly from chance seedlings and spontaneous bud mutations. In fact, this natural intuitive practice forced genetic changes, mostly of genes concerning plant morphology and physiology. Over the course of time such successive selections created most of the pomegranate cultivars grown today in the world. Until several dozen years ago, pomegranates were grown mainly for local use, and selection was oriented towards local standards. Therefore the main cultivars found today reflect local priorities. For example the traditional Indian (Ganesh) and Spanish (Mollar de Elche) cvs. which are characterized by soft seeds and low-acid taste (Holland et al. 2009). However, in recent years several breeding centers have been oriented towards stakeholders from foreign markets, which is expected to result in a wide range of cultivars with traits reflecting the trend in the newly-developed markets in Europe, Asia, the USA and Australia.

15.1.4 Importance and World Distribution

Since ancient times pomegranate consumption by humans and animals has been very divergent and includes usage as a food, medical remedy and ornamental, and for religious purposes. Modern research confirms traditional utilization of pomegranate and show that fruit, flower, bark and leaf tissues contain bioactive phytochemicals suitable for wide-scale medical purposes (Holland and Bar-Ya'akov 2014; Rahimi et al. 2012; Seeram et al. 2006).

Pomegranate culture is generally considered a minor crop. The pomegranate tree requires a long, hot and dry season in order to produce a good yield of large

fruit with optimal color and sugar content and with low likelihood of splitting. The pomegranate tree grows readily in dry, subtropical and tropical regions all over the world. Commercial orchards of pomegranate trees are grown on different scales in the Mediterranean Basin (North Africa, Egypt, Israel, Syria, Lebanon, Turkey, Greece, Cyprus, Italy, France, Spain, Portugal); and Asia (Jordan, Iran, Iraq, Saudi Arabia, Afghanistan, Pakistan, India, China, Bangladesh, Myanmar, Vietnam, Thailand, Kazakhstan, Turkmenistan, Tajikistan, Kirgizstan, Armenia and Georgia). Pomegranates are also grown in North and South America (mainly in the USA, Chile, Argentina, Brazil and Peru), in South Africa, and in Australia (Holland et al. 2009).

The increase in world production over the last two decades reflects the increased demand due to the medical benefits of the various parts of the fruit and tree and the rapid improvement of horticultural agro-techniques for large-scale production of pomegranates. Pronounced progress was also made in methods of fruit processing, mechanical aril extraction, fruit storage period extension, superior agricultural management regarding irrigation methods, low quality water utilization and efficient plant protection (Holland and Bar-Ya'akov 2008). On top of these is the introduction of new cultivars with high fruit quality and a longer production season.

No current complete data exist on pomegranate planted areas and production in the world. The Food and Agriculture Organization (FAO) does not include pomegranate in its reports and at present the United State Department of Agriculture (USDA) and the European Union do not publish pomegranate data (Kahramanoglu and Usanmaz 2016). During the last decade, parallel to world production increase, contradicting information on world production was reported to be 1.5–2.5 million mt (Holland and Bar-Ya'akov 2008; Kahramanoglu and Usanmaz 2016). We estimate (based on personal knowledge) that more than 100,000 ha are grown, respectively, in India, China and Iran. Other countries such as Turkey, Spain and the USA are large producers on the scale of 40,000 ha each. Most of the fruit today is for human consumption either fresh or as juice. However, recent data on the contribution of pomegranate to animal health and milk production may significantly change the picture and positively influence the drive to increase the extent of pomegranate orchards.

15.2 Germplasm Biodiversity and Conservation

15.2.1 Germplasm Distribution

Pomegranate was cultivated in the Transcaucasian-Caspian region thousands of years ago and evidence of pomegranate use in the Middle East is dated to 3000 BC (Goor and Liberman 1956; Stepanyan 2007; Still 2006). From there it was introduced throughout the Mediterranean basin to North Africa and Europe westward and to the rest of Asia eastward. The Greeks and the successor empires distributed the pomegranate all over Europe. The Phoenicians spread it with their ships and by 1000 BC pomegranates were growing in Carthage.

Spanish sailors and Jesuit missionaries brought pomegranate seed with them to the New World, to Mexico and California in the 1500s, and in the 1700s to Spanish Florida.

Pomegranate was taken to the Indian peninsula from Iran about the first century and was reportedly growing in what is now Indonesia in 1416 (Ashton 2006; Holland et al. 2009). Pomegranate was introduced to China around 100 BC during the Han Dynasty by Zhang Qian (Jian et al. 2012).

15.2.2 Germplasm Conservation

15.2.2.1 In Vivo Conservation

Wild, semiwild and cultivated accessions of pomegranate, including commercial cultivars (i.e. cultivated varieties), local traditional varieties, seedlings, landraces and ornamental varieties of local origin and introduced pomegranate are conserved in germplasm collections in many pomegranate-growing countries worldwide. Unfortunately the published data are incomplete and not always accurate. It is highly probable that there is considerable redundancy among accessions within and among collections. Efforts to eliminate redundancy will help to correctly assess the divergence among pomegranate accessions (Holland et al. 2009).

Conservation of genetic resources usually involves traditional methodologies. In general, most collections are kept as living trees in orchards.

Published information on pomegranate germplasm reservoirs as presented below are detailed by country from east to west:

China. Although Chinese scientists published a wide range of research work on pomegranate, germplasm collection data in open sources are incomplete. Those collections published in English include the collection in Yunnan (Yang et al. 2007) and the Genetic Resources Unit of Shandong Institute of Pomology that holds at least 85 accessions (Yuan et al. 2007). In recent years a national pomegranate germplasm repository was established in Yicheng District of Shandong with 289 accessions (Yuan and Zhao 2017). There are several other pomegranate collections (personal communication), but unfortunately information about them is not available in Western languages.

Thailand. A collection of 29 accessions was established in Chiang Mai and Bangkok of which 5 are local while the rest were introduced (Thongtham 1986).

India. At least 14 centers in India are preserving pomegranate accessions. The largest collections which hold more than 60 accessions each are the following. The National Bureau of Plant Genetic Resources Regional Station in Bhowali, Uttarakhand contains 90 accessions (Rana et al. 2007). A collection of 187 accessions was reported at the National Research Center on Pomegranate of the Indian Council of Agricultural Research in Solapur, Maharashtra (Chandra et al. 2010; Verma et al. 2010). A total of 190 accessions are in the Central Institute of Arid Horticulture in Rajasthan; 61

in Mahatma Phule Krishi Vidyapeeth in Rahuri, Maharashtra; 170 in the National Bureau of Plant Genetic Resources in New Delhi; and 52 accessions in Sardakrishi-nagar Dantiwada Agricultural University in Gujarat (Chandra et al. 2010).

Iran. This country, which corresponds with the pomegranate's center of origin, maintains a large germplasm collection, which comprises about 770 accessions at the Iranian National Pomegranate Collection held in Yazd and Saveh. Some 740 are cultivated and the rest are wild and ornamental types (Ebrahimi et al. 2010; Rahimi et al. 2012). Collections are also preserved in the Horticultural Research Centre of the Agriculture Faculty of the University of Tehran (Zamani et al. 2007).

Turkmenistan. This country is part of the region which corresponds with the origin of pomegranate and maintains a collection of more than 1100 accessions in the Turkmenistan Experimental Station of Plant Genetic Resources at Garrygala. The collection contains specimens from the central Asian region as well as introduced specimens (Levin 2006). Unfortunately the current condition of this collection is unknown. A small portion of this collection was transferred to other pomegranate research centers including the USA and Israel.

Uzbekistan. A pomegranate collection is located in the Schroeder Uzbek Research Institute of Fruit Growing, Viticulture and Wine Production in Tashkent (Mirzaev et al. 2004).

Azerbaijan and Tajikistan. The current situation for the germplasm collections in these countries is uncertain as well. Two decades ago, Levin (1995) reported on collections of 200–300 accessions in each country.

Russia. A collection of 800 accessions is reported from the germplasm reservoir at the I. Vavilov Research Institute of Plant Industry, St. Petersburg (Frison and Servinsky 1995).

Ukraine. A germplasm collection of 370 accessions that includes accessions from Central Asia, Europe and the USA is located in Yalta (Yezhov et al. 2005).

Turkey. A collection of more than 180 accessions was established in Alata Horticultural Research Institute, Mersin (Onur and Kaska 1985). Some 158 pomegranate accessions are preserved in the Plant Genetic Resources Department of the Aegean Agricultural Research Institute in Izmir (Frison and Servinsky 1995). A reported 33 accessions are maintained in Cukurova University at Adana (Özguven et al. 1997).

Israel. The Israeli germplasm collection of pomegranate is located at the Newe Ya'ar Research Center and contains more than 150 accessions. Most of the specimens are local and the rest are introduced from all over the world. Some of the data are documented in the Israel Gene Bank for Agricultural Crops in The Agricultural Research Organization in Bet Dagan (http://igb.agri.gov.il/; Harel-Beja et al. 2015; Ophir et al. 2014).

Spain. In Spain, 59 local accessions are preserved at the Escuela Politécnica Superior de Orihuela, Miguel Hernandez University, in Alicante, in a germplasm bank established in 1992 (Martinez-Nicolas et al. 2016). In addition, 87 accessions from 10 different countries are planted in the Spanish germplasm collection at the Instituto Valenciano de Investigaciones Agrarias (IVIA) in Moncada (Badanes et al. 2017).

Tunisia. Two germplasm collections in Gabes and in southern Tunisia containing 63 local accessions were reported by Mars and Marrakchi (1999). A collection was

established in 1990 at the Tunisian National Pomegranate Germplasm Collection at Zerkin (Hasnaoui et al. 2012).

USA. In the early 1900s, the USDA Division of Seed and Plant Introduction station established a germplasm collection at the Plant Introduction Station at Chico, California with over 100 accessions of pomegranates, many of them seedlings originating from early Spanish settlers (Ashton 2006). The U.S. National Clonal Germplasm Repository in Davis, California currently possesses over 280 accessions including those from Chico, originating from 11 countries (Preece 2017).

In addition, partial information identifies Afghanistan, Kabul University, Samadi (2015); Albania, Valias collection orchards belonging to the Agricultural University of Tirana, Koka (2015); Cyprus, Agricultural Research Institute in Nicosia; Egypt; France, French Agricultural Research Centre for International Development at Capesterre Belle Eau; Germany; Greece; Hungary, University of Horticulture and Food Industry in Budapest; Italy, Institute of Agricultural Sciences in Verona; Morocco; Portugal, National Fruit Breeding Station in Alcobaca; and South Africa, Alternafruit SA Ltd. in Wellington as holding germplasm collections of pomegranate (Frison and Servinsky 1995; Mars 2000; Verma et al. 2010).

In some pomegranate-growing countries such as Jordan, the pomegranate has been subjected to elimination in recent decades, because of its replacement with olive trees, and because of a shortage of irrigation water (Owais and Abdel-Ghani 2016) or other agricultural and economic reasons. Although a few local varieties are still propagated, no germplasm reserve exists.

15.2.2.2 In Vitro Conservation

As mentioned above, most of the pomegranate accessions are conserved as living trees. However, several reports indicate that pomegranate could be suitable for conservation as plantlets under tissue culture conditions. In this respect two in vitro methods were reported as relevant to pomegranate conservation: micropropagation and synthetic seeds or micropropagules. The ability to maintain pomegranate somatic tissues in tissue culture could in principle be used for the conservation of pomegranate plant collections in limited space and for distribution of disease-free material between countries.

Micropropagation. One of the promising ways to conserve somatic plant material is by using tissue culture technology. The utilization of in vitro techniques for the establishment of shoot tips, lateral nodes and axillary buds for pomegranate tissue culture is reported in several studies (Teixeira da Silva et al. 2013). In vitro techniques enable fast and disease-free propagation from minute amounts of plant material, increasing the chances for safe propagation of highly-prized material and agility of transport of material from place to place. One example for such a usage was reported by Dessoky et al. (2017) which showed that two local cvs. Taify and Yemeni from Taif, Mecca Province, Saudi Arabia could be micropropagated and preserved by using the slow-growth technique on Murashige and Skoog (1962) medium containing of 2%

sucrose and 2% mannitol (Dessoky et al. 2017). This technique requires additional testing of regenerated trees for trait stability.

Synthetic seeds or seed analogues. The use of somatic embryos or other micropropagules as tissue encapsulation is an interesting method for cryopreservation as described by Sharma et al. (2013). Naik and Chand (2006) showed success in encapsulating pomegranate nodal segments from in vitro proliferated shoot cultures or axenic cotyledonary nodes. Such possibilities could provide efficient and relatively cheap methods for preserving pomegranate germplasm. Reports on the usage of artificial seeds for pomegranate conservation are not yet available but the overall similarity of the regenerated trees to the original trees should be examined.

15.2.3 Germplasm Biodiversity and Phylogeny

Pomegranate varieties display a remarkable variability with a wide range of phenotypic characteristics (Fig. 15.2). These include tree size, breaking dormancy, leaf characteristics, thorniness, flower color, productivity, time of ripening, fruit size, fruit shape, peel thickness, crown size, peel and aril colors, aril size, fruit taste, acid levels, sugar levels, seed hardness, rooting capability, sensitivity to insects and diseases, adaptability to various climatic conditions and tolerance to biotic and abiotic stresses. This impressive variability of phenotypes reflects high genetic diversity. Yet the early studies using molecular markers such as random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR) were inconclusive. Among the genetic markers used for pomegranate are the following: RAPD, AFLP, sequence related amplified polymorphism (SRAP), SSR, inter simple sequence repeats (ISSR) and single nucleotide polymorphism (SNP) (Table 15.1). All these works showed an ability to separate the accessions from each other by the use of genetic markers and found them to be informative and useful (Table 15.1). In some studies low diversity was reported while in other, high diversity was reported (Table 15.1; Norouzi et al. 2012; Parvaresh et al. 2012; Yuan et al. 2007). In most cases it was possible to separate a collection into distinctive groups. Some studies were able to genotype distinct groups by their geographical origin or by their country of origin (Table 15.1; Alamuti et al. 2012; Ebrahimi et al. 2010; Ophir et al. 2014). The main reason for nonagreement between the studies with respect to genetic diversity was probably due to either the low number of molecular markers used or very low number of accessions used (Table 15.1).

About 90% of the studies were based on local germplasm (Table 15.1) and just 10% had accessions from other countries in their study. In those cases there were generally only very few foreign accessions. Interesting studies compared the diversity among cultivated and wild accessions in India (Kanwar et al. 2008; Narzary et al. 2010; Ranade et al. 2009; Zamani et al. 2013). In these profiling experiments it was possible to separate wild, semi-wild and cultivated accessions and demonstrate significant variability among these groups.

Table 15.1 Molecular markers used for the assessment of genetic diversity, phylogeny and cultivar characterization in pomegranate

Marker type	No of accessions	Tree-growing location	Accessions origin	No of marker used	References
RAPD	28	Iran	Iran	13	Talebi Bedaf et al. (2003)
ISSR	–	Iran	Iran	–	Talebi Bedaf et al. (2005)
AFLP	–	Iran	Iran	–	Rahimi et al. (2006)
RAPD	24	Iran	Iran	16	Sarkhosh et al. (2006)
SSR	29	Iran	Iran	5	Koohi-Dehkordi et al. (2007)
RAPD	6	Southern Anatolia, Turkey	Turkey	15	Ercisli et al. (2007)
RAPD	4	India	India	12	Jambhale et al. (2007)
RAPD	55	China	China	15	Lu et al. (2007)
RAPD	11	Iran	Iran	13	Sheidai et al. (2007)
RAPD	25	China	China	12	Yang et al. (2007)
AFLP	85	Shandong, China	China	8	Yuan et al. (2007)
RAPD	24	Iran	Iran	27	Zamani et al. (2007)
RAPD	6	Hatay, Turkey	Turkey	22	Durgaç et al. 2008
AFLP	34	Tunisia	Tunisia	6	Jbir et al. (2008)
RAPD	6	India	India	21	Kanwar et al. (2008)
RAPD	10	Iran	Iran	40	Sheidai et al. (2008)
SRAP	23	China	China	7	Zhang et al. (2008)
RAPD	10	Albania	Albania	5	Ariola et al. (2009)
AFLP	12	Jordan	Jordan	8	Awamleh et al. (2009)
AFLP	17	Sousse, Tunisia	Tunisia	6	Jbir et al. (2009)
RFLP	10	Alicante, Spain	Spain	1	Melgarejo et al. (2009)
RAPD	49	Western Himalayan, India	India	21	Narzary et al. (2009)
DAMD	49	Western Himalayan, India	India	5	Narzary et al. (2009)
RAPD	64	7 states, India	India	8	Ranade et al. (2009)
DAMD	64	7 states, India	India	4	Ranade et al. (2009)

(continued)

Table 15.1 (continued)

Marker type	No of accessions	Tree-growing location	Accessions origin	No of marker used	References
RAPD	10	Iran	Iran	20	Sheidai et al. (2009)
SSR	34	Italy, Spain, Turkey	Italy, Spain, Turkey	9	Currò et al. (2010)
SSR	20	Yazd, Iran	Iran	23	Ebrahimi et al. (2010)
SSR	27	Tunisia	Tunisia	11	Hasnaoui et al. (2010a)
RAPD	12	Gabès, Tunisia	Tunisia	4	Hasnaoui et al. (2010b)
AFLP	33	Italy, Turkey	Sicily, Spain, Turkey		Malfa et al. (2010)
AFLP	67	Yazd, Iran	Iran	8	Moslemi et al. (2010)
ISSR	49	Western Himalaya, India	India	17	Narzary et al. (2010)
RAPD	18	Iran	Iran	40	Noormohammadi et al. (2010)
SSR	60	Iran	Iran	12	Pirseyedi et al. (2010)
SSR	202	Arak, Iran	Iran	7	Basaki et al. (2011)
ISSR	24	Yazd, Iran	Iran	64	Bedaf et al. (2011)
RAPD	24	Yazd, Iran	Iran	29	Bedaf et al. (2011)
RAPD	23	Northeastern Turkey	Turkey	12	Ercisli et al. (2011a)
AFLP	19	Northeastern Turkey	Turkey	4	Ercisli et al. (2011b)
AFLP	21	Yazd, Iran.	Iran	31	Sarkhosh et al. (2011)
SSR	11	Valencia, Spain	Spain, USA, Turkey, Israel	117	Soriano et al. (2011)
SSR	738	Yazd, Iran	Iran	12	Alamuti et al. (2012)
SSR	8	Apulia, Italy	Italy	20	Gadaleta et al. (2012)
SSR	33	Zerkin, Tunisia, Florence, Italy	Tunisia, Italy	12	Hasnaoui et al. (2012)
SSR	32	ex situ collection	Tunis	15	Jbir et al. (2012a)
SSR	18	Tunis	Tunis	4	Jbir et al. (2012b)
AFLP	18	Tunis	Tunis	4	Jbir et al. (2012b)

(continued)

Table 15.1 (continued)

Marker type	No of accessions	Tree-growing location	Accessions origin	No of marker used	References
SSR	42	Henan, China	China	21	Jian et al. (2012)
AFLP	31	Yazd, Iran	Iran	7	Nemati et al. (2012)
RAPD	36	Saveh, Iran	Iran	13	Noormohammadi et al. (2012)
ISSR	36	Saveh, Iran	Iran	6	Noormohammadi et al. (2012)
SSR	36	Saveh, Iran	Iran	2	Noormohammadi et al. (2012)
cpSSR	26	Iran, Davis, CA, USA	Worldwide	16	Norouzi et al. (2012)
SSR	75	Davis, CA, USA.	Worldwide	10	Parvaresh et al. (2012)
AFLP	62	Tehran, Iran	Iran	67	Sarkhosh et al. (2012)
SRAP	63	Iran	Iran	13	Soleimani et al. (2012)
RAPD	47	Zhengzhou, China	China	11	Zhang et al. (2012)
RAPD	10	Albania	Albania	20	Bacu et al. (2013)
SSR	202	Arak, Iran	Iran	7	Basaki et al. (2013)
CTDNA	18	Iran	Iran	2	Hajiahmadi et al. (2013)
RAPD	10	Jammu, India	India	10	Mahajan et al. (2013)
RAPD	13	Jodhpur, India	India	10	Singh et al. (2013)
ITS	13	Jodhpur, India	India	2	Singh et al. (2013)
RAPD	50	North Iran	Iran	18	Zamani et al. (2013)
RAMP	46	7 provinces, China	China	14	Zhao et al. (2013)
SNP	105	Israel	Worldwide	480	Ophir et al. (2014)
RAPD	19	Coruh Valley, Turkey	Turkey	47	Orhan et al. (2014)
AFLP	24	Morocco	Morocco	6	Ajal et al. (2015)
SNP	76	Israel	Isarel	1092	Harel-Beja et al. (2015)
SRAP	12	India	India	30	Kanupriya et al. (2015)

(continued)

Table 15.1 (continued)

Marker type	No of accessions	Tree-growing location	Accessions origin	No of marker used	References
SSR	12	Bengalur, India	India	167	Ravishankar et al. (2015)
SSR	88	ICAR-NRCP, India	India	196	Singh et al. (2015)
RAPD	14	Jordan	Jordan	12	Owais and Abdel-Ghani (2016)
SSR	14	Jordan	Jordan	17	Owais and Abdel-Ghani (2016)
SSR	87	Spain	Worldwide	20	Badanes et al. (2017)

With the development of more robust sets of markers (Ophir et al. 2014; Ravishankar et al. 2015; Singh et al. 2015) the nature of pomegranate variability and diversity has begun to unfold. Still, no studies that provide comparative and comprehensive information regarding the extent of pomegranate diversity and comparison

Fig. 15.2 Pomegranate varieties display a remarkable variability in a wide range of phenotypic characteristics

to the diversity of other plants and fruit trees are yet available. High throughput methods were used to identify SNPs which were utilized for genotyping the Israeli pomegranate collection (Ophir et al. 2014). Because a relatively high number of SNPs was used and because the collection contains cultivars from diverse world geographical sites, this analysis provided better insight into the extent of diversity among pomegranate accessions. Moreover, it was possible to associate molecular markers with phenotypic characteristics and phylogenetic groups (Harel-Beja et al. 2015; Ophir et al. 2014). These studies demonstrated that the Israeli national collection of pomegranate is divided into two main groups. One group originating from Iran, India and China, and another group from Turkmenistan, the Mediterranean Basin, Europe and the USA. These data suggest that pomegranate was spread from Iran in two different directions. The group which spread towards the east (India and East Asia) contains also wild species, suggesting that this group spread earlier in time. The other group which spread to the west might have occurred relatively late. Interestingly, all evergreen accessions belong to the eastern group. These SNPs could also subdivide the collection into different phylogenetic groups such as the group which includes all the Turkmen and Spanish species (pink skin and aril color and soft seeds), the black group which contain black colored pomegranates, the Mediterranean group and the Wonderful cv. group. Yet, despite the relatively high number of molecular markers used in this study, no conclusive information was provided on the extent of diversity among pomegranate varieties. It is worth mentioning that the number of SNPs obtained in the Ophir et al. (2014) study was surprisingly low given that deep sequencing RNAseq methods were used and despite the pronounced phenotypic differences that exist between the accessions that were used for that study. The de novo transcriptome reflects about 70% of the pomegranate genome.

For full comprehension of the pomegranate diversity, the genome sequence of pomegranate is essential. Recently, two studies reported for the first time the de novo assembly of the pomegranate genome (Qin et al. 2017; Yuan et al. 2017). These data provide useful information on pomegranate diversity based on sequencing of as many as possible pomegranate genomes.

15.2.4 Cultivar Characterization

Pomegranate cultivars with widely divergent features are grown around the world. It is impossible to determine their true number because of a great shortage of information regarding the subject, starting with a clear definition of what accessions qualify as cultivars. However, some authors have estimated more than 500 cultivars (Levin 2006). Most of them are local varieties well adapted to their growth habitat. These traditional varieties are known and beloved by both growers and consumers and reflect their cultural priorities as well as growth and environmental conditions. In a large country like India for example, Bhagwa, Alandi, Phule Araka, Mridula, Ganesh, Karadi and Muskat are the main cultivars grown in Maharashtra; Dholka, Muskat Red, Kandhari and Ganesh in Gujarat; Kandhari in Himachal Pradesh; Ganesh, Paper Shelled,

Muskati Red and Spanish Ruby in Andhra Pradesh; Madhugiri, Bassein Seedless, Jyothi, Madhugiri and Paper Shell in Karnataka; Jodhpuri Red, Jodhpuri White and Jalore Seedless in Rajasthan; and other cultivars popular in different states (Efresh-global 2017; Ranpise et al. 2014). Hence, cultivars with a greater distribution, are commonly used even beyond their country of origin. Such are for example cvs. Wonderful (USA), Acco (Israel), Mollar de Elche (Spain), Bhagwa (India) and Hicanzar (Turkey) (Holland and Bar-Ya'akov 2008). A detailed description of pomegranate cultivars was published by Holland et al. (2009).

Wonderful is the most produced pomegranate cultivar in the USA, Israel and some other countries and is among the important cultivars in the European and American markets. Wonderful was an especially good tree found among cuttings by a Mr. Bearrs from Porterville, California, obtained from Florida in 1896 (Ashton 2006). It is interesting to mention in this respect, that there are old reports about the existence of varieties in the Mediterranean region that have a similar phenotype to that of Wonderful such as the Red Lufani (Ophir et al. 2014). Hicaznar is an important red, high producing Turkish cultivar (Özguven et al. 2009). The fruit has a sweet-sour taste and hard seeds and is somewhat similar to Wonderful. Although the name Wonderful is linked to an American distinguished cultivar it is actually a collection of varieties that have a generally similar description with small alterations, more of a landrace or homonym.

Another cultivar to pay attention to is the Indian evergreen cultivar Bagua that bears fruit in India throughout the entire year, depending on the agrotechnique in use. It is a rather small sweet fruit with a strong red skin and aril color and soft seed. It is supplied for local consumption but also exported to Europe. Bhagwa is now the most widely grown pomegranate cultivar in India because of its appealing red color, sweet taste and good yield. However, the cultivar is susceptible to bacterial blight which significantly affects the Indian pomegranate industry and the export of the fruit. Another famous cultivar in India is Ganesh. This cultivar is particularly cherished by the Indians due to its pleasing sweet taste and soft seeds. The light color of the skin and the arils restricts its expansion and use for export. In Spain, the Mollar (Mollar de Elche) cultivar is very popular. Mollar is actually a series of cultivars with similar features that slightly differ from each other and are characterized by large pink color fruit of sweet taste and soft seeds. Mollar is particularly cherished by the Spanish because of its soft seeds and sweet taste. Another popular cultivar in Spain is Valenciana which has slightly better color and precedes Mollar with respect to time of harvest (Melgarejo et al. 1995, 2000).

Most Chinese cultivars are of sweet taste with very low acidity and variable amount of red color starting from white-yellow to dark red (Yuan and Zhao 2017). Of particular interest are the Dabenzi red hard-seeded cultivar which had its genome sequence published recently (Qin et al. 2017) and Tunisi, which is slightly red with soft seeds and is grown in Henan Province.

In Israel, several cultivars (Shani-Yonay, Emek, Acco and Neta-NY) were developed with strong red color, soft seeds and sweet taste. These cultivars are of special interest since they are very early and ripen already in August to September. Emek, Shani-Yonay (Fig. 15.3) and Acco are now grown in several countries in the Mediter-

Fig. 15.3 **New** Israeli cultivars resulted from a continuous breeding project. **a** Emek, **b** Shani-Yonay

ranean region including Spain, Italy and Greece, in the USA and in the Southern hemisphere in South America and South Africa.

15.2.5 Modern Breeding Objectives

The increased economic importance of pomegranate followed by expansion of exports has encouraged and widened breeding efforts and influenced selection criteria. From the beginning of cultivation, pomegranates were selected on the basis of their fruit size and colors, taste and juiciness, and yield level. Other selection protocols were aimed at growth condition aspects, especially soil and climate suitability. During that era the preferences of skin color, aril color and taste differed depending on countries and cultures. Modern breeding takes into consideration all those traditional factors but in addition to highly productive cultivars of high quality attractive fruit it has to face new challenges. There are many attractive cultivars around the world. However, cultivars that combine most of the desired features such as good taste and colors, high content of health-benefit compounds, resistance to major pests

and diseases and good storage ability are rare. Another challenge breeders confront is expanding the shipping season by developing very-early to very-late ripening cultivars. Thus, modern breeding is aimed towards a cultivar that will combine the desired characters as follows:

(a) Productivity: High.
(b) Fruit quality: Large fruit, large and juicy arils, red skin and arils (although light colors are satisfactory in some markets), soft seeds, a balanced taste (fine sugars to acids relation), medium peel thickness.
(c) Physiological fruit harms: No fruit cracking, no sunburn.
(d) Ripening time: Spanning the season from very early to very late.
(e) Pest management: Resistance to major pest (e.g. fruit borers, aphids) and diseases (e.g. tree blight, *Alternaria* spp.) to decrease insecticides and fungicides usage.
(f) Physiological tree harms: Frost resistance, low quality water adaptability.
(g) Postharvest: Long storage.
(h) Health promoting ingredients: High content of desired compounds (e.g. anti-inflammatory, antioxidants, anti-cancer etc.).

Such purposes could be achieved more efficiently, by application of modern technologies, especially molecular genetics methods, such as pomegranate genome sequence, genetic maps, marker assisted selection (MAS) and mutagenesis.

15.3 Improvement Strategies

15.3.1 Trait Inheritance

Trait inheritance analysis in pomegranate is difficult as the parent cultivars are highly heterozygous, and the heterozygosity/homozygosity status of the parents with respect to each trait is usually unknown. Moreover, for almost all of the traits, it is unknown whether the mode of inheritance is recessive or dominant and if it is controlled by a single major gene or by multiple genes. To cope with these obstacles crossbred populations and a time- consuming genetic analysis is required. As a result, very little is known about the heritability of important fruit and tree traits of pomegranates. Few traditional experiments were conducted to study the inheritance of some important features such as acidity, seed hardness and aril color (Holland et al. 2009). Crosses between Daru and Ganesh F2 progenies, revealed that high acidity was always dominant to low acidity, pink aril color was dominant to white color and hard seeds was dominant to soft seeds (Jalikop et al. 2005). Inheritance of taste was studied using several combinations of crosses between the cvs. Fellahyemez, Ernar and Hicaznar. When the sweet-sour Hicaznar was crossed with the sweet cultivar Ernar about 40% of the progenies were sour and when both parents were sweet, about 90% of the progenies were sweet (Ataseven Isik and Celik 2009). A long-term breeding

program which involved F1 and F2 segregating populations of several cultivar combinations at Newe Ya'ar Research Center showed that sour progenies were found among seedlings originated from two sweet cultivars used as parents (Holland et al. unpublished). These results contradict the determination that high acidity is always dominant to low acidity as deduced from the genetic background that was examined by Jalikop et al. (2005) and suggests that acidity heritability is more complex. In the Israeli breeding and mapping project several traits were studied. These included total soluble solids (TSS), fruit weight and perimeter, seed hardness, aril color, aril weight and plant height (Harel-Beja et al. 2015). These traits were found to be inherited as Quantitative Trait Loci (QTLs) and they were mapped on the newly established genetic map of pomegranate. Interestingly one major QTL for seed hardness was found on linkage group 1. Hard seeds were always dominant to soft seeds (Holland et al. unpublished) as found by Jalikop et al. (2005). Another trait's heritability is resistance to bacterial nodal blight that is controlled by recessive genes (Jalikop et al. 2005). Understanding the genetics of natural resistance to pests and diseases is one of the major challenges of future breeding. It will require primarily the screening for natural resistances followed by studying the complexity of these traits.

15.3.2 Role of Biotechnology

The ability to utilize biotechnology in pomegranate research and breeding is dependent on the availability of databases which include genomic and transcriptomic sequences, metabolites content and functional genomics. In addition, tools for the establishment of transgenic plants and genetic manipulation are of crucial importance. Despite the dramatic rise of interest in pomegranate as an important fruit tree, many essential tools required for incorporating biotechnology to pomegranate improvement are still missing. Biotechnology could facilitate pomegranate breeding in many aspects. It could be used to identify genes involved in control of important agricultural traits and to select for desired phenotypes in early stages of seedling development by using marker-assisted selection (MAS). Biotechnology is essential for deliberate manipulation and precise alteration of pomegranate genes for gene function analyses and for generating improved cultivars. It could also be used for increasing the array of traits available for the breeders as heterologous genes from other plants could in principle be used. The availability of efficient methods to propagate pomegranate material is highly important contributions of biotechnology for the pomegranate research. Micropropagation is fast and efficient in vitro technology to rapidly propagate pomegranate material and for conservation of important material. Pomegranate is amenable to tissue-culture propagation technique (Fig. 15.4) and protocols for in vitro propagation were reported for several pomegranate cultivars. Finally, biotechnology is of high importance in developing advanced biological sensors for water status of the plants, and for its interaction with environmental cues such as temperature and light. These in turn will assist the optimization of agricultural technologies for harvest, irrigation and fertilization.

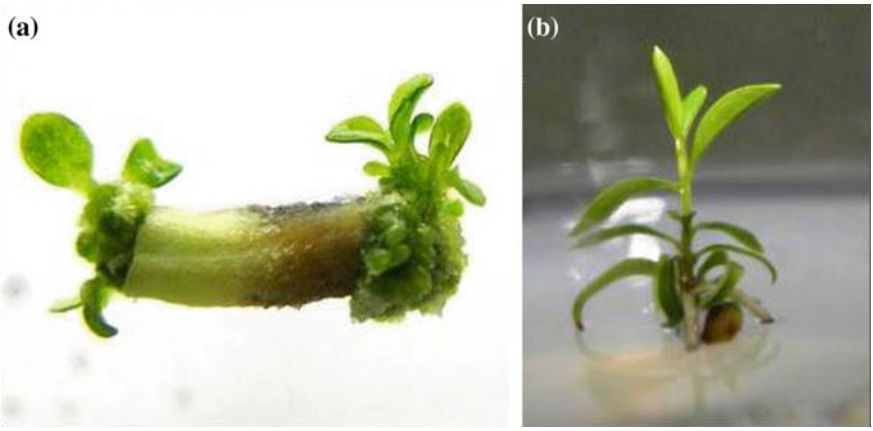


Fig. 15.4 Micropropagation of pomegranate var. *nana* conducted in Newe Ya'ar Research Center. **a** Differentiation of plantlets from stem cuttings originating from the hypocotyls. **b** Rooting of regenerated plantlets on agar medium containing 0.5-strength MS medium (Murashige and Skoog 1962) and 0.2 μM naphthaleneacetic acid

An unprecedented advance in sequencing information is now available for pomegranate. The genome sequences of at list two pomegranate cultivars Dabenzi and Taishanhong were reported (Qin et al. 2017; Yuan et al. 2017), and a general transcriptome of pomegranate was published (Ophir et al. 2014). In addition an increasing number of comparative deep sequencing studies of various pomegranate tissue including peels (Ono et al. 2011), fruit developmental stages (Qin et al. 2017), flower types (Chen et al. 2017), root, leaf, flower, pericarp and the inner and outer seed coats (Qin et al. 2017) and seeds hardness (Xue et al. 2017) were reported. There is no doubt that the sequencing data will contribute to a better understanding of the genetics and biochemistry of pomegranate.

The next challenge will be to reveal the genetic functions that are important for agricultural traits, for breeding and for genetic engineering. A crucial step to advancing in this direction will be the availability of efficient transformation system in pomegranate.

15.3.3 Genetic Transformation

Pomegranate is moderately difficult to micropropagate due to the exudation of phenolics, leading to in vitro failure to regenerate. Several methods involving different tissues and a wide range of chemical combinations have been undertaken to overcome this difficulty with varying results (Teixeira da Silva et al. 2013). A major drawback of this procedure is the difficulty to harden the plantlets once they are transferred from in vitro culture conditions to soil beds. Differing rates of success

of regenerating in vitro plantlets have been reported in pomegranate using different substrates (Teixeira da Silva et al. 2013). These difficulties retard genetic transformation in pomegranate. Unfortunately, in spite of several reports in this direction very few transgenic pomegranate plants have been produced and little progress made in the use of transgenic pomegranates for functional genomics and breeding.

Terakami et al. (2007) reported an *Agrobacterium tumefaciens*-mediated genetic transformation of pomegranate. Regenerated adventitious shoots derived from leaf segments of pomegranate var. *nana* were inoculated with *A. tumefaciens* possessing a known vector. Following inoculation, small segments of the adventitious shoots were regenerated in a selection medium. DNA analysis confirmed the presence of the transgene in the regenerated plant genome, and its inheritance was confirmed in the F1 generation (Terakami et al. 2007). Although not yet suitable for direct improvement of new cultivars, the development of transformation system in the dwarfed pomegranate var. *nana* is expected to be useful as a model system to study genetic manipulation of pomegranate, to identify important pomegranate genes for future exploitation and to decipher the function of genes in pomegranates (Holland et al. 2009).

Four recent studies reported *Agrobacterium*-mediated transformations for two pomegranate cultivars, of which two reached a low rate of transformed plantlets and therefore can be an important step towards the development of transgenic pomegranate cultivars with agronomically-important genes.

The first study, Kaji et al. (2014), developed an *Agrobacterium*-mediated transformation method. In vitro shoot segments of cv. Yousef Khani were inoculated with *A. tumefaciens* harboring a binary vector that carries selection and reporter genes. DNA analysis confirmed the presence and integration of the transgene to the plant genome in three transgenic plants, which were rooted and successfully acclimatized (Kaji et al. 2014). The second study, Hosamani et al. (2016), standardized an efficient in vitro regeneration protocol involving *Agrobacterium*-mediated transformation. Pomegranate production in India is severely harmed by the bacterial blight disease caused by *Xanthomonas axonopodis pv. punicae*. Since conventional treatments failed to control this disease, the transgenic approach appears to be promising. Juvenile explants of Bhagwa pomegranate were co-cultivated with *A. tumefaciens* possessing a vector with the *plant ferredoxin-like protein* (*Pflp*) gene and kanamycin resistance as a selecting marker. *Pflp* is a protein known to provide plants with resistance against bacterial pathogens. The presence of the transgene in the transformant in three transgenic plants out of four putative transformants analyzed was identified, but the integration to the plant genome was not yet proven. Screening has to be done to determine if the transformed plants are resistant to the bacterial blight disease (Hosamani et al. 2016). The third study, Verma et al. (2014), reported that regeneration through callus using embryonic explants was found to be more practicable for producing transgenic tissues than shoot bud or nodal segments as explants and regeneration through cell suspension culture using cotyledonary explants. The work was done on Kandhari Kabuli cv. via *Agrobacterium*-mediated transformation. Successful genetic transformation in the transformed plantlets was confirmed by PCR analysis. The fourth study, Ono et al. (2012), reported the development of a trans-

formation system that allows gene expression. This transformation system is helpful for the research not for cultivar improvement as only roots were reported to be stably transformed. The authors established a pomegranate hairy root culture system that produces hydrolysable tannins. *Agrobacterim rhizogenes* transformed with a binary vector expressing a yellow fluorescent protein gene was used for hairy root induction. The fluorescent protein allowed non-destructive detection of the transgene incorporation. Based on this system the expression of 26 putative UDP-glycosyltransferase genes from pomegranate fruit peel were verified in wild type and hairy roots. In addition, two candidate genes for hydrolysable tannins biosynthesis were identified based on HPLC and differential gene expression analyses of various pomegranate tissues. These four experiments are the only reports available which utilized pomegranate transformation for functional analyses of pomegranate genes.

15.3.4 Genetic Engineering and Genome Editing

Transgenic pomegranate cultivars are not expected to be created in the near future due to severe restrictions on commercial use of genetically-modified plants. Moreover, transformation systems are not yet developed for commercially-important cultivars. However, the development of a transformation system in pomegranate var. *nana* and hairy root culture as specified above is expected to serve for studying important pomegranate genes for future use and for deciphering the function of newly-discovered genes (Holland et al. 2009).

The newly-developed CRISPR-Cas9 genome editing tool was recently used in plants (Yin et al. 2017). Genome-editing technology is of special interest as it allows for editing specific genes within the plant genomes. The usage of genome editing technology has not yet been reported for pomegranate.

15.3.5 Mutagenesis

Somaclonal variation in tissue culture-derived material is 10,000 times more likely to occur than spontaneous mutation in whole plants (Teixeira da Silva et al. 2013). This variation can be used for plant breeding. Nevertheless, there is not a single example of a commercially-grown new cultivar of any major crop species developed as a result of somaclonal variation, including pomegranate (Teixeira da Silva et al. 2013). Other in vitro mutagenesis methods had technical success. Tetraploid pomegranate plants have been produced through colchicine treatment of pomegranate var. *nana* shoots (Shao et al. 2003). The generated plants had shorter roots, wider and shorter leaves and flowers with enlarged diameter as compared to diploid pomegranates. No report on the production of commercially valuable cultivar through this technology is available. Kerkadze (1987) reported on the generation of the Karabakh cv. by using

gamma irradiation. Peerjade et al. (2017) reported on the characterization of progeny derived from gamma- irradiated seed.

15.4 Molecular Breeding

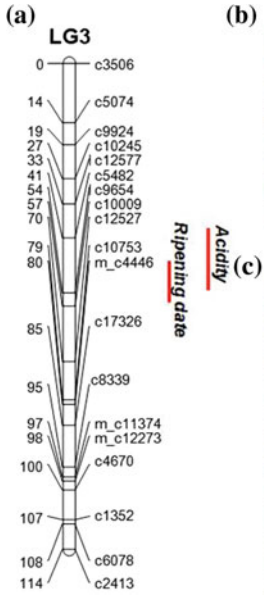
15.4.1 Molecular Marker Assisted Breeding

Traditional fruit-tree breeding based on crosses and progeny selection is time consuming and costly. Molecular marker-assisted breeding can save time and money by an early selection approach. DNA-based markers are used to construct genetic maps, which help to trace markers linked to important quantitative and qualitative traits in fruit trees. A number of DNA based molecular markers were used to reveal the diversity, genetic relations and phylogeny of pomegranate, but also in an attempt to identify varieties by DNA fingerprints.

Sarkhosh et al. (2012) developed the first genetic linkage map for pomegranate, using an F1-segregating population of Malas Danesiyah Esfahani a late-maturing cultivar with dark red peel and arils, sour-sweet taste and semi-hard seeds and Bihaste Danesefid Ravar, an early maturing with yellow peel, white arils, sweet taste, and soft seeds fruit. This AFLP-based genetic map covered 435.5, 344.2, and 335.5 cM of the haploid genomes of Malas Danesiyah Esfahani, Bihaste Danesefid Ravar and the consensus map, respectively. There was no pomegranate genome for comparison and no phenotypic data (Sarkhosh et al. 2012). This early map could not lead to marker assisted breeding at the time of publication. A high resolution genetic map based on 1092 SNP markers combined with phenotypic traits was reported by Harel-Beja et al. (2015). The map is based on an F2 population of pomegranate accessions which represent evolutionary distant pomegranate accessions (Fig. 15.5). This map is the initial step towards the usage of molecular markers for breeding as it contains several traits which are important for breeding. Although, most of the reported traits are QTLs, some major QTLs were found in that research that could be used in breeding. The availability of the pomegranate genome is of high importance as it can dramatically facilitate the efficiency of gene identification and genetic markers identification. For this purpose all published and available genetic markers should be placed on the genome map and standardization of nomenclature of the markers and linkage groups is essential.

15.4.2 Functional Genomics

Gene assignment for particular traits was not described in pomegranate until recently when the gene which encodes for a transcriptional factor WD40 repeat protein involved in anthocyanin biosynthesis and formation of trichomes was reported by



(b)

Trait	No of QTLs	Group	Max. % Explanation
Peel punicalagin	1	LG5	37.7
Peel total phenols compounds	1	LG5	39.2
Peel anthocyanin	1	LG5	18.8
Aril anthocyanins	1	LG5	37.0
Ripening date	2	LG1 and LG3	17.6
Acidity	1	LG3	61.1

(c)

Fig. 15.5 Some major QTLs (**a**, **b**) present on a map derived from a segregating F2 population (**c**) (Harel-Beja et al. 2015)

Ben-Simhon et al. (2011). This function was achieved by using heterologous gene expression for the pomegranate gene and the usage of well-characterized *Arabidopsis* anthocyanin-less and trichome-less mutants in the *WD40* gene. Other functions of the *WD40* gene in pomegranate were not detected, as characterized relevant mutations in pomegranate were not reported. Another gene involved in anthocyanin biosynthesis was published. The *LDOX* gene was shown to be responsible for the white phenotype mutation in pomegranate (Ben-Simhon et al. 2015). This is the first study which used a combined genetic and molecular study for determining the site of the mutation within the *LDOX* gene. Candidate genes for the anthocyanin pathway, the galotanin pathway and flower development were suggested by several transcriptomic studies (Ono et al. 2011; Qin et al. 2017). One of these publication compared different pomegranate fruit developmental stages (Ono et al. 2011). Genes involved in flower development were suggested by comparing different flower types of pomegranate (Chen et al. 2017). Xue et al. (2017) reported that seed formation involves genes related to lignin biosynthesis and metabolism, including some genes encoding laccase and peroxidase, *WRKY*, *MYB*, and *NAC* transcription factors. In the hard-seed pomegranate, lignin-related genes and cellulose synthesis-related genes were highly expressed and in soft-seed pomegranates, expression of genes related to flavonoids and programmed cell death was slightly higher (Xue et al. 2017). These studies did not involve functional analyses and the genes involved in the pathways were assigned only by statistical correlations and gene annotations. Moreover, no correla-

tion was done between gene expression and metabolite concentration. Once an efficient and reproducible transformation system is available, it will be possible to utilize the CRISPR mutagenesis system to generate specific mutations in the pomegranate genome.

15.5 Current Breeding Projects

Pomegranate improvement projects have been initiated in some countries over the past century but has become more intensive in recent decades. These projects are mainly based on breeding by deliberate crossing or selections among local seedlings. However, details about only a few breeding projects have been published and not many new cultivars are spreading worldwide.

India. India invested intensively in pomegranate breeding starting in the early 1900s (Chandra et al. 2010). Breeding of pomegranates was done for disease resistance (Jalikop et al. 2005, 2006), low acidity and high fruit quality under a hot arid environment (Samadia and Pareek 2006), fruit yield (Manivannan and Rengasamy 1999), juice production (Jalikop and Kumar 1998), aril color (Wavhal and Choudhari 1985), fruit weight, flesh color, seed size and juice content (Karale et al. 1979), total soluble solids (Choudhari and Shirsath 1976) and seed softness (Jalikop and Kumar 1998). The Ganesh cv. is an evergreen, pink and soft-seeded selection from cv. Alandi released in 1954 (Chandra et al. 2010; Jalikop 2003). Ruby cv. is the product of complex successive crossings between Indian and foreign varieties and is an early-maturing red and soft-seeded cultivar, released in 1997 by the Indian Institute of Horticultural Research (IIHR) (Chandra et al. 2010; Jalikop and Kumar 2000). Some other minor varieties that resulted from clonal selections or crosses were also released during the last century (Chandra et al. 2010).

Two of the main cultivars in India, Mridula and Bhagwa are evergreen, red and soft-seeded cultivars. Mridula was released in 1994 and Bhagwa in 2003, by Mahatma Phule Krishi Vidyapeeth University in Maharashtra. Both cultivars are the result of selections from progenies derived from a series of crosses between the Indian cv. Ganesh and the red Russian cv. Gul-e-Shah Red. (Chandra et al. 2010; Ranpise et al. 2014). Phule Arakte cv is also a selection from F2 progeny of Ganesh and Gul-e-Shah Red cvs that was released in 2003 by Mahatma Phule Krishi Vidyapeeth University, Rahuri as a new evergreen heavy yielder of good quality red fruit (Ranpise et al. 2014).

The National Research Centre on Pomegranate (NRCP), Indian Council of Agricultural Research (ICAR) in Solapur released four new cultivars derived from unspecified parents of a project started in 2008. The new cultivars are not yet commercialized. NRCP Hybrid 4 and NRCP Hybrid 12 are designated for making anardana spice, and NRCP Hybrid 6 and NRCP Hybrid 14 show better yield and quality than Bhagwa (Dighe 2014).

Disease-resistant wild Daru have been hybridized with commercial varieties at the Indian Institute of Horticultural Research (IIHR) aiming at a bacterial blight disease

resistant cultivar. One unspecified selection was documented as disease-resistant and now is being tested in some sites. This new selection is expected to be released in two years when the selection is done (Satish Kumar 2016).

The IIHR in Bangalure released a new anardana spice pomegranate, Amlidana cv which derived from a cross between Ganesh cv and the dwarf Nana variety. Goma Khatta cv, which is a hybrid of Ganesh and Daru cvs, is also an anardana pomegranate bred type superior to Daru (Ranpise et al. 2014).

Iran. Although there are many reports on the study of various aspects in pomegranate physiology and genetics (Alamuti et al. 2012; Bedaf et al. 2011; Ebrahimi et al. 2010; Masoud et al. 2005; Moslemi et al. 2010; Noormohammadi et al. 2010; Rahimi et al. 2012) we do not have any updated information on pomegranate breeding projects in Iran.

Turkmenistan. Hybrid seedlings with good frost resistance were achieved following successive crosses, are mentioned without specifying cultivar names (Levin 2006). Desertnyi and Parfianka are choice pomegranate cultivars from the Turkmenistan collection of Dr. Gregory Levin that at present are commercialized in the USA (Bay Flora 2017).

China. Several pomegranate breeding projects yielded new Chinese cultivars over recent decades. They were obtained by breeding using local varieties, selecting mutants or chance seedling. These cultivars include early Linxuan 8 and late Lintong 14 (Zhang 2004), early Taishan Dahong (Sun et al. 2004), the large fruit bud sport Baiyushizi (Zhu et al. 2004), the soft-seeded large fruit Quingpiruanziw (Diao 2004), the highly productive disease-resistant mutant Hongyushizi (Zhu et al. 2005), a compact tree-selected seedling, early ripening and productive Mengliaihong (Pu et al. 2005), the crossed early-ripening Yushiliu 4 (Zhao et al. 2006), two good quality fruit-selected seedlings Zaoxuan 018 and Zaoxuan 027 (Wang et al. 2006), the mutant soft-seeded, productive and disease resistant Hongmanaozi (Zhao et al. 2007), the early mutant good-quality fruit Taihanghong (Zhao 2007), and some others that were reported during the 1900s (Holland et al. 2009; Jalikop 2010).

Israel. A breeding project in Israel was initiated 15 years ago to exploit the advantages of the early ripening, good color, and soft-seeded Israeli cultivars (Fig. 15.6). The project is aimed towards extending the pomegranate season (Fig. 15.7) through producing very-early and very-late ripening cultivars together with high production of good quality red fruits. Breeding was initiated by selecting seedlings from open pollination of known cultivars from which the cv. Emek was released. Emek is a high-yielding very-early cultivar that ripens in mid-August. It has a large fruit with red skin and bright red arils, soft seeds and a sweet and low-acid taste (Holland et al. 2014). Two other Israeli cultivars are selections made out of a seedling collection held at Newe Ya'ar Research Center. Shani-Yonay cv. is a productive early ripening, sweet, red pomegranate (Holland et al. 2007). Kamel is essentially very similar to cv. Wonderful with respect to fruit quality and tree growth habit. Its most distinguished trait is the dark red skin color, which appears about a month earlier than in Wonderful. Emek, Shani-Yonay and Kamel are registered under the terms of the Israeli Plant Breeders' Rights Law and are registered or under registration procedures in various countries (Holland et al. 2009). In an effort to develop early cultivars to supply the

Fig. 15.6 A young breeding plot. **a** Planting year. **b** One year after planting

European market early in the pomegranate season, a new cultivar named Neta-NY under registration procedures was recently developed and reported. This cultivar has very soft seeds, intense internal and external red color and its ripening precedes Emek by 10 days. This cultivar is now under evaluation in commercial conditions.

In the context of global warming, a new breeding project was recently initiated in Israel. Crosses were done aiming to obtain very early ripening cultivars that tolerate

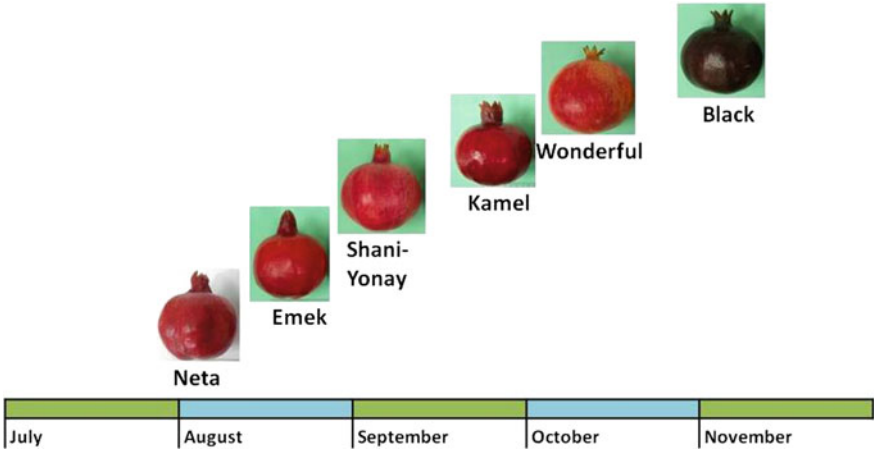


Fig. 15.7 The pomegranate season in Israel was extended as a result of the breeding project

the negative effect of heat on skin and the aril's anthocyanin content. The selection was done in the desert region of Israel and first results showed selections that proved to be heat resistant, but are still not of commercial quality.

Turkey. A total of 47 pomegranate cultivars were released as a result of various selection and adaptation studies in Turkey. Hicaznar is the only cultivar with broad production and export. It has a red skin color, dark red arils, a sour taste and a high yield and is especially suitable for transport and storage. To eliminate its sour taste and hard seed, breeding was initiated to develop sweet, juicy, soft, large red arils, and dark red skin color. This first hybridization work in Turkey included crosses between Hicaznar and the cultivars Ernar (early sweet) and Fellahyemez (late sweet). Desirable fruit and tree characteristics were tracked in the progeny, but a new cultivar is not yet released (Yazici and Sahin 2016).

Since 1990s an ongoing breeding program is underway in the Aegean region in Turkey. Three sweet, soft-seeded with red or dark skin color cultivars were registered: early ripening Tezeren 35, early-mid ripening Efenar 35 and late ripening Dr Ercan 35 (Kucuk et al. 2017). Selection of 76 pomegranate accessions from the Hatay region was reported by Caliskan and Bayazit (2013). In this work several cultivars specifically suitable for juice or for fresh consumption were described.

Italy. Selection work in Sicily produced six local pomegranate varieties. Those selections displayed a considerable amount of variation (Barone et al. 2000).

Tunisia. Selection of pomegranate was reported in south-eastern Tunisia. The authors mainly characterized local accessions looking for most desirable commercial traits and found some trees that have an attractive appearance, low acid content and high soluble solids, and were most suitable for that region, but did not specify them. These genotypes were marked as parents for the desired traits (Mansour et al. 2011).

Spain. Mollar de Elche, which is the main cultivar in Spain, has undergone a selection for new productive, sweet, soft-seeded and large-fruited cultivars. Pinon-

tierno de Ojos and Casta del Reino de Ojos are less important cultivars but have good qualities and large soft seeded fruit cultivars were selected from their populations. These selections yielded new cultivars: Mollar de Elche 14, Mollar de Elche 15, Pinontierno de Ojos 2, Pinontierno de Ojo 7 and Casta del Reino de Ojos 1 (Martinez et al. 2006).

Purple Queen, MR100, Meli and Kingdom are new protected pomegranate cultivars released recently by the companies Caliplant S.L. and Citrus Genesis International Ltd., suitable for the fresh market, processing and juice industries (Rubio 2014).

Villamon et al. (2017) published a new cultivar Rugalate, a hybrid between Mollar de Elche and Wonderful that bears large red sweet fruit that mature between Mollar de Elche and Wonderful. This hybrid still has not been tested commercially (Villamon et al. 2017).

USA. During the twentieth century, some new cultivars were released commercially in the USA, which broadens the variety of grown cultivars but none of them have displaced Wonderful. Among the new ones are Early Wonderful, similar to Wonderful but ripens about two weeks earlier; the dark red Granada cv. that ripens one month earlier than Wonderful (Tanner 2016); sweet pale pink soft-seeded arils Eversweet cv.; very large sweet fruited Ambrosia cv. (Bay Flora 2017; Tanner 2016); the red, large late fruiting Eve cv. from the University of California; and the dark burgundy skin and arils of cvs. Sharp Velvet and Sweet which have compact tree forms with very sweet fruit (Bay Flora 2017).

The late sour-sweet red cv. Angel Red (formerly Smith USA Plant Patent #16,578, 2016) was discovered by G.R. Smith as a chance seedling growing near a cv. Granada orchard located close to Visalia in the San Joaquin Valley of Central California (USPP16,578 P3 30/5/2006).

An ongoing breeding project by the University of Florida utilizes 15 varieties, including Wonderful and varieties from Iran, Turkmenistan and other sources, with the aim of possible commercial fruit production. Plants were made available to pomegranate growers, but no new cultivars have yet been designated (Deng 2015).

A new cultivar named Red Jay is a sport found on Wonderful by J.A. Yurosek near McFarland, California and is characterized by sweet red fruits that ripen in mid-August in California (USA Plant Patent #27,981, 2017).

Mexico. Several cultivars have been obtained by hybridization and selection at the Instituto Nacional de Investigaciones Forestales Agricolas y Pecuarias (INIFAP). Four improved cultivars were found suitable for cultivation in central semiarid Mexico (Mondragon-Jakobo et al. 2015).

15.6 Conclusions and Prospects

15.6.1 An Overview of Current Status

Despite its status as a healthy nutritious fruit and the interest of the scientific community in the beneficial activities of its metabolites, pomegranate has remained a minor fruit. Consequently the number of pomegranate breeding centers in the world is relatively limited and includes mostly Iran, India, and some Mediterranean countries such as Tunisia, Israel, Turkey and Spain. Pomegranate breeding has also been initiated recently in Florida USA. Most of the cultivars grown today are the result of chance seedling selections. It will take several years before new cultivars, originating from breeding centers, will prove themselves and replace the older cultivars. In spite of the great variance in pomegranate fruit phenological characteristics it appears that the genetic variability of pomegranate is rather limited. Overall recent studies indicate that the pomegranate germplasm is divided into two main groups. One branch of germplasm appears to have spread from Iran to India and China and the other to Central Asia and the Mediterranean and westward. Genetic studies can differentiate between wild and cultivated pomegranates. Only a few new cultivars were released to the markets in recent years and even fewer cultivars are expected to commercially succeed. Despite its resilience, the pomegranate tree requires skilled professional care in order to produce high yields and good quality fruits. This requires best practices of irrigation, fertilization, pest and disease management and in-depth understanding of present and potential markets. Several countries such as Iran, India, Turkey, Spain and Israel are exporters of pomegranate fruit. Others, such as Chile, Peru and South Africa have recently begun to export. Countries with a long tradition of pomegranate consumption such as Italy and Spain are gradually changing their pomegranate cultivation to increase the proportion of new cultivars with high red anthocyanin colors and sweet taste.

15.6.2 Recommendation for Future Research

The future of pomegranate culture depends on research aimed to improve the trees and enable them to cope with the demands of modern agriculture and markets. Much knowledge is required to increase the yield, fruit quality, and natural resistance to pests and diseases. Pomegranate culture requires the investment of considerable effort to develop water resource as the tree is fruiting during the hot summer; the most suitable climate for pomegranate is one with a long, hot and dry season. Commercial harvest requires many days of work for fruit picking. Fruit improvement will require, on the one hand, trees that will produce fruit with high nutritional and healthful compounds, pleasing taste and appealing color for the consumer and, on the other hand, trees that will be easier for the farmers to grow. This means high-yielding trees with natural resistances to pest and diseases such as bacterial blight, *Deudorix*

livia, carob moth and other moths. While some solutions to the problems will come from agrotechnical practices such as developing new machines for fruit harvesting, irrigation and fertilizations, others will require more radical solutions such as MAS and genetic engineering. The great advance in establishment of genetic maps that include significant mapped traits and genome sequencing are expected to greatly facilitate breeding.

Appendix 1

Major institutes involved in research related to pomegranate

Institute	Research activities	Contact information and website
Kabul University, Kabul, Afghanistan	Horticulture, germplasm collection	e-mail: samadigr@yahoo.com; http://ku.edu.af/en
Department of Biotechnology, Faculty of Natural Sciences, University of Tirana, Albania	Genetics, in vitro propagation	e-mail: ariolabacu@yahoo.com; http://www.unitir.edu.al/
Agricultural university of Tirana	Germplasm collection	e-mail: Tatjanakokai@hotmail.com; http://ubt.edu.al/sq/
College of Horticulture, Nanjing Agricultural University, Weigang, Nanjing, P.R. China	Genetics	e-mail: fanggg@njau.edu.cn; http://www.nauacademicjobs.com/colleges.html
Zhengzhou Institute of Pomology of Chinese Academy of Agricultural Sciences, Zhengzhou, P.R. China	Genetics	email: Shangyin Cao 13937192127@163.com; http://en.zzgss.cn/
College of Horticulture, Henan Agricultural University, Zhengzhou, P. R. China	Genetics	e-mail: jbhu220@yahoo.com.cn; http://www.henau.edu.cn/
Shandong Institute of Pomology, Tai'an, China	Genetics	e-mail: zhyuan88@hotmail.com
Institute of Horticulture, Anhui Academy of Agricultural Sciences, Hefei, China	Genetics, chemistry	e-mail: yiliuxu@163.com
Fruit Trees Division, Agricultural Research Institute, Nicosia, Cyprus	Germplasm collection	e-mail: s.ioannidou@ari.gov.cy; http://www.moa.gov.cy/moa/ari/ari.nsf/contact_en/
University of Horticulture and Food Industry, Budapest, Hungary	Germplasm collection	https://szie.hu/budai-campus

Institute	Research activities	Contact information and website
Conservation Biology and Molecular Taxonomy, National Botanical Research Institute (CSIR), Lucknow, Uttar Pradesh, India	Genetics	e-mail: ranats@nbri.res.in; http://www.nbri.res.in/
ICAR-National Research Center on Pomegranate, Kegaon, Solapur, Maharashtra, India	Horticulture, genetics, breeding' germplasm collection	e-mails: rchandranrcp@gmail.com; jyotisharma128@yahoo.com; nripendras72@gmail.com; http://nrcpomegranate.icar.gov.in/Staff
Mahatma Phule Krishi Vidyapeeth Agricultural University, Rahuri, Maharashtra, India	Germplasm collection	http://mpkv.ac.in/
Central Arid Zone Research Institute, Jodhpur, Rajasthan, India	Genetics, germplasm collection	e-mail: sksingh1111@hotmail.com; http://cazri.res.in/
ICAR-NBPGR (National Bureau of Plant Genetic Resources), Regional Station Jodhpur, Rajasthan, India	Germplasm collection	e-mail: nbpgr.jodhpur@icar.gov.in; http://www.nbpgr.ernet.in/Regional_Stations/Jodhpur_Rajasthan/did/36.aspx
Division of Fruit Crops, Indian Institute of Horticultural Research, Bangalore, Karnataka, India	Horticulture, cultivars, breeding, genetics	e-mail: jalikop@iihr.ernet.in; http://www.iihr.res.in/
Department of Horticulture, College of Agriculture University of Agricultural Sciences, Bijapur, Karnataka, India	Horticulture	http://www.uasd.edu/
Department of Biotechnology, Dr Y S Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh, India	Genetics	e-mail: vipasha.verma@gmail.com; http://www.yspuniversity.ac.in/
Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinaga, Gujarat, India	Germplasm collection	http://www.sdau.edu.in/
Department of Horticultural Sciences, Faculty of Agriculture, University of Tehran, Karaj, Iran	Genetics	e-mails: zzamani@ut.ac.ir; sarkhosha@gmail.com; http://can.ut.ac.ir/en/sciences%20and%20engineering/default.aspx
Department of Horticultural Science, Faculty of Agriculture, Bu-Ali Sina University, Hamedan, Iran	Transformation	e-mail: Ershadi@basu.ac.ir; http://www.basu.ac.ir/
Iranain National Gene Bank, Mahdasht Road, Karaj, Iran	Germplasm collection	e-mail: mzahravi@yahoo.com; http://medomed.org/featured_item/national-plant-gene-bank-of-iran/

Institute	Research activities	Contact information and website
Agricultural and Natural Resources Research Center of Markazi Province, Arak, Iran	Germplasm collection	Sakine Faraji; http://manrrc.ir/
Department of Horticultural Sciences, Faculty of Agriculture and Natural Resources, Arak University, Arak, Iran	Genetics	e-mail: a-khadivi@araku.ac.ir
Department of Plant Breeding, Science and Research Branch, Islamic Azad University, Tehran, Iran	Genetics, germplasm collection	Tayebe Basaki; http://www.riau.ac.ir
Department of Horticultural Sciences, Faculty of Agriculture, Vali-e-Asr University of Rafsanjan, Iran	Genetics	e-mail: h_karimi1019@yahoo.com; en.vru.ac.ir/
Ferdowsi University of Mashhad, Faculty of Agriculture, Center of Pomegranate Research, Mashhad, Iran	Genetics	e-mail: zahra.nemati@rocketmail.com; https://en.um.ac.ir/
Department of Agricultural Biotechnology, College of Agriculture, Isfahan University of Technology, Isfahan, Iran	Genetics	e-mails: mtalebi@cc.iut.ac.ir; sayedt@cc.iut.ac.ir; https://www.iut.ac.ir/en/
Agriculture and Natural Resources Research and Education Center of Yazd, Yazd, Iran	Germplasm collection, breeding	e-mail: Asman2000@gmail.com, Mohammad Reza Vazifehshenas
Pomegranate Research Station, Saveh Agriculture Research Center, Saveh, Iran	Germplasm collection, cultivars, genetics	Seyed Ziaedin Tabatabaei
Unit of Deciduous Fruit Tree Sciences, Newe Ya'ar Research Center, Agricultural Research Organization, Israel	Cultivars, breeding, genetics, germplasm collection, horticulture	e-mail: vhhollan@agri.gov.il; http://www.agri.gov.il/en/people/733.aspx
Department of Plant Pathology, Agricultural Research Organization, Volcani Center, Bet Dagan, Israel	Horticulture	e-mail: dezra@agri.gov.il; http://www.agri.gov.il/en/people/653.aspx
Department of Post Harvest, Agricultural Research Organization, Volcani Center, Bet Dagan, Israel	Horticulture	e-mail: rporat@agri.gov.il; http://www.agri.gov.il/en/people/702.aspx
Fruit Storage Research Laboratory, Kiryat Shmona, Israel	Horticulture	e-mail: fruitlab@netvision.net.il
Migal - Galilee Research Institute, Kiryat Shmona, Israel	Chemistry	e-mail: rachel@migal.org.il; http://www.migal.org.il/rachel-amir
Istituto Coltivazioni Arboree, Università di Palermo, Palermo, Italy	Cultivars	Francesco Sottile; http://www.unipa.it/Dipartimento-di-Colture-Arboree/

Institute	Research activities	Contact information and website
Department of Environmental and Agro-Forestry Biology and Chemistry, Section of Genetic and Plant Breeding, University of Bari Aldo Moro, Bari, Italy	Genetics	e-mail: agata.gadaleta@uniba.it; http://www.uniba.it/
University of Tsukuba, Tsukuba, Japan	Transformation	e-mail: ssumiko@sakura.cc.tsukuba.ac.jp; http://www.tsukuba.ac.jp/
Department of Plant Production, Faculty of Agriculture, Mu'tah University, Karak, Jordan	Cultivars	e-mail: owais@mutah.edu.jo; https://www.mutah.edu.jo/
Lebanese Agricultural Research Institute, Zahle, Lebanon; Faculty of Agricultural Sciences, The Lebanese University, Dekwaneh, Beirut, Lebanon	Cultivars	e-mail: lamis.chalak@gmail.com; https://www.ul.edu.lb/faculte/
Programa de Frutales, Campo Experimental Bajio, INIFAP, Celaya, Guanajuato, Mexico	Cultivars, breeding	e-mail: jaccobo77@hotmail.com; http://www.inifap.gob.mx/circe/SitePages/internas/bajio.aspx
University College of Agriculture & Environmental Sciences, The Islamia University of Bahawalpur, Bahawalpur, Pakistan	Cultivars	e-mail: muhammad.nafees@iub.edu.pk; http://www.iub.edu.pk/
Plant Science and Microbiology Department, Universitas Miguel Hernández, Elche (Alicante), Spain	Horticulture, genetics	e-mail: rafa.font@umh.es; http://www.umh.es/contenido/Investigacion/:uor_106/datos_en.html
Estacion Experimental Agraria de Elche, Elche, Elicante, Spain	Horticulture	e-mail: bartual_jul@gva.es; http://www.ivia.gva.es/
Instituto Valenciano de Investigaciones Agrarias, Moncada, Valencia, Spain	Genetics	e-mail: badenes_mlu@gva.es; http://www.ivia.gva.es/
Departamento de Produccíon Vegetal y Microbiologı´a, Escuela Politécnica Superior de Orihuela, Universidad Miguel Hernández, Orihuela, Alicante, Spain	Cultivars	e-mails: juanjose.martinez@umh.es; pablo.melgarejo@umh.es; https://www.umh.es/
Department of Horticultural Science, University of Stellenbosch, Stellenbosch, South Africa	Horticulture	e-mail: opara@sun.ac.za; https://www.sun.ac.za/english/faculty/agri/horticulture
Tunisian national germplasm collection of pomegranate, Zerkin	Germplasm collection	–
University of Sousse, Chott Mariem, Sousse, Tunisia	Cultivars, genetics, chemistry	e-mails: mb.mars@yahoo.fr; trifimokhtar@yahoo.fr; http://www.uc.rnu.tn/

Institute	Research activities	Contact information and website
Laboratory of Molecular Genetics, Immunology & Biotechnology, Faculty of Sciences, University El Manar, Tunis, Tunisia	Genetics	e-mail: Amel.SalhiHannachi@fsb.rnu.tn;
Laboratory and Dry Land Crop Oasis, Institute of Arid Regions, Elfje Mednine Tunis, Tunisia	Selection, genetics	e-mail: mansourelhem@yahoo.fr; www.ira.agrinet.tn/
Department of Horticulture, Faculty of Agriculture and Natural Sciences, Recep Tayyip Erdoğan University, Rize, Turkey	Cultivars, breeding	e-mail: keziban.yazici@erdogan.edu.tr; https://erdogan.edu.tr/
West Mediterranean Agricultural Research Institute, Antalya, Turkey	Cultivars, breeding	e-mail: sahinalpaslan@tarim.gov.tr;
Çukurova University, Faculty of Agriculture, Department of Horticulture, Adana, Turkey	Cultivars, horticulture, genetics	e-mail: ashen@cu.edu.tr; http://www.cu.edu.tr/eng/detay.aspx?pageId=812
Department of Horticulture, Faculty of Agriculture, University of Mustafa Kemal, Antakya, Hatay, Turkey	Cultivars	e-mail: ocaliskan@mku.edu.tr; http://www.mku.edu.tr/
Department of Horticulture, Ataturk University Agricultural Faculty, Erzurum, Turkey	Horticulture, genetics	e-mail: sercisli@atauni.edu.tr; https://www.atauni.edu.tr/
Alata Horticultural Research Institute, Erdemli-Mersin, Turkey	Germplasm collection	e-mail: cenapyilmaz@yahoo.com; https://arastirma.tarim.gov.tr/alata
Turkmenistan Experimental Station of Plant Genetic Resources, Garrygala, Turkmenistan	Germplasm collection	–
The Nikita Botanical Gardens of the Ukraine Academy of Agrarian Sciences, Yalta, Crimea, Ukraine	Germplasm collection	http://nikitasad.ru/
Paramount Farming Company, Bakersfield, California, USA	Horticulture	e-mail: EricM@paramountfarming.com; http://www.wonderfulorchards.com/
Department of Plant Sciences, University of California, Davis, California, USA	Genetics	e-mail: ltian@ucdavis.edu; http://www.plantsciences.ucdavis.edu/plantsciences/visitors/map.htm

Institute	Research activities	Contact information and website
USDA/ARS Horticulture and Plant Breeding Unit, Horticultural Research Laboratory, Ft. Pierce, Florida, USA	Cultivars	e-mail: Ed.Stover@ars.usda.gov; https://iapreview.ars.usda.gov/main/site_main.htm?modecode=60-34-05-00
The National Clonal Germplasm Repository, Tree Fruit & Nut Crops & Grapes, USDA, ARS, UC Davis, Davis, California, USA	Germplasm collection	e-mail: John.Preece@ars.usda.gov; https://www.ars.usda.gov/pacific-west-area/davis-ca/natl-clonal-germplasm-rep-tree-fruit-nut-crops-grapes/
Horticulture and Landscape Architecture, Perdue College of Agriculture, West Lafayette, Indiana, USA	Horticulture	e-mail: hwetzste@purdue.edu; https://ag.purdue.edu/
Richard R. Schroeder Uzbek Research Institute of Fruit Growing, Viticulture, and. Wine Production, Tashkent, Republic of Uzbekistan	Germplasm collection	Mirmahsud M. Mirzaev

Appendix 2

Pomegranate varieties and their cultivation regions

Country	Cultivation regions	Varieties
Afghanistan	Kandahar	Soor Shakari, Speen Shakari, Sheen, Sheen I, Soor Maranjani, Speen, Sedana, Speen Khozh, Soor I, Pundpostai
Afghanistan	Helmand	Tursh Kaghazi, Gurmah, Speen Khozh I, Sarakhwagh, Speen I
Afghanistan	Farah	Shirinak, Shirinak I,Fakhri, Fakhri I
Afghanistan	Heart	Kadu Anar, Shirin, Fakhri
Afghanistan	Kapisa	Tursh I, Maikhosh, Khog Sedana, Kacha Tuush, Speen Danadar
Afghanistan	Nangarhar	Khog Bocha, Khog I, Pestakai, Danadar Pestakai, Taki Tursh, Srah trush, Tangali Tursh, Rankai Pastaki, Tursh Bocha, Tashgurghani, Speen Tursh, Tursh Bedana
Afghanistan	Balkh	Shir-shakar, Zati, Kadu Anar I, Post Ghasp
Afghanistan	Samangan	Kasagi, Surkhak, Safeid Nazok, Shirin Anar, Shirinak
Albania	General	Tivarash, Devedishe, Majhoshe
Algeria	General	Quares, Lahlou
Azerbaijan	General	Nasimi, Aleko, Azerbaojan, Bala-Miursel, Giulosha, Guilosha Pink, Kaim-Nar, Krmyzy-Kabukh, Nazik-Kabukh, Shakh-Nar
China	Sichuan	Qingpiruanzi
China	Yunnan	Tianlvzi, Houpitianshazi, Tianguangyan, Lvpi, Hongmanao

Country	Cultivation regions	Varieties
China	Xinjiang	Piyaman, Yechengdazi, Louke 4
China	Shandong	Daqingpitian, Dahongpao, Dahonpitian, Damayatian, Taishansanbai, Taishanhong, Tunisi, Qingpidali
China	Zhejiang	Tunisruanzi, Yiseliesuantian, Qingpi
Croatia	General	Barski Slatki, Ciparski, Glavas, Sladun
Egypt	General	Arab, Manfaloty, Nab Elgamal, Wardy
Georgia	General	Pirosmani, Gruzinskii No. 1, Gruzinskii No. 2, Vedzisuri, Lyaliya, Tengo, Imeretis Sauketeso, Bukistsikhe, Khorsha, Zugdidi, Erketuli, Forma No. 1, Forma No. 15, Forma No. 70, Shirvani, Apsheronskii Krasnyi, Burachnyi, Rubin, Frantsis, Sulu-nar, Kyrmyz Kabukh, Shir-anar, Shakh-anar, Gyuleisha Krasnaya, Apsheronskii Krasnyi, Sulunar, Imeretis Sauketeso
Greece	General	Ermioni, Persefoni, Pluto, Porfyrogenniti
India	Maharashtra	Bhagwa, Alandi, Phule Araka, Mridula, Ganesh, Karadi, Muskat
India	Karnataka	Madhugiri, Bassein Seedless, Jyothi, Madhugiri, Paper Shell
India	Gujarat	Dholka, Muskat Red, Kandhari, Ganesh
India	Himachal Pradesh	Kandhari
India	Andhra Pradesh	Ganesh, Paper Shelled, Muskati Red, Spanish Ruby
India	Rajasthan	Jodhpuri Red, Jodhpuri White, Jalore Seedless
India	Haryana	Chawla, Nabha, Country Large Red, Ganesh, Muskat Red, Paper Shell
India	Tamil nadu	Velludu, Kabul Red, Yercaud-1
India	West Bengal	Ruby, Mridula, Ganesh
Iran	General	Malas-e-Saveh, Malas-Torsh-Saveh, Rabab-e-Neyriz, Malas-e-Yazdi, Sishe Kape-Ferdos, Naderi-e-Budrood, Ardesstani Mahvalat, Bajestani Gonabad, Ghojagh Ghoni, Khazr Bardaskn, Galou Barik, Bajestan, Zagh, Shavar Daneh Ghermez, Sefid, Togh Gardan, Esfahani Daneh Ghermez, Alack, Alak Shirin, Alak Torsh, Maykhosh
Iran	Esfahan	Naderi, Ravandi, Malas, Shahvar, Zagh, Shirin
Iran	Tehran	Galou Barik, Malas, Ghojagh, Shirin, Arousak
Iran	Sout-khorasan	Shisheh-cap
Iran	Khorasan-e-Razavy	Shisheh-cap, Bajestani, Khazar, Ardestani, Ghand(Meshki), Malas, Leili
Iran	Khozestan	Malas, Shirin
Iran	Zanjan	Dousti, Shahvar, Malas, Meykhosh, Syah
Iran	Semnan	Galou Barik, Sorkhak, Shahvar, Malas
Iran	Sistan and Balouchestan	Bazmani, Sangani

Country	Cultivation regions	Varieties
Iran	Fars	Rabab, Bereit, Farough, Kadrou, Ghojagh, Malas, Syah, Shahvar, Rami, Abr
Iran	Ghom	Ghojagh, Shahpasand
Iran	Kerman	Keyvani
Iran	Kermanshah	Malas
Iran	Mazandaran	Kolbad, Shekar, Malas, Lamsari
Iran	Markazi	Malas, Agha Mohammad Ali, Alak, Syah, Shahvar
Iran	Yazd	Malas, Meykhosh, Shahvar, Zagh, Gel, Togh Gardan, Souski
Iraq	General	Ahmar, Aswad, Halwa, Salakhani
Israel	General	Wonderful, Shani-Yonay, Acco, Emek, Kamel, 116-17, Rosh Hapered, Malisi, Black
Italy	Sicily	Dente di Cavallo, Neirana, Profeta, Racalmuto, Ragana, Selinunte
Italy	Apuglia	Chinonna, Cisternino
Japan	General	Haku Botan
Jordan	General	Khdaree, Hmadee Hmaree, Khdaree Sfaree, Maleese, Hlawi, Khashaby, Khdaree Hello, Bradee Sharabee Asfar, Hasmasi, Hmaree Hmadee, Bradee Sharabee Ahmar, Zeklabee, Zokom Albagel, Khratee Ahmar Hello, Khdaree Hmadee, Lfani Sharab, Zarori, Esari
Libya	General	Khadouri
Mexico	General	Apaseo
Morocco	Meknes	Gjeigi, Dwarf ever Green, Grenade Jaune, Gordo de Javita, Djeibali, Onuk Hmam
Pakistan	General	Kandahari anaar
Saudi Arabia	General	Mangulati
Saudi Arabia	Taif	Taify, Yemeni
Spain	Murcia	Albar de Blanca, Agridulce de Ojós, Borde de Ojós, Casta del Reino, Piñonenca de Blanca, Piñón duro de Ojós, Piñón tierno de Blanca, Piñón tierno de Ojós, San Felipe de Blanca
Spain	Alicante	Mollar de Elche, Borde de Albatera, Mollar de Albatera, Mollar de Crevillente, Mollar de Orihuela, Valenciana de Albatera
Spain	Córdoba	Puente Genil
Tajikistan	General	Kai-Achik-Anar

Country	Cultivation regions	Varieties
Tunisia	General	Gabsi, Tounsi, Zehri, Chefli, Mezzi, Jebali, Garoussi, Kalaii, Zaghouani, Andalousi, Bellahi, Beyounsi, Florepleno Panache, Gabsi Khadouri, Garoussi Sahel, Kalali, Nabli
Turkey	General	Hicaznar, Katırbaşı, Aşınar, Fellahyemez, Eksilik, Mayhoş II, Mayhoş IV, Tatlı Mayhoş, Çekirdeksiz, Lefan, Ekşi Göknar, Silifke Aşısı, Çevlik, Beynarı, Ekşi Kırmızı, Ernar, Gliksiz,, Hatay, Izmir 1, Izmir 1264, Izmir 1265, Janarnar, Katrbas, Yufka Kabu
Turkey	Siirt	Zivzik
USA	California	Wonderful, Early Wonderful, Early Foothill, Granada, Ruby Red, Balegal, Cloud, Fleshman, Crab, Francis, Green Globe, Home, King, Phoenicia, Sweet, Utah Sweet, Purple Heart, Eversweet
Uzbekistan	General	Achik-Dona, Kazake-Anar, Kai-Achik-Anar, Kzyl-Anar
Vietnam	General	Vietnamese

The order of the varieties listed does not indicate their relative importance

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Part III
Tropical Fruits

Chapter 16
Genetics and Breeding of Fruit Crops in the Annonaceae Family: *Annona* spp. and *Asimina* spp.

Jorge Lora, Nerea Larranaga and José I. Hormaza

Abstract The Annonaceae is the largest family in the early-divergent Magnoliid clade of angiosperms with a limited number of species producing edible fruits. The species of agronomic interest in the family belong to two genera, *Annona* and *Asimina*. Several of those species have been cultivated and used as a food source by pre-Columbian cultures in the Americas. Their cultivation has continued to the present day and now they are incipient but prosperous crops in several countries. The most widely cultivated species in the family are *Annona cherimola* (cherimoya), *A. squamosa* (sugar apple), *A. muricata* (soursop), *A. cherimola* × *A. squamosa* (hybrid atemoya) and *Asimina triloba* (pawpaw). With the exception of cherimoya, which is a distinct subtropical species, most of the fruit crops in the genus *Annona* originate from warm lowland tropical regions and they have naturalized in different regions with subtropical and tropical climates. The pawpaw is the most widespread and the only species in the *Asimina* genus that produces fruits of significant interest as a food source and the northernmost representative of the Annonaceae. In this review several aspects of genetics and breeding, mainly in cherimoya and pawpaw, are discussed.

Keywords *Annona* · Annonaceae · *Asimina* · Atemoya · Cherimoya · Pawpaw Soursop · Sugar apple

J. Lora · N. Larranaga · J. I. Hormaza (✉)
Department of Subtropical Fruit Crops, Instituto de Hortofruticultura, Subtropical y Mediterránea La Mayora, (IHSM La Mayora-CSIC-UMA), 29750 Algarrobo-Costa, Málaga, Spain
e-mail: ihormaza@eelm.csic.es

J. Lora
e-mail: jlora@eelm.csic.es

N. Larranaga
e-mail: nlarranaga@eelm.csic.es

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16.1 Introduction

The Annonaceae is the largest family in the early-divergent Magnoliid clade of angiosperms with approximately 108 genera and 2400 species with a mainly pantropical distribution (Chatrou et al. 2012). A limited number of species, belonging to just two genera [*Annona* and *Asimina*, since *Rollinia* has been included into *Annona* (Rainer 2007)], produce edible fruits. Several of those species have been cultivated and used as a food source by pre-Columbian cultures in Central and South America (Popenoe et al. 1989) and, in the case of *Asimina*, in North America (Hormaza 2014). Cultivation of *Annona* species has continued to the present day and now they are incipient but prosperous crops in several countries with tropical and subtropical climates. The most widely cultivated species in the family are *Annona cherimola* (cherimoya), *A. squamosa* (sugar apple), *A. muricata* (soursop), *A. cherimola* × *A. squamosa* (hybrid atemoya) and *Asimina triloba* (pawpaw). However, other species that have possible commercial interest have been considered, but their horticultural development is still at its infancy: *Annona macroprophyllata* (ilama), *A. reticulata* (custard apple), *A. purpurea* (soncoya), *A. glabra (*pond apple*)*, *A. scleroderma (*cawesh*)* or *A. mucosa* (biribá). In this review we will discuss several aspects of genetics and breeding mainly in cherimoya, probably the most extensively cultivated species of the family. *Annona* fruits have a flesh with a sherbet-like texture and a flavor that has been compared with a mixture of banana and pineapple and was noted by author Mark Twain as *the most delicious fruit known to men* (Lora et al. 2011a). Even the young Charles Darwin praised the cherimoya during the last stopover of the Beagle on the South American continent in Lima (Peru) in 1835 before heading for the Galapagos Islands: *There are two things in Lima which all travelers have discussed; the ladies 'tapadas', or concealed in the saya and manta, and a fruit called chilimoya (sic). To my mind the former is as beautiful as the latter is delicious* […] (Darwin 1835). The first report on cherimoya was recorded by Jose de Acosta, a sixteenth century Spanish Jesuit missionary and naturalist who wrote that *all the flesh inside is soft and tender as butter and white and of an exquisite taste and some believe that it is the best fruit in the Indies* (Acosta 1590).

Besides their interest as fruit crops, attention in Annonaceae has recently increased dramatically due to the presence of the polyketide secondary metabolites acetogenins in this family. Acetogenins are found only in this family and have promising new cytotoxic, antitumoral, antimalarial and pesticidal properties (Alaly et al. 1999; Liaw et al. 2010) that could explain the use of ground cherimoya seeds as insecticide in various American countries as well as the utilization of different parts of this crop and others in the family in traditional medicinal applications (Morton 1987).

16.2 Origin and Botany

16.2.1 Origin, Production and Dissemination

The genus *Annona* includes around 160 species of mainly shrubs or small trees (Chatrou et al. 2012) which are distributed primarily in the tropical areas of America, with a few species native to Africa. The genus *Asimina* includes 8 species native to North America of which *Asimina triloba* is the most widespread and the only species in the *Asimina* genus that produces fruits of significant interest as a food source and the northernmost representative of the Annonaceae (Hormaza 2014).

With the exception of cherimoya which is a distinct subtropical species, most of the fruit crops in the genus *Annona* originate from warmer lowland tropical regions and have become naturalized in different regions with subtropical and tropical climates. Cherimoyas grow wild in the subtropical strata (1000–2300 m elevation) of the Neotropics characterized by dry and wet seasons. The distribution of this species has been well studied in the interandean valleys of northern Peru and southern Ecuador. It has also been found on the Pacific slopes of the Andes in northern Peru (Popenoe 1920). The origin of *A. cherimola* is still the subject of controversy. Mesoamerica and the central Andean region (northern Peru and southern Ecuador) are the two alternative centers of origin for the species. Since De Candolle (1882), most horticulturists have favored the Andean origin. Further support has also been based on terracotta vase renderings, presumably of cherimoyas, from the Mochica and Chimu cultures of Peru, 100–700 AD (Bonavia et al. 2004; Museo de la Nación 1995) although those renderings may also be ascribed to *A. squamosa* or *A. muricata*, which were introduced in the northern coastal area of Peru before or during the Mochica period. Berry (1929) suggested that fossil seeds of *A. cherimola* were found in the Ancon sandstones of Santa Elena Peninsula in southern Ecuador. Regretfully seed anatomy can hardly be considered a discriminating test within *Annona* (Koek-Noorman 1987). At the Guitarrero Cave in Ancash (8000 BC), inhabited by hunter-gatherers, a varied list of food plants were recorded (Museo de la Nación 1995): beans, peppers, pumpkins (zapallo, pallar) and caigua but no *Annona* seeds are mentioned. Several of these species were probably obtained from mesothermic valleys nearby where cherimoyas could have been growing. This suggests that, at this early date, no Annonas had reached the middle interandean valleys. At a later date (2700 BC) at Los Gavilanes Cave (Huarmey valley, south of Ancash) *Annona* seeds were found together with maize, yucca, peanuts and other food seeds (Museo de la Nación 1995). This shows the early introduction of mesothermic crops from the eastern slopes of the Andes and from the Amazon basin where several of these species originated. Towle (1961) mentions cherimoya fruits from Ancón (2000 BC) near Lima, as well as seeds from La Rinconada, near Lima, but, again, seeds cannot be considered a good discriminating test within *Annona* (Koek-Noorman 1987).

The Spanish chronicler Bernabé Cobo was the first to support a Mesoamerican origin of cherimoya since he claimed to have sent cherimoya seeds from Guatemala to Peru in 1629, where he thought that the fruit was unknown at that time (Bonavia et al.

2004; Cobo 1653). However, *Annona cherimola* is the only representative of a group of closely-related species in the *Atta* section of Annonaceae that occur exclusively in Mesoamerica, and this has led other researchers (Pozorski and Pozorski 1997) to support Mesoamerica as the center of origin. This, together with recent molecular studies that have shown a high diversity in Mesoamerican materials compared to South American samples (Larranaga et al. 2017), suggests that Central America could be the center of origin of the species and South America an important domestication center of secondary diversification. Interestingly, different common names are used for *A. cherimola* in Central and South America. In South America the crop is known in Spanish as *chirimoya*, a word probably derived from a Quechua word which could mean *cold fruit or seed* (Pozorski and Pozorski 1997). In Central America, the common word for cherimoya and other closely related species is *anona*. In fact, Linnaeus used the vernacular Central American designation to name the genus but referred to it as derived from the Latin word *annona* that can be translated as *the harvest of a year* (Linnaeus 1737).

Regardless of the origin, cherimoya fruits were consumed in the Andean region in antiquity (Popenoe et al. 1989), and movement of germplasm through Mesoamerica, central Mexico, and northern South America took place in pre-Columbian times. Cherimoya seeds can be kept dry for several years and could easily have been exchanged between Mesoamerica and northern South America. The Andean cultures developed extensive irrigation systems from approximately 2000 BC and this could have allowed the expansion of the crop to drier habitats north and south of the interandean valleys of northern Peru and southern Ecuador in pre-Columbian times. This was also the case in other woody perennial crops, such as avocado. Avocado seeds, a recalcitrant species, have been found in the Nazca Valley (southern Peru) far from its center of origin and diversification in Mesoamerica (Towle 1961). Soursop is native to warm tropical areas in South and Central America and it is now distributed throughout tropical and subtropical parts of the world, including Southeast Asia and some African countries.

An exception to the mainly tropical and subtropical distribution of the Annonaceae is the genus *Asimina*, the only genus of the family with species adapted to cold climates (Hormaza 2014). *Asimina triloba*, the North American pawpaw, is the most widespread species of the genus, indigenous to 26 states in the eastern United States, ranging from southern Ontario (Canada) to Northern Florida. The earliest written report of pawpaw was made in 1541 by a Portuguese officer who was a member of Spaniard Hernando de Soto's expedition through the southeastern United States where they observed consumption of the fruit by native Americans (Hackluyt 1609).

16.2.2 Morphological Description

Cherimoya is a fast-growing, evergreen tree, briefly deciduous in Mediterranean climates for a couple of months at the end of winter just before flowering. The tree can reach a height of 7–8 m. The root system is shallow. The leaves are single and

alternate, and the axillary buds are hidden beneath the short leaf petioles. The flowers are fragrant and solitary or in groups of 2 or 3. They appear mainly with new growth flushes but also on old wood and have 3 fleshy, greenish outer petals and 3 smaller, pinkish inner petals. The fruit is compound conical or heart-shaped. The skin, thin or thick, may be smooth with fingerprint-like markings or covered with conical or rounded protuberances. The white flesh is sweet and juicy. Each segment of flesh surrounds a single hard black bean-like seed (Morton 1987). The other cultivated species in the genus are somewhat similar to cherimoya in tree height and flowering behavior. The largest fruits are produced by the soursop.

Pawpaw trees can reach up to 10 m tall and have a pyramidal habit in sunny locations. The fruits are botanically berries, sweet, with a unique exotic taste that resembles a combination of banana, mango, and pineapple.

16.2.3 Flowering, Pollination and Fruit Set

Phenological stages have been studied for *Annona cherimola* (Cautín and Agustí 2005) and *A. squamosa* (Liu et al. 2014). *Annona cherimola*, like most species in the Magnoliales, has hermaphrodite flowers, with a pyramidal gynoecium and an androecium surrounded by 2 whorls of 3 petals, the internal small and scale-like and the external greenish and fleshy. The gynoecium is composed of up to 300 fused carpels forming a syncarp that occupies the center of the conic receptacle. Each carpel contains a single ovule. The androecium is located below the gynoecium forming a helicoidal structure with up to 200 stamens. Cherimoya shows protogynous dichogamy (Schroeder 1971), a common characteristic in the *Annonaceae* (Gottsberger 1999) and in other early divergent angiosperm lineages with hermaphrodite flowers (Endress 2010), where female and male structures do not mature simultaneously, generally preventing self-fertilization in the same flower. Moreover, flowers of the same genotype usually open synchronously and, consequently, transfer of pollen between different flowers of the same genotype is difficult (Lora et al. 2010). The cycle of the flower is completed in 2 days; in the first day of the cycle the flower is in preanthesis with the petals tightly closed and the flower passes to the female stage about midday under the environmental conditions of Southern Spain (Fig. 16.1). This phase lasts for about 30 h and the second day the flower switches to the male stage, when the anthers dehisce, around 17:00–18:00 h, depending on temperature. After anther dehiscence, the stigma rapidly dries up losing receptivity. The length of the receptive phase varies depending on environmental conditions. Thus, stigmatic receptivity is extended with increasing humidity and decreasing temperatures and shortened with decreasing humidity and increasing temperatures and, under some conditions, the stigmas can be receptive even when the flowers have switched to the male state (George et al. 1989; Lora et al. 2011b).

Recent studies on the final stages of pollen development in *Annona cherimola* have shown a rapid and active pollen development prior to pollen shedding, resulting in the release of both bi- and tricellular pollen at anther dehiscence and the ratio

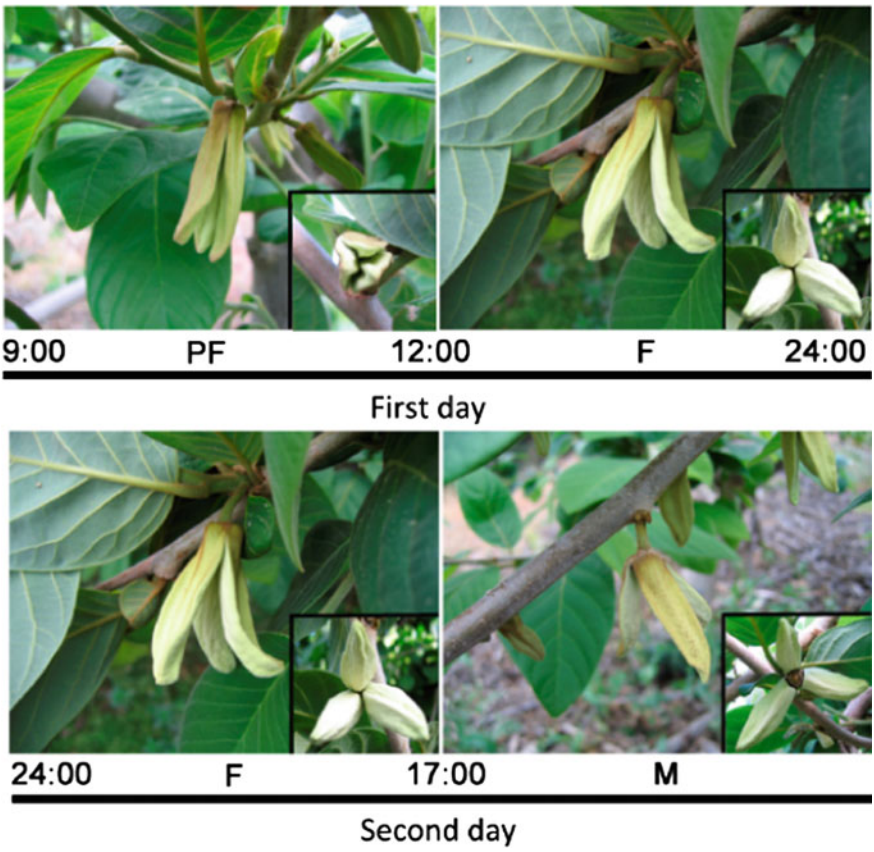

Fig. 16.1 Flower cycle of *Annona cherimola*. The flowers start to open during the first day of the flowering cycle in a prefemale/preanthesis stage (PF). The flowers stay in a female stage (F) up to the second day of the flowering cycle. Anther dehiscence occurs at 17–19 h of the second day of the cycle when the flower switches to the male (M) stage

between both types of pollen is greatly influenced by temperature (Lora et al. 2009). Individual pollen grains are released after contact with the stigmas or to an artificial germination medium. Similar to other tricellular pollen, cherimoya pollen has a short viability and high sensitivity to desiccation and, consequently, pollen storage is difficult (Lora et al. 2006).

Most species in the Annonaceae are pollinated by beetles. Nitidulid beetles (Nitidulidae) that breed and feed on decaying fruits or sap flows, are the main pollinators of commercial *Annona* species, mainly cherimoya and atemoya (Peña et al. 2002). Research in Spain has shown that hemiptera insects of the genus *Orius* could also be effective as pollinating vectors in cherimoya (Guirado et al. 2001). Other beetles, such as scarab beetles (Scarabaeidae), weevils (Curculionidae), cucujids (Cucujidae), rove beetles (Staphylinidae) or anthicids (Anthicidae) can also par-

ticipate in pollination of fruit crops in the genus *Annona* (Peña et al. 2002). An appropriate tree management maintaining higher humidities and shading below the trees can help to promote the presence of pollinating insects, reducing the need for hand-pollination.

Inadequate pollination is one of the most important factors limiting commercial production of *Annona* species in most locations. This is due to the lack of overlap between male and female stages and, as in cherimoya, together with the fact that the crop is propagated away from its areas of origin without its natural pollinator agents. As a result, hand pollination with pollen and stamens together was first proved successful by Schroeder (1943) and currently is a common practice for commercial production in all countries where the crop has been introduced (Schroeder 1971). Moreover, hand-pollinated flowers produce fruits that exhibit usually better symmetry than those derived from insect-pollinated flowers that often results in the production of asymmetric fruits with low commercial value. Due to the short longevity of cherimoya pollen, growers have to collect it daily to perform hand pollination, although pollen can be stored at low temperatures for several months (Lora et al. 2006). Fruit crops of the genus *Annona* are fleshy and aggregated with several seeds formed mainly by reticulate endosperm and a tiny embryo.

Different exocarp types are distinguished in cherimoya (Bioversity International and CHERLA 2008; Schroeder 1945): *laevis* (smooth), *impressa* (slight depressions), *umbonata* (small protusions), *tuberculata* (medium protrusions) and *mamillata* (large protrusions). Usually forms *laevis* and *impressa* are preferred by consumers.

In the case of pawpaw, flies and beetles are the main pollinators and cross-pollination seems to be required for proper fruit set although some cultivars seem to be self-compatible (Pomper and Layne 2005). The progamic phase between pollination to fertilization recently described in this species showed a similar structure to other members of the family, but with an uncommon very long stigmatic receptivity associated with a long flowering cycle (Losada et al. 2017).

16.2.4 Cultivation

Cherimoya cultivation has expanded into areas with frost-free Mediterranean climates where it is grown under irrigation. Early Spanish explorers introduced cherimoya to Spain, and from Spain it was extended to other southern European countries. It is cultivated in California (USA), Chile, Argentina, Madeira (Portugal), Peru, Ecuador, Bolivia, South Africa, Australia, Italy, Spain, Morocco, Algeria, Egypt and Israel. In South Africa, Australia, Egypt and Israel most commercial plantings are of the hybrid atemoya (*Annona cherimola* × *A. squamosa)* which tolerates higher ambient temperatures during flowering.

The most important producing countries have developed cultivars (clonal selections) well adapted to their climatic conditions: Spain (Fino de Jete, Campas) Guirado et al. (2003); California (USA) (White, Bays) Grossberger (1999); Chile (Concha Lisa, Bronceada) and Peru (Cumbe) Grossberger (1999).

Reliable data are not available for production of other cultivated species of the Annonaceae since in many cases they are grown as a backyard fruit for domestic consumption. Soursop is cultivated in different countries with tropical climates such as Angola, Brazil, Colombia, Costa Rica, Cuba, Jamaica, India, Mexico, Panama, Peru, Venezuela and different countries in Southeastern Asia. The main producing countries are Mexico, Venezuela and Brazil. Sugar apple and atemoya are also cultivated commercially in some countries with tropical climates, mainly in Southeast Asia, Taiwan, Brazil and Australia.

Regarding pawpaw, in spite of its high potential as a new high-value niche fruit crop, it is still only in the early stages of commercial production and mainly found in local markets in the USA. Most fruits are still collected from wild stands or produced in small family orchards.

16.3 Utilization of Genetic Resources

Conservation of genetic resources can be performed both in situ and ex situ, complementary approaches that, especially for the in situ component, should be integrated into a sustainable development strategy in the regions where biodiversity is present (Larranaga and Hormaza 2016). In situ conservation should also include the preservation of traditional cultivated fields (*on farm* conservation) together with the accompanying traditional agricultural methods and knowledge of local farmers. Unfortunately, no in situ conservation programs for cherimoya have been implemented. However, there is an urgent need to develop in situ strategies especially in the recently-proposed center of origin of the crop in Central America where genetic erosion is taking place at a rapid rate (Larranaga et al. 2017). On the other hand, most current ex situ germplasm collections in the Annonaceae show significant limitations in the number of accessions and in the genetic variability and quality of the samples conserved. Except for cherimoya, germplasm collections of species of *Annona* are rare. In cherimoya, a germplasm bank was established in 1985 at the IHSM La Mayora (Málaga, Spain). It now holds a total of 10 species in the Annonaceae with 310 cherimoya entries from twelve different countries. Several plant and fruit characteristics are being studied in this collection in order to identify superior genotypes, following the descriptors elaborated by Bioversity International and CHERLA (2008). A selection of the most informative traits has been made, including three leaf traits (blade shape, length and width), two flower traits (petal length and width), seven fruit traits (weight, exocarp type and weight, weight of all fresh seeds, number of seeds, soluble solids and acidity) and one seed trait (tenacity of the seed in its epithelium). Molecular markers, such as SSRs are routinely used to characterize the collection. The collection is also the base for a breeding program in this species. The other important germplasm collection of cherimoya is that maintained by INIA of Peru in Ayacucho. Some other small collections of this and other species in the genus have been reported but not much information is available. In the case of the pawpaw, Kentucky State University, Frankfurt, Kentucky, holds the USDA National

Clonal Germplasm Repository for *Asimina* species with around 2000 accessions and a number of high-quality pawpaw cultivars with large fruits and high productivity have been selected since the 1950s (Pomper and Layne 2005). Although protocols for in vitro micropropagation and regeneration in cherimoya and other species of the family have been developed (Encina et al. 2014) no germplasm collections have been established in vitro.

16.4 Current Status and Breeding Efforts

16.4.1 Breeding Objectives

Annonaceae breeding programs have been mainly carried out in cherimoya and atemoya and, to a lesser extent, in pawpaw. Some of the fruit characteristics of cherimoya and atemoya, such as resistance and shape of the skin of the fruit, longer postharvest life, pulp quality (°brix and acidity), and number of seeds are of agronomical importance and, consequently, objectives of the breeding strategies. Skin resistance, evaluated as resistance with a penetrometer, is an important fruit trait to avoid damage caused during transportation. In this sense the skin shape that shows smooth or slight protrusion and called *laevis* or *impressa,* respectively (Bioversity International and CHERLA 2008) are valuable traits for breeding programs. Another fruit trait to consider is longer postharvest life. Cherimoya is a climacteric fruit and shows a rapid ripening process. Cherimoya fruits can be stored for 15–20 days under refrigeration at 8–10 °C, but below 8 °C they can suffer cold damage (Guirado et al. 2003). Regarding pulp quality, °Brix above 20 is considered optimal and is generally found in commercial cultivars such as Fino de Jete in Spain (Guirado et al. 2003). Yield is also an important trait although in addition to obtaining a cultivar that provides good yield and fruit traits, optimization of cultivation techniques can improve fruit production; an example is a second pruning at the end of July that provides flowers in August and a second cherimoya production in spring in Spain (Farré et al. 1999; Guirado et al. 2003). Disease resistance is not a priority in this crop. Regarding pests, it is of interest to select genotypes resistant to seed borers that are affecting different crops of the Annonaceae in the Americas.

Temperature is a main limiting factor for the tropical species of the family such as soursop, custard apple, sugar apple and atemoya which suffer below 15 °C temperatures. On the other hand, climate change is another threat to the current areas of cultivation of cherimoya both in South (van Zonneveld et al. 2012) and Central America (Larranaga et al. 2017). Strategies should be developed to take this issue into account in new plantations.

Pawpaw production challenges have been reviewed by Pomper and Layne (2005). A review on pawpaw breeding and selection (Peterson 2003) has identified the most interesting breeding traits of vigor, yield and diverse fruit quality traits such as larger fruit size, smaller and fewer seeds, appearance, uniformity of fruit and mild, sweet flavor with no unpleasant aftertaste together with a longer shelf life.

16.4.2 Breeding Programs

A cherimoya breeding program initiated in the 1990s at the IHSM La Mayora in Spain has produced advanced selections to complement the main cultivar in the region, Fino de Jete. A new selection, Alboran is currently under registration. This variety has a lower seed index than Fino de Jete and excellent organoleptic properties during the winter and spring months. Another trait of interest in the breeding program is the search for seedless cherimoya genotypes since a low seed index is highly appreciated and an important fruit trait that is being considered in several breeding programs. One of the options for this is the availability of a spontaneous mutant of *Annona squamosa*, Thai seedless (Ts), that produces fully seedless normal-sized fruits (Lora et al. 2011a). The reasons behind the seedless character in Thai seedless apparently is a deletion of the *INNER NO OUTER* (*INO*) locus (Lora et al. 2011a) that is involved in the outer integument development of the ovule (Fig. 16.2). The ongoing breeding program to introduce this trait currently is in progress at the IHSM La Mayora; different crosses using Thai seedless as the male parent and Fino de Jete as the female parent have been successfully carried out. Breeding programs are more scarce in the countries of origin of cherimoya with highly diverse germplasm, but a low commercial development of cherimoya that can significantly improve with the improvement of crop production and subsequent marketing and promotion by producers (Van Damme and Scheldeman 1999; Vanhove and Van Damme 2013).

In the case of atemoyas, two hundred years ago, the botanist St. Hilaire realized the possibilities of breeding *Annona* and (then) *Rollinia* species by selection, but was not until the early 1900s when Wester obtained atemoya hybrids between cherimoya and sugar apple, and initiated a breeding program in Florida (Wester 1913). In Australia a breeding program in this crop was established with 8000 and 15,000 breeding lines in 1992 and 1998, respectively, using inter-varietal and interspecific crosses to successfully develop hybrids with red skin color and pink flesh, and crosses that bear fruit with a symmetrical shape, flesh recovery and excellent flavor (George et al. 1999, 2002, 2005). An alternative approach was also performed to obtain seedless cultivars producing tetraploids using gamma radiation or colchicine and then crossing to create diploids to produce seedless triploids (George et al. 1999). Some atemoya breeding programs have also been established in Brazil that also include the use of the spontaneous seedless *A. squamosa* mutant Thai seedless mentioned above (Mendes et al. 2012).

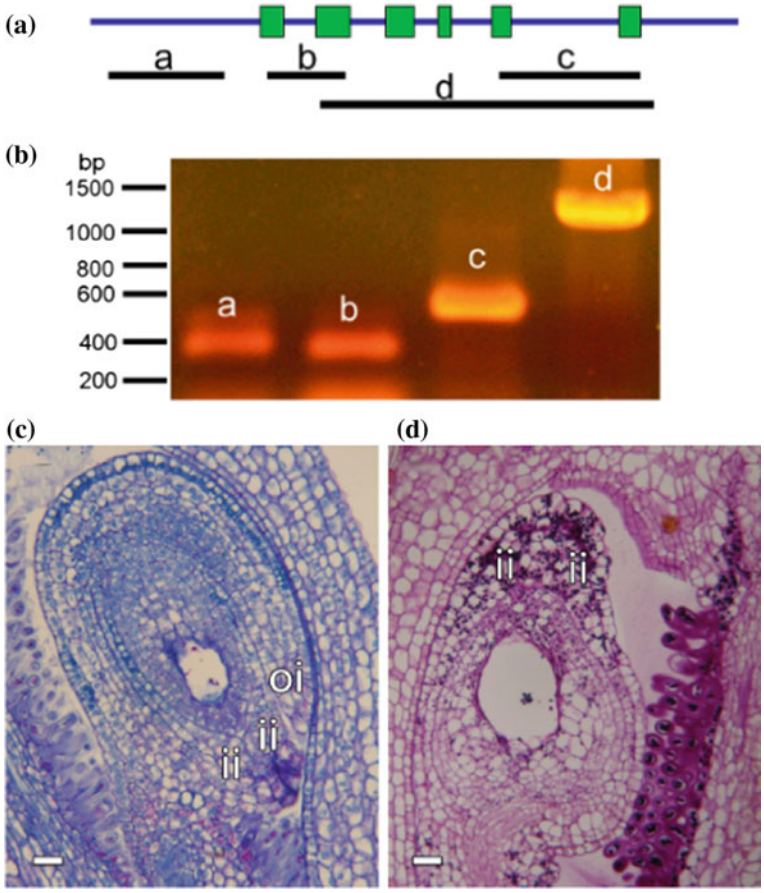

Fig. 16.2 The natural mutant Thai seedless and wild type of *Annona squamosa* (Lora et al. 2011a) A, B. Gene structure of the *INNER NO OUTER* showing the DNA fragments **a** that were only amplified in the wild type of several *Annona* species including *A. squamosa,* **b** and *A. cherimola*, **c** Anatropous ovule in wild type *A. squamosa*, **d** Orthotropous ovule in Thai Seedless *A. squamosa,* **e** showing only the inner integument (ii), **c** PAS and toluidine blue staining of 2 μm resin sections, **d** PAS staining of 2 μm resin sections. Inner integument (ii). Outer integument (oi). Bar = 20 μm

Pawpaw breeding began in about 1900 at Kentucky State University, Frankfort, with several cultivars selected from the wild and propagated until the 1980s. This large breeding program has resulted in several advanced selections such as KSU-Atwood™, NC-1 Overleese, Potomac, Shenandoah, Sunflower, Susquehanna and Wabash (Peterson 2003; Pomper et al. 2008). Information on new cultivars and different pawpaw activities, such as annual state festivals can be found at the Kentucky State University pawpaw program website (www.pawpaw.kysu.edu).

16.5 Biotechnology

16.5.1 Micropropagation and Genetic Transformation

Traditional methods of vegetative propagation are inefficient in most *Annona* species, due to the low morphogenetic potential and the low rooting rate. Thus, micropropagation in vitro is an alternative to overcome these problems. Micropropagation has been performed on cherimoya, sugar apple, soursop and atemoya (Rasai et al. 1995). Initially, plantlet differentiation was described in *A. muricata* (Benjoy and Hariharam 1992). Lemos and Blake (1996a) reported micropropagation of *A. squamosa* from axillary shoots and Lemos and Blake (1996b) developed a micropropagation protocol for juvenile and adult *A. muricata* tissue with good acclimatization percentages. Encina et al. (1994) developed a protocol for micropropagation from juvenile nodal explants of Fino de Jete cherimoya with good acclimatization percentages achieved. Padilla and Encina (2004) were able to optimize the protocol to micropropagate adult cherimoya genotypes and also performed micrografting (Padilla and Encina 2011). In order to improve plant recovery rate during acclimatization, mycorrhizal inoculation with *Glomus deserticola* has also been applied to cherimoya produced in vitro (Azcon-Aguilar and Barea 1994; Azcón-Aguilar et al. 1996).

In vitro techniques can also accelerate breeding programs. Haploid plants have been induced from anther callus of *Annona squamosa* (Nair et al. 1983) and in the same species triploids developed from endosperm have been reported (Nair et al. 1986).

Recently, a binary RNA interference vector that inhibits *INO* gene expression is being introgressed in cherimoya via *Agrobacterium tumefaciens* (Garcia et al. 2012) using micropropagation approaches (Encina et al. 1994; Padilla and Encina 2003). We have been able to optimize a method for genetic transformation of cherimoya using hypocotyls and the *INO* gene has been silenced in the transformants but we must wait for several years in order to study the flower and fruit phenotype (Padilla et al. unpublished data).

16.5.2 Molecular Phylogeny

Among the 2400 species in the Annonaceae family, are tropical and subtropical trees, shrubs and lianas distributed all around the world (Chatrou et al. 2012). Based on the APG III classification (Stevens 2001), this family is placed in the order Magnoliales close to five other families (Myristicaceae, Degeneriaceae, Himantandraceae, Magnoliaceae and Eupomatiaceae) and, together with the orders Canellales, Laurales and Piperales, shape the Magnoliid complex (Zanis et al. 2002). Annonaceae emerged in the Upper Cretaceous period about 82–98 millions of years ago when the taxon was distributed in Laurasia and north of Godwana (Doyle and Le Thomas 1997; Richardson et al. 2004; Scharaschkin and Doyle 2005; Su and Saunders 2009). Some genera

in the family, such as *Guatteria*, *Annona*, *Duguetia*, *Uvaria* and *Polyalthia* have more than 100 species (Chatrou et al. 2004).

Since Fries (1959) classified the Annonaceae, many studies have been carried out to investigate the systematics of this group based on palynology (Doyle and Le Thomas 1994; Goodrich 2012; Gottsberger 2012; Saunders 2012), morphology (Couvreur et al. 2009; Doyle and Le Thomas 1996; Maas and Westra 1985a, b; Su and Saunders 2006) and molecular markers. Among the latter, different chloroplast sequences, such as rbcL, matK, ndhF, trnL, trnT-L, trnL-F, trnS-G, atpB-rbcL, psbA-trnH, ycf1, rpl32-trnL or ndhF-rpl32 (Chaowasku et al. 2014; Chatrou et al. 2012; Erkens et al. 2012; Larranaga and Hormaza 2015; Mols et al. 2004; Pirie et al. 2005; Richardson et al. 2004; Su et al. 2010; Thomas et al. 2012) have been used. Within the *Annona* genus, a phylogenetic analysis demonstrated that the flanking regions of some already-developed microsatellites could perform as well as some of the most used chloroplast markers in phylogenetic analysis (Chatrou et al. 2009).

16.5.3 Cultivar Fingerprinting and Diversity Analyses

Traditionally, the use of phenotypic characters has been the basis for the identification and characterization of species and cultivars. Some examples in cherimoya are studies by Hermoso-González et al. (1999) and Andrés-Agustín et al. (2006). However, identification based exclusively on phenotypic traits is labor intensive and inaccurate due to the influence of the environment, subjectivity in the evaluation of traits and the limiting number of discriminating traits. Therefore, molecular markers have been increasingly used to optimize plant genetic resource management (Larranaga and Hormaza 2016). However, few molecular works have been carried out in cultivated Annonaceae species and most of them have been performed in pawpaw and cherimoya.

In pawpaw different works have been carried out with isozymes, RAPDs, AFLPs and ISSRs to identify genotypes and to study genetic diversity (Huang et al. 1997, 1998, 2000, 2003; Pomper et al. 2003; Wang et al. 2005).

In the genus *Annona*, Ronning et al. (1995) and Rahman et al. (1997) studied variability and heritability among some species using RAPD and STS markers, respectively, while Ahmad et al. (2010) used ISSR and RAPDs. In cherimoya, isozymes (Ellstrand and Lee 1987; Pascual et al. 1993; Perfectti and Pascual 1996, 1998a, b, 2004, 2005), RAPDs (Ronning et al. 1995) and AFLPs (Rahman et al. 1998) have been used with the same objectives although involving a low number of loci and/or genotypes. Escribano et al. (2004, 2008a) reported a set of microsatellite loci, a preferred technique for the molecular characterization of plant species due to their genetically (codominancy and high level of polymorphism) and technically (reproducibility and automatization potential) interesting properties (Wünsch and Hormaza 2002). These markers have been used to fingerprint the world's largest cherimoya germplasm bank the maintained at the IHSM la Mayora and to study the genetic diversity of materials conserved ex situ and directly collected in situ in the putative

areas of origin in southern Ecuador, northern Peru and Mesoamerica (Escribano et al. 2007; Larranaga et al. 2017; van Zonneveld et al. 2012). Moreover, they have allowed the development of a core collection representing most of the diversity conserved in the germplasm collection (Escribano et al. 2008b). The interspecific transferability of microsatellite markers has allowed further studies in other species of the genus such as the one carried out by Kwapata et al. (2007) in *A. senegalensis*. An additional set of microsatellite markers has been developed in *A. crassiflora* (Pereira et al. 2008) and used to assess its diversity in the Brazilian Cerrado (Collevatti et al. 2014) and transferred to *A. coriacea* (Ribeiro et al. 2014). *Annona crassiflora* has also been analyzed with RAPD markers (Cota et al. 2011). A few studies performed on *A. squamosa* have also permitted evaluation of the structure and genetic diversity of several genotypes of this species with isozymes, RAPDs or AFLPs markers (Bharad et al. 2009; Guimarães et al. 2013; Zhichang et al. 2011).

16.5.4 Genomics

Since the early 2000s, next generation DNA sequencing methods, sometimes divided into second and third generation automatic sequencers, depending on whether prior amplification of the DNA template is needed or not (Gleen 2011), have made a qualitative change in genetic analyses. These technologies provide several advantages and have revolutionized many areas in biology since they allow genome-wide characterization and profiling of mRNAs, small RNAs, transcription factor regions, structure of chromatin and DNA methylation patterns, microbiology and metagenomics (Ansorge 2009). In recent years, the decreasing cost of these technologies is permitting their use in non-model organisms and some examples can already be found in the *Annona* genus. Gupta et al. (2015) performed a massive pyrosequencing of transcriptome from early stages of fruit development (0, 4, 8 and 12 days after pollination) of two different genotypes of *A. squamosa*. They differed significantly in the number of seeds in fruits, which permitted them to point out candidate genes related to seed development as well as provide transcriptome information that could serve to study other fruit-related traits in this or sister species. Blazier et al. (2016) sequenced, assembled and annotated the cherimoya plastid genome in order to compare it with other plant species and study the genes for the plastid-encoded RNA polymerase. Genotyping by sequencing technologies are currently being used in cherimoya to link molecular markers to traits of interest in the Spanish cherimoya germplasm collection (Talavera et al. unpublished data).

16.6 Conclusion and Prospects

Although cherimoya (*Annona cherimola*) and soursop (*A. muricata*) are probably the most popular fruit crops in the Annonaceae, other species in the family are

promising as new crops in some areas of the world. In any case, all the species in the family remain underutilized and additional efforts are needed in order to expand their production range. Recent results indicate that in the case of cherimoya the highest diversity is present in areas in which it has not been conserved. Strategies for both in situ and ex situ conservation of germplasm should be established and studies made to identify the hotspots of diversity in other members of the family. As in other crops, next generation sequencing approaches which can generate hundreds to thousands of molecular markers, will make a qualitative change in breeding different species in the Annonaceae. Although currently genomic resources for species in the family are scarce, this will probably change in the coming years due to the feasibility of molecular studies at reasonable cost and the main limitation in the future will probably switch to the availability of thorough and reliable phenotypic information. This highlights the urgent need for curation, analysis and exchange of germplasm of species in the Annonaceae among collections worldwide to ensure the preservation of genetically and phenotypically diverse material for future generations.

Appendix 1

Major institutes engaged in research on fruit crops in the Annonaceae family

Institute	Location
Department of Agriculture and Fisheries	PO Box 5083, SCMC, Nambour, Queensland 4560, Australia
EMBRAPA Cerrados	Km 18 da Br 020, P.P. Box 08223. Planaltina-DF, Brazil
Instituto Nacional de Pesquisa da Amazonia	INPA, 69000, Manaus AM, Brazil
Universidade Estadual de Montes Claros/ Unimontes	Campus Janauba, Av. Reinaldo Viana, 2630, 39440-000 Janauba, MG, Brazil
Universidade Federal de Minas	Gerais, Brazil
Universidade Federal Alagoas	Maceio, AL, Brazil
Universidad Catolica de Valparaiso	Quillota, Chile
Lingnan Normal Univ, Life Sci & Technol Sch	Zhanjiang 524048, Guangdong, China
CORPOICA	C.I. La Selva, Rio Negro, Antioquia, Colombia
Universidad de Costa Rica (UCR) Estacion Experimental Fabio Baudrit Moreno	Alajuela, Costa Rica
Universidad Agraria de La Habana	San Jose de las Lajas, Mayabeque, Cuba
Instituto Nacional de Investigaciones Agropecuarias (INIAP)	Quito, Ecuador
Universidad Nacional de Loja	Loja, Ecuador
Botanical Garden and Herbarium	University of Ulm, 89081 Ulm, Germany

Institute	Location
National Agri-Food Biotechnology Institute (NABI)	Department of Biotechnology (DBT), Mohali, Punyab, India
The Volcani Center	Bet Dagan, Israel
CRUCO-UACH	Av. Periférico Independencia Poniente No.1000. C.P. 58000. Morelia, Michoacán, Mexico
Universidad Autónoma Chapingo	Chapingo, Mexico
Universidad Autonoma Nayarit	Ciudad Cultura S-N, Tepic 63000, Nayarit, Mexico
Universidad Ciencias & Artes Chiapas	Tuxtla, Gutierrez Chiapas, Mexico
Universidad Veracruzana	Xalapa 91000, Veracruz, Mexico
University of Calabar	Dept Genet & Biotechnol, Calabar, Nigeria
Instituto Nacional de Innovacion Agraria (INIA)	Peru
Institute for Subtropical and Mediterranean Horticulture la Mayora (IHSM la Mayora - CSIC - UMA)	29750 Algarrobo-Costa, Málaga, Spain
Chiayi Agricultura Experimental Station	Chia-yi, Taiwan
Kentucky State University	Frankfort, Kentucky, USA
Subtropical Horticultural Research Unit	USDA, Miami, USA
Tropical Agricultural Research Station	USDA, Mayaguez, Puerto Rico, USA

Appendix 2

Genetic resources. A list of the main cherimoya cultivars in different countries (Grossberger 1999; Guirado et al. 2003)

Country	Main cultivars
Chile	Concha Lisa, Bronceada
Peru	Cumbe
Spain	Fino de Jete, Campas
USA (California)	White, Bays

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Chapter 17
Breeding of Coconut (*Cocos Nucifera* L.): The Tree of Life

Yaodong Yang, Amjad Iqbal and Rashad Qadri

Abstract Coconut (*Cocos nucifera* L.) is a continuous fruiting perennial tropical fruit and oil crop that is mainly cultivated in the humid and sub-humid coastal tropics. It is a perennial multipurpose palm with great importance in sustaining the life of the people who grow them for various economical uses. As coconut is known as the *tree of life*, efforts have been made to sustain its production to fulfill the demands of the people. In this regard, coconut genetic resources have been widely exploited through selection, hybridization for a number of desirable traits that resulted in the development of many productive varieties. With increasing demand from the coconut sector, particularly the nontraditional coconut products, it is indeed crucial to identify and screen the potential varieties. However, due to the biological nature of the coconut, the traditional way of breeding might not be feasible; therefore, molecular-aided breeding can be the best alternative. Molecular-aided breeding can play an important role in future coconut breeding programs with the advancements in biotechnology. The strategies suggested for future breeding programs, include development of new high yielding, stress tolerant and disease resistant varieties with the benefits of providing high value-added products, like inflorescence sap or high oil content, and varieties with slow vertical growth. With the availability whole genomic sequences of coconut more opportunities exist for the development of molecular markers, thus encouraging the use of molecular-aided breeding in future coconut breeding programs.

Y. Yang (✉)
Hainan Key Laboratory of Tropical Oil Crops Biology, Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences, Wenchang 571339, Hainan, China
e-mail: yyang@catas.cn

A. Iqbal
Department of Agriculture, Abdul Wali Khan University Mardan, Mardan, Pakistan
e-mail: amjadiqbal@awkum.edu.pk

R. Qadri
Institute of Horticultural Sciences, University of Agriculture Faisalabad, Faisalabad, Pakistan
e-mail: waseemrana_83pk@yahoo.com

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17.1 Introduction

Coconut (*Cocos nucifera* L.) is a continuous fruiting perennial tropical fruit and oil crop that is adapted to the humid and sub-humid coastal tropics. It has multiple purposes; i.e. stem for timber, leaves for thatch roofing and handicrafts, coconut sap for the production of vinegar, wine or sugar, kernel oil and coco cream/milk, husks for fiber/coir, coconut water for beverages, vinegar and wine and the coconut shells for charcoal and so on. Due to its importance in sustaining the life of the people, who grow them for various economical uses, therefore, coconut is often regarded as a *tree of life* (Burkill 1966). Coconut is planted in more than 92 countries of the world. Coconut cultivation covers 12 million ha of plantation area, of which 86% is grown in the Asia-Pacific region (FAOSTAT 2014).

Coconut has been cultivated for thousands of years in the tropical zone around the world. However, coconut farmers are suffering due to a decrease in farm productivity cum income and unstable market for traditional dried kernel products (Batugal et al. 2009). This situation has arisen due to availability of other vegetable oils, ageing and senile coconut palms, abiotic and biotic stresses and lack of affordable, yet high-yielding and adapted coconut varieties. One of the ways to increase farm productivity and competitiveness of this important smallholder crop is to develop improved varieties having the capacity for high productivity, resistance to pathogens and adaptation to specific growing environments.

Many cultivars, varieties and types were produced during the long-term natural evolution, artificial selection and domestication of the coconut (Perera et al. 2009). It is important to conserve coconut genetic resources that can serve as the fundamental basis for a strong breeding program. However, the diversity in coconut is under threat because of genetic erosion, which in turn is due to decreasing hectarage caused by urbanization, crop shifts and natural and human-made calamities. Many coconut-growing countries are developing without any concrete planning to conserve coconut diversity (Batugal et al. 2005). Despite its agronomic importance, most of the previous studies regarding germplasm assessment have revolved around morphological and agronomical traits. Molecular biology techniques have been scarcely used in the assessment of genetic resources and for the improvement of important agronomic and quality traits in *coconut* . Therefore, it is important to adopt new strategies for assessing the breeding material and setting up of new breeding programs to accelerate the creation of the elite varieties for large-scale coconut plantations.

The coconut plantation area (Table 17.1) and production (Table 17.2) in the major coconut growing countries from 2011 to 2014 are quiet stable with a slight decrease in 2014. In the past decades, coconuts were mainly grown because of the consumer demands for copra, crude coconut oil (CNO) and its derivatives at the international level. However, in recent years, new products so-called *nontraditional* products enter-

ing the global market. The market growth of these products, mainly coconut water extracted from mature or immature nuts, virgin coconut oil (VCO) cold pressed from the fresh kernel, coconut sugar taken from the sap tapped from the inflorescence, is exponential. The introduction of such products raise the demands of raw materials, which encourages growers, thus strengthening the coconut sector. Moreover, keeping the present demands of the market in mind, it is necessary to characterize the biodiversity of coconut trees immediately on the basis of new demands, rather than on copra alone. It is indeed crucial to identify promising coconut varieties that are best suited to improve the existing varieties. These varieties could be used in replanting programs, selection or genetic improvement that will generate high-yielding coconut hybrids for the future. Replanting coconut trees with quality planting material is urgent and essential (Prades et al. 2016).

This chapter presents the current strategies and developments in coconut breeding, and the new strategies and technologies that may be adopted to facilitate the breeding efforts of the coconut-producing countries. It also describes the efforts being made to collect the various ecotypes to conserve the coconut genetic diversity and facilitate their use for developing the desired ideotype. The ideotype, for most breeders, would be high-yielding varieties with wide adaptability and potential resistance to pests and disease. With increasing expectations from the coconut sector, rapid multiplication of quality coconut is of paramount importance for the fast moving consumer goods (FMCG) industries.

17.2 Origin and Botanical Aspects of Coconut

17.2.1 Origin and Distribution

Coconut belongs to the palm family (Palmae or Arecaceae), which has about 190 genera and over 2800 species. The Cocoeae tribe with 27 genera and nearly 600 species, includes several economically-important palms, such as *Cocos nucifera* (coconut), *Elaeis guineensis* (African oil palm) and *Bactris gasipaes* (peach palm). Coconut is a diploid with 32 chromosomes ($2n = 32$) and a large genome size of about 2.4 Gb (Xiao et al. 2017) nucleotide pairs and is the sole species of the genus *Cocos*. It is propagated by seed nut traditionally and is a slow growing tree (with reproductive maturity at 3–10 years depending on the genotype and environment). Hybrid cultivars of coconuts are generally a product of intra-specific crosses.

It is believed that coconut is an ancient species with no possibility of mating or gene exchange with any related species. It is believed that all coconut cultivars constitute a single inter-mating gene pool and is one of the few major crop species that lacks close relationship with their wild relatives. Phylogenetic studies show the date of differentiation of the genus Cocos, that are: 26.71–22.20 Mya (Gunn 2004), 44.4–23.9 Mya (Meerow et al. 2009, 2014) and 23.61–3.92 Mya (Baker and Couvreur 2013a, b). Current theories mainly suggest that it must have originated in the Indonesian

Table 17.1 Area of coconut plantation worldwide, 2010–2014 (in 1000 ha)

Country	2010	2011	2012	2013	2014
A. APCC Countries	**10,778**	**10,793**	**10,988**	**10,974**	**10,928**
F.S. Micronesia	17	17	18	18	18
Fiji	60	60	65	60	62
India	1895	1896	2071	2137	2141
Indonesia	3739	3768	3782	3654	3610
Jamaica	15.4	15.6	15.8	14.8	15.9
Kenya	78	82	86	177	177
Kiribati	19	19	19	20	20
Malaysia	106	106	101	88	88
Marshall Islands	8	8	8	8	8
Papua New Guinea	221	221	221	221	221
Philippines	3576	3562	3574	3551	3502
Samoa	102	104	97	99	99
Solomon Islands	38	38	38	38	38
Sri Lanka	395	395	395	395	440
Thailand	232	216	214	209	206
Tonga	34	34	33.8	33.8	31
Vanuatu	96	96	92	92	92
Vietnam	147	155	157	158	159
B. Other Countries	**1243**	**1242**	**1253**	**1250**	**1268**
Africa	**389**	**388**	**397**	**394**	**455**
Benin	12	12	12	12	12
Ghana	55	55	55	55	27
Ivory Coast	30	30	30	30	38
Madagascar	33	33	33	33	34
Mozambique	70	70	70	70	81
Nigeria	39	39	39	39	39
Tanzania	134	134	134	131	128
Others	16	15	24	24	96
Americas	**620**	**620**	**622**	**622**	**581**
Brazil	273	273	279	279	251
Dominican Rep.	35	35	34	34	49
Mexico	179	179	176	176	169

(continued)

Table 17.1 (continued)

Country	2010	2011	2012	2013	2014
Venezuela	18	18	18	18	10
Others	115	115	115	115	102
Asia	**130**	**130**	**130**	**130**	**186**
Bangladesh	39	39	39	39	44
China	29	29	29	29	32
Myanmar	42	42	42	42	48
Others	20	20	20	20	62
Pacific	**104**	**104**	**104**	**104**	**46**
French Polynesia	20	20	20	20	22
Others	84	84	84	84	24
Total	12,021	12,035	12,241	12,224	12,196

Sources Asian and Pacific Coconut Community (APCC) http://www.apccsec.org/

islands and later spread to become pantropical, although the date of its spread to the Pacific has been under considerable debate (Harries 1990, 1995). However, there is also evidence to support the American origin, since palm species (especially coconut palm) are found in Colombia (Cook 1901). In general, coconut appears to have had a diffuse origin in the region of time and place—in the Indian Ocean islands, insular Southeast Asia (including New Guinea) and western Melanesia, including the Philippines. Several early authors (Beccari 1917; De Condolle 1886) had indicated one part or the other of this vast area as the center of domestication of the coconut. It is reasonable to assume that early humans, while developing habitats in coastal areas, must have slowly domesticated any wild form present at that time.

Coconut has been distributed to all parts of the tropical world, such as Central and South America, East and West Africa, South and Southeast Asia and the Pacific Islands. From its putative center of origin, coconut has been disseminated both east and west by floating in the sea and by human dissemination (Ohler 1984). It is reasonable to assume that the spread of coconuts was based on small initial sample size, considering the bulk of the seed material and the sample size might be limited to one or two nuts. In order to understand the process of spread, further studies on the historical and pre-historical knowledge of coconut are needed. Current knowledge appears to be weak in many countries and there has been no attempt to carry out a thorough check of the world literature relevant to this subject as suggested by Bourdeix (1999).

The coconut can now be found on practically every suitable tropical and subtropical worldwide coastline, and has been transported far from the coast in many regions, where rainfall is adequate and the elevation does not exceed 1000 m. Plantations were developed throughout the tropics by the end of the nineteenth century. Coconut populations that are now considered endemic to the Atlantic Coast of Africa, America and around the Caribbean region are basically the same as the coconuts in

Table 17.2 Production of coconut as a whole nut, 2010–2014 (in 1000 nuts)

Country	2010	2011	2012	2013	2014
A. APCC Countries	**56,494,498**	**56,307,021**	**64,047,154**	**62,762,120**	**61,511,181**
F.S. Micronesia	40,000	40,000	45,000	45,000	39,549
Fiji	165,000	160,000	179,500	148,000	200,000
India	16,918,000	16,943,000	23,351,000	22,680,000	21,665,000
Indonesia	16,150,000	16,189,000	16,269,000	16,463,000	16,354,000
Jamaica	95,300	95,500	95,700	97,400	98,500
Kenya	177,144	181,041	185,024	246,416	258,737
Kiribati	57,000	57,500	58,000	56,200	54,600
Malaysia	550,000	563,000	624,000	625,000	653,000
Marshall Islands	34,000	29,500	33,000	35,000	35,000
Papua New Guinea	1,495,000	1,495,000	1,495,000	1,482,592	1,483,000
Philippines	15,510,000	15,245,000	15,862,000	15,354,000	14,696,000
Samoa	200,000	180,000	262,000	267,000	267,000
Solomon Islands	100,000	100,000	100,000	100,000	100,000
Sri Lanka	2,619,000	2,707,000	2,927,000	2,513,320	2,870,000
Thailand	997,704	845,000	806,000	838,000	1,001,000
Tonga	86,900	86,100	81,600	81,762	75,100
Vanuatu	481,250	450,000	447,000	493,980	415,110
Vietnam	818,200	940,380	1,226,330	1,235,450	1,245,585
B. Other Countries	**9,153,575**	**9,074,202**	**8,016,381**	**8,169,672**	**8,325,180**
Asia	***969,744***	***956,779***	***919,021***	***951,183***	***984,935***
Bangladesh	101,750	100,000	81,400	81,400	81,720
Brunei Darussalam	431	430	258	260	270
Cambodia	81,926	82,000	55,000	58,000	62,090
China	330,698	325,000	271,118	280,379	310,208
Maldives	640	3644	1368	565	310
Myanmar	428,075	420,000	490,717	510,412	509,177
Pakistan	9900	9750	10,010	10,007	10,340
Singapore	151	155	150	160	130
Timor Leste	16,173	15,800	9000	10,000	10,690
Pacific	***276,907***	***278,950***	***251,255***	***248,132***	***245,274***
American Samoa	5430	5300	7000	7500	7440

(continued)

Table 17.2 (continued)

Country	2010	2011	2012	2013	2014
Cocos Islands	8780	8850	8837	8824	8559
Cook Islands	2285	2250	1820	1850	1770
French Polynesia	101,925	105,000	83,840	82,000	82,560
Guam	61,468	62,500	52,000	50,000	50,190
Nauru	2079	2000	2600	2700	2780
New Caledonia	19,043	18,625	19,000	19,000	19,000
Niue	3000	3000	3300	3200	3210
Palau	64,692	63,250	63,258	63,258	60,095
Tokelau	3466	3500	4300	4300	4320
Tuvalu	1961	1975	2100	2200	2010
Wallis	2778	2700	3200	3300	3340
Africa	***1,958,844***	***1,960,735***	***1,973,332***	***2,017,843***	***2,029,370***
Benin	23,382	23,500	18,780	18,780	18,730
Cameroon	5892	5750	5250	5100	5350
Cape Verde	6931	7000	5600	5600	5700
Comoros	88,952	87,000	90,000	92,000	90,640
Congo	23,574	20,940	3500	3550	3600
Equatorial Guinea	6931	5000	7000	7500	7270
Ghana	365,002	365,000	345,000	366,183	380,380
Guinea—Bissau	52,563	52,750	42,226	43,000	43,060
Guinea	41,679	51,400	50,950	50,000	49,850
Ivory Coast	154,135	155,000	190,000	195,000	182,170
Liberia	8310	8125	6600	6650	6740
Madagascar	97,614	95,000	76,930	78,000	77,660
Mauritius	2075	2000	1688	1500	1440
Mozambique	313,713	313,750	270,000	260,000	244,060
Nigeria	263,815	265,000	264,814	265,000	267,520
Reunion	647	645	350	350	300
São Tomé Principe	32,431	31,750	38,300	54,100	63,600
Senegal	5430	5325	4260	4500	4550
Seychelles	3663	3550	2800	2900	2580
Sierra Leone	3498	3500	4250	4100	4120
Somalia	13,863	13,750	11,037	11,000	10,980
Tanzania	427,432	427,500	520,000	530,000	545,800
Togo	17,312	17,500	13,997	13,030	13,270

(continued)

Table 17.2 (continued)

Country	2010	2011	2012	2013	2014
America	**5,948,080**	**5,877,738**	**4,872,773**	**4,952,514**	**5,065,601**
Barbados	2440	2350	2000	2200	2300
Belize	1286	1300	768	1041	904
Brazil	3,445,108	3,450,000	2,931,531	2,890,286	2,919,110
Colombia	126,683	127,000	114,585	114,828	118,778
Costa Rica	9242	9035	38,000	38,000	37,940
Cuba	125,429	123,900	70,854	64,619	63,261
Dominica	11,707	9000	7966	8000	7660
Dominican Republic	147,773	127,500	160,935	286,934	333,630
Ecuador	24,238	11,500	17,372	17,500	15,940
El Salvador	68,361	68,500	60,210	60,250	67,396
Grenada	7810	7650	6500	6330	6260
Guatemala	47,396	46,500	22,988	23,000	23,000
Guyana	95,930	93,900	75,320	75,350	78,750
Haiti	28,593	27,950	28,017	28,000	27,940
Honduras	18,458	18,500	14,830	14,850	14,220
Martinique	1329	1300	1100	1050	1020
Mexico	1,414,522	1,385,000	1,091,800	1,064,400	1,118,750
Nicaragua	7534	7400	6000	6000	6330
Panama	17,133	16,800	14,575	14,800	14,770
Peru	28,549	27,950	33,577	34,593	34,597
Puerto Rico	4368	4300	4100	4150	4190
Saint Lucia	16,427	16,000	14,900	15,000	13,010
St. Kitts and Nevis	1455	4300	3012	3000	2800
St. Vincent	5252	5350	2037	2314	2259
Suriname	8236	8050	4090	10,786	12,880
Trinidad Tobago	20,794	20,335	16,349	16,500	16,480
Venezuela	261,669	256,000	129,031	148,403	121,096
Total	**65,648,073**	**65,381,223**	**72,063,535**	**70,931,792**	**69,836,361**

Sources Asian and Pacific Coconut Community (APCC) http://www.apccsec.org/

East Africa, India and Sri Lanka (Harries 1977). Additionally, coconuts in the Pacific Coast of America are related to the Pacific Islands and Southeast Asian coconuts on the basis of fruit component analysis data (Harries 1978; Vargas and Blanco 2000; Zizumbo-Villarreal et al. 2005).

17.2.2 Botanical Description

Descriptions of the morphological characteristics and reaction to abiotic and biotic stresses of an accession are the most useful information for plant breeding programs. These data can be used to identify diverse areas and associated specific traits and to explore the relationship among accessions. However, the use of morphological characterization data for locating diversity has a number of limitations. Morphological characteristics, like differential heritability, pleiotropic and epistatic effects, polygenic control and interaction of genotypes with environment are the main hurdles in making estimates of genetic variation. In many cases, long-term crossing and inheritance studies will be needed for precise estimation. As most of the genetic variation is hidden and is not apparent at the phenotypic level, the morphologically-similar material may in fact be different at the genetic level, which is indeed another hurdle. Despite these drawbacks, morphometric methods have been used, which is an advantage in coconut as well as other crops (Akpan 1994; Ashburner and Rohde 1994; Sugimura et al. 1997).

Coconuts are generally classified as tall or dwarf (Santos et al. 1996). The talls, sometimes referred to as var. *typica* (Nar.), are widely distributed, can grow more than 50 cm annually, flower at 6–10 years with an economic life of 60–70 years. Dwarfs, sometimes referred to as var. *nana* (Griff.) are believed to be mutants from tall types with shorter stature (about 8–10 m when 20 years old). They have a shorter productive life of 30–40 years, but usually start flowering in the third year at less than 1 m height. Occasionally, natural crosses that occurred sporadically in traditional populations between the talls and the dwarfs produced *intermediate* types that are phenotypically distinct from either the talls or the dwarfs. These open-pollinated hybrids may become fixed as semi-talls, which have the same mating behavior as dwarfs, but grow fast.

The coconut grows from a single shoot meristem or terminal bud (commonly called the *cabbage* or *heart* in all the Palmae) at the apex of the plant. The stem is erect, unbranched and cylindrical with no outer cambium layer, unusually sturdy and can tolerate adverse conditions, like strong wind, typhoon and ring barking because of its numerous vascular bundles. It is anchored by numerous adventitious roots that are produced from the *swollen* basal (known as *bole*) part of the stem. The top of the trunk is surmounted by a radiating compact crown (which can either be spherical, semi-spherical, drooping or erect) of large, thick cuticle paripinnate leaves. The petiole or leaf stalk is directly attached to the stem and naturally falls when it reaches the end of its mature phase leaving leaf scars on the trunk. A rough estimate of the age can be made by counting the leaf scars on a coconut trunk. A coconut palm may

produce 14 leaves a year but in old age an average of 12 is probably reasonable as a basis for estimating age, but this may also vary depending on the genotype (Child 1974).

The dwarfs may start to flower as early as 3 years, while talls start to flower in 5 years under favorable conditions. A flower cluster or inflorescence enclosed in a sheath (called a *spadix*) is produced in each leaf axil in continuous succession. Each inflorescence consists of 20–30 branches (rachillae), but some may abort due to unfavorable climatic conditions and/or poor cultural management. The coconut palm is monoecious, i.e. its inflorescence carries both staminate (male) and pistillate (female) flowers (Frankel and Galun 1977). The female flowers (also known as *button nuts*) are situated at the base of each rachilla, while most of the male flowers can be found on the apical portion of the inflorescence and beside the pistillate flowers. After fertilization, the button nuts develop into fruits, forming a bunch that matures into a nearly round or spherical-shaped nut in about 12 months. The ripe nut is a large drupe with the solid white kernel (endosperm) encased in a hard shell (endocarp). At the basal end of the fruit, there are three pores, *eyes,* one of which is soft where the plumule or the shoot appears during germination. A coat of fibrous mass (mesocarp), that is, the husk from which coir is obtained, further protects the shell. For young or green coconut, the mesocarp is white and firm and the kernel is thin and jelly-like. Water or liquid endosperm is found inside the cavity of the kernel, which diminishes towards the end of maturation.

17.2.3 Pollination Behavior

Generally talls shed pollen prior to stigma receptivity. They are generally considered allogamous. Nevertheless, selfing is possible through inter-spadix pollination because of the overlap between the female phase of an inflorescence and the male phase of the next inflorescence. The pace of production of inflorescences varies according to genotype and environment with a great seasonal variation; so does the selfing rate. On the other hand, the dwarfs are generally considered autogamous/homogamous as stamens and pistils mature simultaneously. Thus, dwarfs can shed and receive pollen at the same time resulting in inbreeding. Apart from their short stem, most of the dwarfs show a combination of common characteristics: preference to autogamy, sensitivity to environmental stresses, small-sized organs, precocity and rapid production of inflorescence (Thomas and Josephrajkumar 2013). Because of the last three characteristics, the dwarfs play an important role in hybridization programs. However, the genetic determinant of coconut dwarfism is still unknown.

The inflorescence primordium can be detected about 4 months after the first leaf primordium is differentiated, whereas the male and female flowers can be seen after 22 months thereafter. The opening of the fully grown spathe occurs 1 year later (Thomas and Josephrajkumar 2013).

The male flowers are the first to open, beginning at the top of each spikelet and proceeding towards the base. After each flower opening, the pollen is shed, and

male flowers abscise, the whole process taking just 1 day. The male phase, however, takes about 20 days in most palms but this may vary according to season and variety (Thomas and Josephrajkumar 2013).

A female flower remains receptive for 1–3 days. Depending on the environmental conditions and variety, the female phase may begin a few days or later after the spathe has opened and lasts 3–5 days in tall palms and about 8–15 days in dwarfs. A normal inflorescence may have 10–50 female flowers. With natural pollination, 50–70% usually abort and fall off, especially those which emerge during severe dry weather. The remaining flowers develop into fruits, which take about 12 months to mature (Thomas and Josephrajkumar 2013).

The length of the male and female phases is affected by climate and usually do not overlap in the tall types, such that self-pollination rarely occurs. In some dwarfs, particularly the Malayan Dwarf, overlapping of the male and female phases and between spadices usually takes place, promoting selfing.

The bisexual nature of the talls and the dwarfs allow manipulation of pollination to secure the desired level of genetic introgression with the talls as a source of heterozygous genotypes, while the dwarfs provide the progenitors of homozygosity (Santos et al. 1996; Thomas and Josephrajkumar 2013).

17.3 Coconut Genetic Resources

17.3.1 Coconut Germplasm Collection and Classification

Although the coconut is monotypic, considerable diversity can be observed in the existing populations in term of stature (tall or dwarf); leaf configuration (formation); size, shapes and color of the nuts; pests and disease (resistance); precocity of flowering, vertical stem growth, fruit setting or number of bunches, number of nuts, weight and composition of the fruit and so on. These core characters have been used for quick characterization and evaluation of coconut germplasm by conservation centers (Batugal et al. 2005; Perera et al. 2009). However, the genetic bases of these phenotypic variations are still to be studied in coconut.

The International Coconut Genetic Resources Network (COGENT) is an association of 38 coconut growing countries promoted by the International Plant Genetic Resources Institute (IPGRI, now Bioversity International). COGENT has coordinated the collection of important coconut varieties worldwide and their conservation in national coconut field gene banks with important duplicates maintained at the COGENT's multi-site International Coconut Genebank (ICG). The French Agricultural Research Centre for International Development (CIRAD) is serving as the primary source of materials for genetic characterizations. Together with a polymorphic microsatellite marker kit (Baudouin and Lebrun 2002), a book on Coconut Genetic Resources (Batugal et al. 2005) published by IPGRI and the CGRD database provide a comprehensive coverage on the coconut genetic resources available throughout the

world. COGENT has also initiated the establishment of four large multi-site international coconut gene banks in Indonesia, India, Papua New Guinea and Ivory Coast as well as 28 national gene banks in 24 countries, in addition to the existing small international collections in Tanzania, Ivory Coast, The Philippines and India. Based on COGENT's International Coconut Genetic Resources Database (CGRD), the total number of germplasm accessions collected and conserved increased from 761 in 1994 to 1416 accessions in 2003 (Batugal et al. 2005). The quantitative and qualitative descriptions of major traits on most of the accessions are available in the CGRD and the germplasm is are available in ICG and national gene banks. More accessions are currently being incorporated into CGRD and ICG for germplasm improvement and utilization purposes. These accessions provide new options to breeders to incorporate germplasm into their breeding programs. The genetic resources in coconut are widely exploited through selection, hybridization for a number of desirable traits and have resulted in the development of many varieties.

Previously, there was no proper standardized nomenclature for coconut, whereas now each variety is given a unique international name, usually consisting of the group, whether it is a tall or dwarf. Besides, geographical or cultural reference is added; for varieties of uniform color, for instance, the different color forms of the dwarf group consists of the color information. For examples the cultivars are separated as West African Tall, Sri Lanka Tall, Mozambique Tall, Malayan Tall, originating from different countries, and San Ramon, Tagnanan Tall, Markham Valley Tall, originating from various regions within a particular country. Examples of such cultivars in the dwarf group are Cameroon Red Dwarf, Sri Lanka Green Dwarf, Malayan Yellow Dwarf and Madang Brown Dwarf (Batugal et al. 2005). Furthermore, germplasm collections, field gene banks and the COGENT database have certain other entries termed as populations or accessions for the collections of coconut populations within a particular coconut cultivar, identified in different geographic locations. Examples of such populations are Panama Tall Monagre and Panama Tall Aguadulce, and West African Tall Mensah and West African Tall Akabo, Sri Lanka Tall Ambakelle and Sri Lanka Tall Kasagala. They are also sometimes referred to as ecotypes if the particular accession or population was a result of an environmental selection over several generations. In these populations or accessions the morphological differences are either not noticeable or very slightly detectable, but their adaptability for particular ecological niches or for a particular pest or disease resistance which cannot be predicted from morphology.

Within the main varieties, numerous groups of coconut do exist which Liyanage (1958) referred to as *forms* of coconut and Bourdeix et al. (2005) referred to as *variants* that are phenotypically highly distinctive. Some examples are Bodiri, Nawasi, Dikiripol and Porapol in Sri Lanka (Liyanage 1958), Laccadive Micro Tall in India (Bourdeix et al. 2005), Spicata in different countries, Makapuno in The Philippines and Nim in Thailand.

Instead of the above varietal groups, Harries (1978) proposed two main types of tall coconuts; one that has large, long, angular, thick-husked and slow-germinating nuts with less free water content, the Niu kafa type or wild coconut, which evolved naturally and was disseminated by ocean currents. Another has more spherical nuts

with an increased proportion of endosperm, reduced husk thickness, early germination and resistance to disease, the Niu vai type or domestic coconut, which was selected under cultivation for increased nut water content and was disseminated by humans. Harries (1978) further suggests that introgression of these two types and further selection and dissemination by humans afforded the wide range of varieties and pantropical distribution of coconut that can be seen today.

Among all collections, the more popular parental accessions for hybridization are the West African Tall (WAT), Rennell Island Tall (RIT), Vanuatu Tall (VTT), Malayan Yellow Dwarf (MYD), Malayan Red Dwarf (MRD), Malayan Green Dwarf (MGD) and Cameroon Red Dwarf (CRD).

17.3.2 Coconut Germplasm Conservation: Ex Situ and In Situ

Both ex situ and in situ germplasm conservation are important for genetic diversity and should be regarded as complementary to each other (Engels and Wood 1999; Ramanatha Rao 1999).

Ex situ refers to conservation outside the natural habitat in facilities, such as seed banks, field gene banks, in vitro collections, botanic gardens, with germplasm conserved in the form of plants, seeds, pollen, tissues, cells or DNA. In contrast, in situ conservation is conserving germplasm in the natural habitat where the target species is found, and in habitats, such as farms and home gardens, where the species have developed their distinctive properties as a result of long-term selection by humans. In the case of coconut, seeds are bulky and heavy, making them costly to transport. They are also highly recalcitrant (Chin and Roberts 1980). These characteristics limit the amount of material that can be collected and restricts the geographic range of collecting missions. These limitations may have serious consequences for genetic resource conservation, since it is recognized that a large amount of the untapped genetic diversity in coconut is located in remote areas, such as atoll islands. The key to solving these problems, however, lies in recognizing that only the embryo is needed to propagate a coconut palm. Various efficient in vitro culture protocols are available which allow the production of whole plantlets from coconut zygotic embryos inoculated in vitro (Batugal and Engelmann 1998; Engelmann 2002).

Recommended standardized coconut conservation techniques are applied in ex situ gene banks to adequately represent genetic diversity and allow consistent characterization and workable regeneration. Ex situ conservation is crucial to coconut conservation, particularly for varieties disappearing from farmers' fields. It forms a buffer between users and the fast-evolving in situ genetic diversity. For that purpose, multi-site International Coconut Genebanks (ICGs) have been established by COGENT. Ex situ germplasm conservation and management includes: targeted collecting; establishing and maintaining field collections; regenerating old accessions using controlled hand-pollination; characterization and identity verification studies;

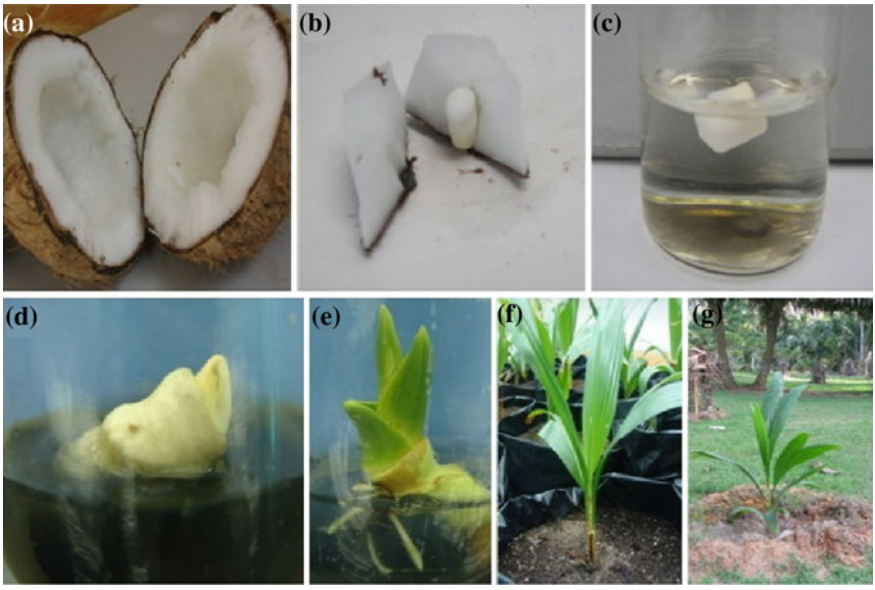

Fig. 17.1 Coconut embryo culture. **a**–**b** Embryo isolation, **c** Embryo disinfection, **d** Embryo germination, **e** Shoots and rooting, **f** Nursery, **g** Field plantation

evaluation for priority traits; information management; safe exchange of germplasm; and sometimes germplasm pre-breeding. Such conservation relies solely on field gene banks, where the growing material readily provides seednuts or pollen, and is available for distribution, characterization, evaluation and training. Field collections however, remain vulnerable to pests and diseases, natural disasters and land pressure.

In vitro tissue culture is another method of ex situ conservation for coconut. Germplasm is internationally transferred mostly as in vitro cultivated embryos (Fig. 17.1a–g) and sometimes as seednuts. Farmers mainly receive coconut planting material as seednuts and seedlings. In vitro collections are not yet used for safety duplication of the coconut field collections or for rapid multiplication and dissemination of disease-free planting material. No in vitro multiplication protocol exists yet, so constraints such as contamination result in low regeneration rates. The material also demands regular sub-culturing and may be subject to somaclonal variation. In such cases, rejuvenation and verification of the trueness-to-type of the conserved germplasm has to be performed periodically.

Conservation of coconut pollen is an additional option. Pollen can be dried and stored under vacuum for a short period of time (2–6 months) in a domestic deep freezer. Freeze-drying experiments showed no viability loss after 3 and 6 months (Whitehead 1966) of storage at room temperature. Coconut pollen is highly tolerant to desiccation and preliminary experiments have demonstrated that it could be successfully cryopreserved. Long-term storage of coconut pollen under cryop-

reservation would represent an important additional technique for genetic resources conservation.

17.4 Breeding Goals and Achievements

17.4.1 Breeding Goals for Coconut

Coconut is an enduring crop, grown in a range of regions and countries with a diversity of soil, climate, pests and diseases. Coconut breeding in global terms is a great challenge and was initiated in 1916 at the Nileshwar Coconut Research Station, Kerala, India. Like other crops, coconut genetic improvements have been largely focused around high yield, improvement in oil and copra yield, resistance to drought, insects and pathogens. The weight of meat/copra is important because of high demand by the processing and manufacturing industries (Perera et al. 2009). Also, the number of nuts is one of the attractive parameters to the grower who sell coconuts on a number basis. Other objectives that are currently under consideration, include improvement in the protein content, quality of the fatty acids, coconut oil and fat. In fact, the breeding objectives outlined by formal institutions involved in coconut research and development have influenced the evaluation and maintenance of coconut germplasm. Coconut research centers have access to a very small fraction of the variability that actually exists within the species (de Nuce de Lamonthe 1991). Much of the diversity, which is not accessible to coconut researchers, is in the hands of small farmers who grow coconuts in diverse environment for varied uses.

The explanation of yield in terms of yield improvement in coconut is difficult. Because the number of nuts per bunch and size of the nuts is negatively correlated in coconut, one cannot enhance both traits simultaneously by improving naturally-occurring coconut varieties by selection alone. The breeding programs aiming at a higher nut number have succeeded through the production of dwarf-tall hybrids that surpass the yield of tall coconut cultivars by over 45% (Perera 2005). The low copra content in hybrids is more than compensated for by the number of nuts per coconut tree. Certainly, copra content per hybrid nut is generally low compared to tall nuts, therefore, hybrid nuts are less in demand by manufacturers due to incurring high labor costs during processing. On the contrary, tall-tall hybrid (CRISL98) did not attract the farmers in Sri Lanka due to low number of nuts per palm. Hence, in the last few decades, breeding goals have been centered on the improvement of hybrids that produce a large number of nuts having a thick kernel. In this regard, the hybrid coconut Kapruwana developed from large-fruited parent San Ramon (originating from The Philippines) and thick kernel parent Dwarf Green (originating from Sri Lanka) is the best example. The performance of Kapruwana regarding the nut number is equal to the CRIC65 hybrid and the copra content per nut is comparable to the CRISL98 hybrid. Another important issue in coconut breeding is precocity in flowering, as the vegetative phase of commercially grown tall coconut varieties take about 8–10 years.

Significant progress has been made in the precocity of coconuts by combining early-flowering behavior of dwarfs with commercially-grown tall coconut cultivars (Perera et al. 2009).

The objective of coconut breeding for improving oil contents or oil rich in a particular fatty acid is still at an early stage, but there is great potential for success. With the continuing changes in global climate, droughts have become frequent in many coconut-growing countries and hence a constraint in coconut production. Breeding for drought tolerance has become one of the goals in coconut breeding in many countries (Nair et al. 2016). Selection of cultivars and individual genotypes that can resist the drought conditions should be used as parents in breeding programs in order to improve local varieties.

Likewise, many major and minor coconut pests exist that can cause severe damages to coconut palms. Although there are efficient and effective chemical and biological control methods in place, yet they are unsuccessful to control some pests, including coconut mites (*Aceria guerreronis*). Coconut mites are microscopic pests that live beneath the perianth of the nut causing critical damages to developing nuts. Hence, one of the breeding goals in Sri Lanka and India is to develop/produce cultivars with tolerance to Aceria mite (Perera 2005, 2006). Screening of coconut varieties for tolerance to Aceria mite has been undertaken with the development of hybrids resulted from the crosses between tolerant parents (Perera 2006).

Breeding for disease resistance has also been an important subject in various countries, where millions of coconut palm were destroyed by lethal yellowing disease (Caribbean and Latin America) and root wilt disease (India). Indubitably, such phytoplasma-causing diseases have been given priority due to their occurrence and potential to spread quickly (Nair et al. 2016)

Nowadays coconut farmers prefer to cultivate short-stature palms because of the unavailability of trained pickers or climbers and hence breeding for short stature is also a current breeding objective.

17.4.2 Current Status of Breeding

Coconut belongs to the monocotyledons group of flowering plants and is considered a natural and valuable gift to humankind due to its abundant uses by millions of people. Coconut palm is grown throughout the tropical world particularly in Asia, Oceania, West Indies, Central and South America and West and East Africa. Coconuts grown all over the world have evolved through mass selection and open pollination. Globally coconut breeding is a great challenge and was started in 1916 at the Nileshwar Coconut Research Station Neleshwar, Kerala, India. However, it was not until 1996 that the first international scientific gathering on coconut breeding was held in Cote d'Ivoire to standardize breeding programs and research techniques (Batugal and Ramanatha Rao 1998).

Most advanced breeding centers, like the Marc Delorme Research Station in Cote d'Ivoire have focused on local cultivar evaluation versus exotic varieties. On the other

hand, it was observed that locally-produced hybrids were more prominent during national evaluations (Batugal 2004). Moreover, offspring were evaluated through: morphological and genotypic characterization of breeding accessions; evaluation of exotic/locally breeding materials for general and specific combining abilities; selection/acquisition of mother palms and sources of pollen; pollination of selected mother palms to produce the desired crosses; and production of hybrid nuts for field testing in many countries.

Different coconut hybrids were introduced by selected countries after performance evaluation. Introduced or exotic cultivars are mostly found in research stations for characterization and on-farm evaluation or for testing as breeding materials. From 1950 to 1993, more than 400 hybrids were developed worldwide under established coconut improvement programs using various breeding strategies (Bourdeix 1999). There were also instances where spontaneous hybrids, due to natural out-crossing, were identified and selected by farmers. In the 1990s, with the availability of characterized germplasm from gene banks, hybridization to develop improved varieties intensified. Research stations reported copra yields of 2–6 t ha $^{-1}$ from their hybrids. However, hybrids are seldom grown commercially due to the lack of information on their availability, insufficiency of planting materials and high input requirements. Many countries with national breeding programs tend to develop hybrids from *proven* single crosses based on results known from advanced breeding programs, to eliminate the need for combining ability tests of potential progenitors, which would require at least 12 years. However, the coconut is highly polymorphic, i.e. its performance is determined not only by its genes but also by environmental factors. Hence, results of progeny testing from the same parental population vary within and across countries (Batugal et al. 2005) (Table 17.3).

17.4.3 Major Constraints in Coconut Production

Lack of adapted, high-yielding varieties; low level of yield stability and low farmer productivity are major constraints to sustainable world coconut production (Batugal 1999).

Hence, the major coconut growing countries focus on breeding superior varieties with high yield, high oil content, disease resistance and tolerance to biotic stress. Yields are generally measured in terms of the number of nuts and copra/nut. Other yield parameters may include nut size, weight of whole nut, dehusked nut, shell and water content. The oil content is derived by drying the kernel, extracting the oil using oil expellers and determining the percentage of oil over dry matter. Breeding for disease resistance is given more emphasis in African and South American countries because of the presence of the phytoplasma (causing lethal yellowing disease). Vanuatu is highly involved in developing cultivars with resistance against coconut foliar decay. Some countries like India, Sri Lanka and Tanzania pay attention to drought tolerance since coconuts are generally grown in marginal, rainfed areas. The Coconut Research Institute in Hainan (China) is developing cold-resistant varieties

Table 17.3 Summary of coconut breeding program in some countries

Country	Breeding program	Goals and performance
China	Crossing between Malayan Yellow Dwarf (MYD) and the Local Hainan Tall (HAT) variety	Yield nuts (80/palm/year) and copra (4 t ha-1 yr-1), which showed early flowering (3–4 years) and 3–4 fold increase in terms of harvest
Côte d'Ivoire	Hybrids: PB 213 (WAT × RIT), PB 214 (WAT × VTT), PB121 (MYD × WAT), PB 132 (MRD × TAT or Tahitian Tall), PB123 (MYD × RIT) and PB111 (CRD or Cameroon Red Dwarf × WAT)	Flowering very early (40–57 months after field planting). 100 to 132 nuts/palm/year which is 34–138% higher compared with the control, i.e. the WAT. copra yields ranged from 3.15–4.8 mt ha^{-1}
India	Crossing between Crossing between Chowgat Orange Dwarf (COD), West Coast Tall (WCT), Laccadive Ordinary (LCT), Gangabondam (GBGD), MYD, SS Apricot (SSAT) and East Coast Tall (ECT)	All the hybrids performed better than the traditional cultivar with an average nut yields of 98–156 per palm. COD × WCT (Chandra Sankara), WCT × SSAT (Kera Sowbagya) and WCT × MYD (Kera Sree) produced the highest copra yields, i.e. more than 4 t ha-1per yr-1
Mexico	Initial hybrids were mainly derived from crosses between MYD and improved Pacific Tall populations and Intra-population crosses of selected Pacific Tall	Developing hybrids resistant to lethal yellowing disease
Philippines	Crossing between different local cultivars (Catigan Green Dwarf, Tagnanan Tall, Baybay Tall, Laguna Tall, Bago-Oshiro Tall) and the exotic varieties (Malayan Red Dwarf and Polynesian Tall)	Shown early bearing, these hybrids yield 117–155 nuts per palm and 4–6 mt copra per hectare. Among the nine hybrids, PCA 15-2 (cross between Malayan Red Dwarf × Tagnanan Tall) and PCA15-3 (cross between Malayan Red Dwarf × Baybay Tall) are outstanding, giving the highest number of nuts (144–155/palm) and copra yield (6 t ha-1)
Sri Lanka	Sri Lanka Green Dwarf [SLGD] × Sri Lanka Tall [SLT]) SLT × SR and a first generation inbred (SLT × SLT)	Yields ranged from 80–125 nuts per palm and from 3.6–4.0 mt copra ha-1. The yield hybrid nuts have almost double to that of local cultivar

(continued)

Table 17.3 (continued)

Country	Breeding program	Goals and performance
Tanzania	Hybrids with the local East African Tall (EAT) as the sole pollinator. Mother palms involved Malayan Green Dwarf (MGD), CRD, Pemba Red Dwarf (PRD), MYD, MRD and improved EAT populations	F1 progenies were evaluated for their resistance to lethal disease and tolerance to drought stress
Thailand	Hybrids: Sawi Hybrid No.1 (an introduced hybrid known as PB 121 or MAWA), the locally developed hybrids Chumphon Hybrid No.60 (Maphrao Yai or Thai Tall × West African Tall) and Chumphon Hybrid No. 2 (MYD × Thai Tall)	Nut and copra yields of the recommended hybrids ranged from 80–126/palm and from 3.4–4.2 t ha-1, respectively
Vanuatu	Crossing between the local cultivars Vanuatu Tall (VTT), Vanuatu Red Dwarf (VRD), and the introduced varieties Renell Island Tall (RIT), Brazilian Green Dwarf (BGD) and Malayan Red Dwarf (MRD)	The BGD crossed with either RIT or VTT produced the best copra yields of 4.4–5.2 mt/ha but they were also found to be very susceptible to CFD. The VRD × VTT hybrids had lower copra yields (3.3–3.7 mt/ha), but were found to be more tolerant against CFD. Both the traditional and improved VTT types had the lowest reported copra yields of 2.6–2.8 mt/ha, yet comparable with the hybrid MRD × RIT
Vietnam	Hybrids in Dong Go Experimental Centre (Eo × Ta; Tam Quan × Ta; Tam Quan × BAOT) and in Binh Thanh Experimental Station (MYD × Renell Tall; MYD × Palu Tall; MYD × Ta)	High yields and early flowering

for its low-temperature (about 10 °C during the winter) growing areas in Southern China. The Caribbean and Pacific countries are interested in developing cyclone tolerant varieties because their open positions make them wind disaster-prone areas (Batugal et al. 2009; Perera et al. 2009).

17.4.4 Impact of Climate Change on Production and Breeding Strategies

Climate change is projected to increase global annual mean temperatures in the range of 1.8–4 °C; increase the variability in rainfall; and enhance frequency of extreme weather events such as heat waves, cold waves, droughts and floods (IPCC 2007). All these changes could potentially influence agricultural production and many production systems may become vulnerable. Weather extremes will become predominant. Coastal and hilly areas are believed to be more vulnerable to climate change compared to other terrestrial areas.

Plantation crops, mainly coconut, rubber, tea, coffee, oil palm, areca nut, cashew and cacao, are grown in ecologically-sensitive areas such as coastal belts, hilly areas, and areas with high rainfall and high humidity. Among these coconut is a major multi-utility crop that plays a significant role in the economy of the countries, including 10 million farming communities in India. Climate change will affect coconut plantation through higher temperatures, elevated CO_2 concentration, precipitation changes, and increased weed growth, higher incidence of pests and disease, and increased vulnerability of organic carbon pools. Unlike in seasonal crops, the impact of climate change will have long-standing ill effects in coconut since it is a perennial crop. In general, various approaches are used to mitigate risks associated with seasonal climate variability, including the adoption of the tolerant crop varieties and best management practices (Hebbar et al. 2013).

Around 180,000 islands encompass a fifth of the world's biodiversity and certainly more than 50% of coconut diversity. At least two-thirds of coconut plantations are located in coastal zones and the majority of coconut growing countries are islands. Growing more resilient varieties is essential for regions that are projected to be negatively impacted by climate change. Coconut may be one of the best-suited crop in climate change situations and has a cosmopolitan growing habitat covering the largest region of the world. It can withstand temporary waterlogging conditions from floods and tides with special adaptability against the strong winds, storms and cyclones (Kumar and Aggarwal 2013). Moreover, as it is salinity-tolerant crop, it is greatly adaptable for the coastal zones of tropical and equatorial regions.

Global climate change result in the change in CO_2 concentration, effect of ozone on plants, productivity fertilization effect, UV (ultraviolet) radiation, temperature and stress and new diseases on coconut growth. Global climate change may also alter microorganisms in soil-agriculture ecosystems (Kumar and Aggarwal 2013).

Much attention should be paid to identify the coconut varieties with resistance to the biotic and abiotic stresses and to set up the breeding program for creation of new varieties with greater tolerance to extreme climate conditions.

17.4.5 Breeding Strategies and Methods

The estimated gap in coconut yield in terms of nuts and copra around the world ranges from 33 to 84%. Such gaps can be overcome by introducing high-yielding hybrids and/or improved varieties with the aid of appropriate management and cultural practices. Generally, hybrids perform well compared to conventional varieties, but they are planted on a limited area (14%) in various coconut producing countries. Traditionally, coconut genetic resources have been collected and conserved in order to create cultivars with quality traits. These conserved genetic resources are generally used as: (i) basic planting material to improve the coconut production in the country or region; (ii) to evaluate the material through phenotypic and genotypic characters; (iii) a population base for breeding superior hybrids/varieties (Batugal et al. 2009).

Origin-based germplasm could be evaluated as: traditional varieties or landraces, exotic varieties, modern varieties or hybrids as a result of national breeding program (Perera et al. 2009). However, most coconut-producing countries have a mixed population of landraces and hybrids/varieties, but they are intensively involved in improving the existing cultivars through mass selection and hybridization.

17.4.6 Breeding for Yield Improvement

The breeding strategies that have been applied to the yield improvement of the coconut palm can be divided into two main sections: intra-varietal breeding strategies (mostly based on the mass selection methods) and hybridization between varieties to exploit the hybrid vigor potential.

17.4.6.1 Intra-Varietal Breeding

Mass Selection Mass selection is the most fundamental method for coconut breeding (Liyanage 1955). The majority of the world's coconuts are derived from mass selection, which is informally done by all growers. At the end of the nineteenth century, large plantations were established by importation of fruits from a region known for its production (Ziller 1962). The seednuts were selected according to their specific characteristics: some preferred large and heavy fruits (Zuniga et al. 1969), others medium-sized fruits preferably round-shaped (Apacible 1968). The genetic structure of the coconut populations has been modified by successive selections based on fruit characteristics.

There are three variants of mass selection based on the reproductive system used, i.e. mass selection using open-pollination, selfing or inter-crossing (Bourdeix 1988). Mass selection using open-pollination has been practiced the most. The advantage of the method is its simplicity; the seednuts are collected from the palms that present attractive characteristics at a certain time or over a period. The progenies resulting

from open-pollination are the basis of an improved population that will then undergo other selection cycles. This method leads to variable results. Even in the most favorable cases, the drastic selection necessary to obtain an improvement considerably reduces seednut production potential. One generation of multiplication is inevitable; therefore, it is better to use this generation for the evaluation of the parents based on the performance of their progeny. The only advantage of mass selection using open-pollination is its simplicity. Mass selection using inter-crossing appears more effective, as it allows for a strict selection of pollinators while retaining the potential for large seednut production.

In Sri Lanka, a national replanting program has been in operation for the last four decades. Most of the planting materials were produced from a pool of about 50,000 good mother palms selected from farmers' fields and experimental stations (Peries 1998). In such a program, investigations about the selfing rate is strongly required. If the best palms have a higher selfing rate, a single generation of selfing usually reduces the yield of fruits 20–30%. In tall types, selfing generally induces an average yield decline of 15–30% without appreciably increasing production homogeneity. There are practical solutions to restrict selfing during the open-pollination process. The first option could be as simple as avoiding the harvesting of seednuts during the 3–6 months period within the year when the production of inflorescences is fastest. The second alternative is to remove one of the inflorescences within two successive inflorescences to minimize overlaps of pollen dehiscence and stigmatic receptivity. The level of selection pressure at the nursery stage is also very important (Perera et al. 2009).

17.4.6.2 Inter-Varietal Breeding

Different crosses between genetically diverse varieties are made using hand pollination. The first known coconut hybrids were created by crossing the MRD with the Niu Leka Dwarf in Fiji (Marechal 1926). Hybridization between dwarf and tall coconut varieties was first done in India (Patel 1938) The progeny of the crosses are evaluated in the field in comparison with recommended cultivars as controls and tested in different agro-ecological zones and in different soil types in order to select new coconut hybrids suitable for particular environments or soil types. Since production of seeds by inter-varietal crosses is laborious and time consuming and generates only a very few seeds in relation to the general demand for improved seeds, the isolated seed garden concept is also adopted for production of inter-varietal hybrids (Batugal et al. 2009).

There are three main types of coconut hybrids, ranked by order of economic importance: dwarf × tall (D × T), tall × tall (T × T) and dwarf × dwarf (D × D) (Batugal et al. 2005; Perera et al. 2009).

17.4.7 Breeding for Biotic and Abiotic Stress Resistance

17.4.7.1 Disease and Pets Resistance

Lethal yellowing is a disease caused by phytoplasma which rapidly destroy large coconut areas in several regions. It appears to have been first observed in the nineteenth century in the Cayman Islands, Cuba and Jamaica (Arellano and Oropeza 1995) and has been present in the region since then. The genetic strategy was adopted in Jamaica where the Malayan Yellow Dwarf (MYD) and its hybrid with the Panama Tall (PNT) or Maypan were used extensively for replanting the affected regions in the 1980s. This strategy appeared to be successful until the beginning of this century when the disease extensively destroyed the supposedly resistant materials. MYD and PNT were chosen in an extensive series of trials (Been 1981). Various cultivars and hybrids were also tested in Ghana, and Sri Lanka Green Dwarf and the Vanuatu Tall were not affected by the disease. However, active search for a broader range of partially-resistant cultivars remains necessary. Material from Southeast Asia and Mexico seems to offer the most promising material to date, which are all of Pacific origin.

Eriophyid mite, rhinoceros beetles and red palm weevil are main pests for coconut causing major economic losses in most cultivation areas. Among them, the eriophyid mite and rhinoceros beetles are serious pest in almost all growing regions. It has been shown that round and dark green coconut exhibit better tolerance against the eriophyid mite than the elongated coconuts and those of other colors (Moore and Alexander 1987). The gap between fruit and tepal for round fruits is smaller than elongated and angled fruits which may result in less mite incidence, since the entry of mites depends on the tightness of tepals to the fruits at the early stages of fruit development. All coconut cultivars are prone to damage by rhinoceros beetles, but the hybrids resultant from Chowghat Orange Dwarf is known to be more susceptible (Nambiar 1988).

17.4.7.2 Drought and Cold Tolerance

The coconut palm generally grows well in locations where the annual rainfall is between 1300 and 2500 mm and above. A prolonged dry season lasting up to four months may adversely affect the palms; this kind of climatic aberration occurs periodically in various important coconut growing zones, such as in Kerala (India), Sri Lanka or the West African coast. Many phenotypic and physiological traits will affect drought tolerance in coconut. Cultivars with more roots and a fine root density show more drought resistance than others (Cintra et al. 1993). On the other hand, leaf stomatal frequency, stomatal index, chlorophyll fluorescence, epicuticular wax content, activities of lipases and proteases are major physiological traits for identifying drought tolerant coconut cultivars (Rajagopal et al. 1988; Repellin et al. 1994). Federated Malay States, Java Giant, Fiji, Laccadive Ordinary and Andaman Giant

cultivars have been identified as drought-tolerant varieties based on morphological and physiological studies and have been utilized in the many coconut breeding programs for high yielding hybrids like Laccadive Ordinary Tall x Chowghat Orange Dwarf and Malayan Yellow Dwarf x West Coast Tall. Those hybrids have been found to be drought tolerant (Kasturi Bai et al. 2006; Rajagopal et al. 2005).

Due to the natural habitat of coconut in China, Hainan Tall, Yunnan Tall and Guangdong Tall are more cold tolerance than the other varieties. However, cold damage to coconut occurs from time to time during the winter. It has been shown that flowering and fruit set showed cold damage symptoms when the temperature falls below 13 °C for a few weeks. During an investigation of the meteorological indices for coconut cultivation in China, results showed that the spear leaf damage, drying of leaves and uneven or wrinkled kernel inside the nuts were symptoms due to cold (Mao 1986). A few coconut varieties are reported to be cold tolerant and Hainan Tall of China and Kamrup Tall in India are the most significant (Chowdhury et al. 2001; Mao and Lai 1993).

17.4.7.3 Breeding for Other Traits

Coconut special varieties are collected from coconut-growing counties, most of which are important spontaneous mutations with desirable traits having the potential to be used in breeding programs (Arunachalam et al. 2001). A mutant with spicata (multiple spicata coconut-MSC) results in many flowers on an unbranched inflorescence (Sankaran et al. 2015), the thairu thengai mutant with soft endosperm (Jerard et al. 2013), the kaithathali with an edible husk (Samsudeen et al. 2006), sweet kernel coconut and flavored coconut are some of the novel mutants which have immense potential (Nair et al. 2016). Makapuno is a single recessive mutation, originally reported from The Philippines, where the endosperm becomes soft as butter. This mutant has been successfully exploited through controlled pollination among makapuno-bearing palms followed by embryo culture. The same mutant was also found in Indonesia (called kopyor), and in Thailand (called kathi) which is a coconut sport or naturally occurring coconut mutant which has an abnormal development of the endosperm. The result of this abnormal development is a soft jelly-like coconut flesh (Sukendah et al. 2009).

Dwarf coconut with slow vertical growth is another important variety for coconut breeding from which the extreme dwarf mutants from natural populations can be used, besides the identified prominent dwarf cultivars. One such extreme dwarf mutant identified in India from Lakshadweep coconut populations has shown extreme dwarfism compared to the conserved dwarf cultivars among the genetic resources (Nair et al. 2016).

Aromatic coconut is another type of mutant confer the coconut with special aromatic flavor. In Thailand, the Aromatic Green Dwarf was known as pandan which is a single point mutant in BADH2 gene which result in the accumulation of flavor component 2AP (Saensuk et al. 2016; Vongvanrungruang et al. 2016). Brazil Green Dwarf (BGD) is also renowned for its very sweet juice of tender nuts.

17.5 Biotechnology and Molecular-Aided Breeding

The coconut is one of the essential fruit and oil crops in the humid tropical regions of the world. Factors, such as large stature, prolonged generation, recalcitrant seeds and minimal multiplication rate (Ashburner 1995) make coconut breeding and selection more difficult. Therefore, extensive resources to evaluate coconut breeding strategies are the demand of today. In this regard, molecular markers have shown positive results in enhancing the efficacy and productivity of coconut germplasm. Besides, the molecular markers helped in the identification of genotypes and selection of coconut genotypes with quality traits. However, the absence of applicable molecular techniques impedes the developments in molecular-based coconut breeding. To facilitate molecular based studies in the Palmae, Fan et al. (2013) assessed the transcriptome of coconuts from the main coconut producing countries through de novo assembly and RNA-seq technology. The data were also used in the past to develop gene-based SSR markers for coconut germplasm evaluation and associated studies (Xia et al. 2014; Xiao et al. 2013).

Thanks to progress in the next generation DNA sequencing (Hardwick et al. 2017) and other omics tools (Lieben 2017), large amounts of data can be produced efficiently, even at the scale of animal and plant breeding populations. Compared to traditional phenotypic data collected from the field or greenhouse trials, genetic marker data sets can be very large. For example, genotyping 2000 individuals for 10,000 SNP markers is feasible for many breeding programs. The data set resulting from this genotyping will contain more than 20 million data points.

In plant biotechnology significant progress has been made in the last decade in terms of technological advances (Kumar and Salar 2017; Sihag et al. 2017). Precise breeding by use of molecular markers and other genomics tools have helped breeders in the introgression of desirable genomic regions in various crop plants (Jangra et al. 2017). To improve crop productivity through functional genomics and molecular-aided breeding, it is obligatory to have access to the whole high-quality map-based sequence of various crop plants. Furthermore, the advances in other omics approaches will lead to molecular switches regulating tolerance mechanisms and other desirable traits in crop plants. This will eventually lead toward attaining sustainability in agriculture.

The recent advances made in plant genomics (Xiao et al. 2017), transcriptomics (Fan et al. 2013; Huang et al. 2014; Nejat et al. 2015; Saensuk et al. 2016), proteomics (Huang et al. 2016), and their utility in addressing the unresolved problems in agriculture, which include biotic as well as abiotic stresses, will attract a large number of readers.

17.5.1 Molecular Markers and Molecular-Aided Breeding

Biotechnology refers to the use of either whole living organisms or their functional component(s) to produce products with desirable traits in the shortest possible time. With conventional breeding, the development of new crop varieties with quality trait(s) was taking more than two decades. But nowadays, biotechnology has revolutionized the system by producing the new crop varieties having quality attributes in the shortest possible time (<10 years) with marker-assisted selection (MAB) (Fig. 17.2a, b). Besides, plant biotechnology has become the key tool for comprehensive research studies in recent times with the successful developments in one of the tools, i.e. marker-assisted breeding. MAB seems to be the benchmark in feeding the fast-growing population of the world. MAB enables the crops to overcome some of the major constraints in terms of price and environmental stress (Jiang 2013). Plant breeders had made notable advancements through breeding programs, yet it is critical that this carries on to enhance yield and other agricultural goods. However, current breeding programs seem to make a great impact, but MAB can enable the breeders to achieve their future goals quickly and sufficiently.

17.5.1.1 Molecular Markers Types

Genetic markers can be used to identify variations in gene sequences that are responsible for a trait, thus serving as tags or probes in order to locate a gene, chromosome, nucleus, cell, tissue or an individual. Numerous markers have been developed over the past few decades, which have been used as indicators in breeding and selection of desired traits.

Morphological Markers The use of morphological markers by breeders in plant selection for the transfer of desirable traits has a long history. Morphological markers include leaf shape and color, flower color, shell color, seed shape and color, pubescence color, fruit shape, stem length and size, etc. Genetic polymorphism can be easily identified and manipulated by morphological markers, however, its use is limited and cannot be associated with many important traits (including late expression, dominance, deleterious effects, pleiotropy and epistasis, which frequently reduce the usefulness of such markers). More than 300 morphological markers are reported to be available for genetic studies in rice (Khush 1987) Many morphological marker stocks are also available for maize (Neuffer et al. 1997) and soybean (Palmer and Shoemaker 1998).

Cytological Markers Cytological markers are mainly associated with the variation in chromosome number, shape, size and banding pattern. Moreover, quinacrine hydrochloride produces Q bands and Giemsa stain produces G bands, which can act as a marker to differentiate between normal chromosomes and mutated ones. Besides, they can be used in physical mapping, which in turn provide a foundation for genetic linkage mapping with the help of molecular techniques that is useful in

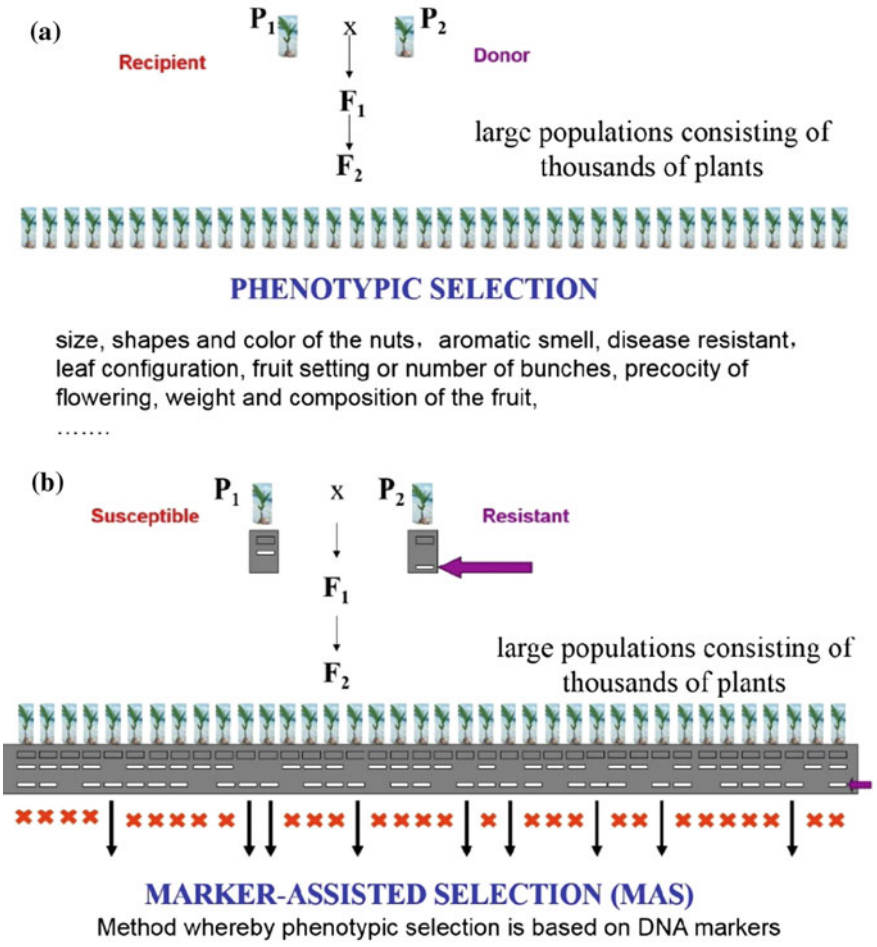

Fig. 17.2 Comparison of conventional breeding and molecular breeding. **a** Conventional breeding, **b** Marker-assisted selection

plant breeding. In coconut, leaf phenols were used as a cytochemical component marker for a coconut polymorphism study (Jay et al. 1989).

Protein Markers Isozymes are enzymes that exhibit the same catalytic activity with different charge to mass ratio (*e/m*). These isozymes can be produced by different alleles as a result of point mutation, i.e. substitution of amino acid. The limitations in isozymes analysis can be linked with the low number of available markers; therefore, it is seldom used in plant breeding. Different isozymes were used for coconut diversity and phylogenetic analysis (Geethalakshmi et al. 2005; Zizumbo-Villarreal et al. 2002) and leaf proteins were also used as markers for the same purposes (Cardeña et al. 1998).

Restriction Fragment Length Polymorphism (RFLP) RFLP is based on the hybridization of probe DNA with that of plant DNA. Although RFLP provides precise and accurate data, it yet lacks high throughput potential. RFLP has been beneficial to check the genetic diversity in coconut germplasms (Lebrun et al. 1998).

Random amplified Polymorphic DNA (RAPD) RAPD markers are based on polymerase chain reaction (PCR), which is the first among the new generation of markers that utilizes random primers to start with the intensification of random pieces of plant DNA. Although the technique is simple and does not involve in-depth knowledge of the genome to be screened, it is not popular due to inconsistent results among various populations and laboratories. RAPD has been developed and used for germplasm evaluation for coconut (Ashburner et al. 1997; Manimekalai and Nagarajan 2006; Paul et al. 2008; Upadhyay et al. 2004).

Amplified Fragment Length Polymorphism (AFLP) In AFLP, the first step is to cut the sample DNA into small pieces by the action of enzymes, followed by the amplification of fragments by PCR. Although AFLP offers intensive information concerning markers, it still lacks the ability to study high throughput marker-assisted selection. AFLP were used rigorously to study the genetic relationship that might exist between the local accessions of coconut from Sri Lanka (Perera et al. 1998; Teulat et al. 2000).

Simple sequence repeats (SSR) Microsatellites (SSRs) as a tandem DNA repeats are commonly distributed in eukaryotic genomes covering both coding and non-coding regions. These markers are usually polymorphic and codominant which is why they have importance in the construction of genetic maps, locus mapping of quantitative traits and diversity analysis (Qiu et al. 2006; Singh et al. 2013). SSR markers are, therefore, used to explore the various levels of coconut genetic diversity and genetic structure of their population (Liu et al. 2011; Meerow et al. 2003; Perera et al. 1999, 2000, 2003; Rajesh et al. 2008; Xia et al. 2014; Xiao et al. 2013).

Insertion or Deletion Markers (InDel) Insertion or deletion of bases in the genome of an organism can cover genetic variations in base pairs (measuring from 1 to 10,000 base pairs in length) of the whole genome. That is the main reason, InDels can be utilized as genetic markers in natural populations (mainly in phylogenetic studies). Such a property enables InDel to be used in species-identification procedures that undergo multiple insertion or deletion of bases in their genomes. InDel markers have been developed in rice and soybean for genetic mapping (Song et al. 2015; Yamaki et al. 2013). No study has yet been done on InDel markers for coconut.

Single Nucleotide Polymorphism (SNP) SNP is responsible for point mutations and thus causes massive differences between individuals. Intrinsically, all species consists of enormous amounts of latent SNP markers, therefore, significant amounts of sequence data are needed from the parent genotypes to develop useful SNP markers. The huge advantage of SNP markers is due to its potential screening through microarray rather than electrophoresis (Mauro et al. 2006). In studies of aromatic

coconut, one point mutation was discovered and a SNP marker was developed to discriminate the aromatic and non-aromatic phenotype of coconut (Saensuk et al. 2016; Vongvanrungruang et al. 2016).

17.5.1.2 Marker-Assisted Breeding

Marker-assisted breeding provides a better option to select the parental lines with a desirable combination of genes efficiently and with great precision and accuracy. A molecule is a genetic tag that represents a specific site in a DNA sequence and can be used in transferring genes from parent to offspring. The genetic markers can be based either on DNA or protein. Protein markers are mainly based on the divergence in the proteins that are synthesized by those genes. A plant often consists of many copies of the same proteins (known as isozymes) with small changes in size and shape, which is the basis of their separation by various techniques (chromatography, electrophoresis, etc.). In such cases, the genes that are responsible for the coding of that specific isozyme can be identified through inheritance of that particular isozyme. DNA-based markers, on the other hand, identify the differences in the sequences of the various cultivars in a breeding line within the gene or between the genes. Such differences can be regarded as a polymorphism, which can be detected by various marker-assisted tools within the chromosomes. In general, MAB encompasses numerous breeding strategies in the modern era, including marker-assisted backcrossing (MABC), selection (MAS), recurrent selection (MARS) and genome-wide selection (GWS)/genomic selection (GS) (Collard and Mackill 2008; Crouch 2000; Jiang 2013; Suslow et al. 2002).

Marker-assisted selection (MAS) and strategies MAS depends on traditional genetics and advanced molecular biology that allow the selection of genes responsible for a particular trait of interest. The main aim of the MAS is to identify the genes with significant effects and target them in the selection process. There are certain traits that can be regulated by a single gene, yet some quantitative characters are highly dependent on many genes. Out of those genes, some of them might play a minor role, while some of them can perform a major role and are known as major genes (Jiang 2013). MAS in combination with traditional selection techniques serves as a valuable tool in the selection of parents with desirable traits of interest, including yield, drought resistance, and insect and pathogen resistance. In MAS, an individual can be selected as a parent for the next generation on the basis of DNA testing and their history. The resulted information from the DNA testing along with the previous performance of the parent is used to identify the progeny with the desirable or undesirable traits at early stages of development.

Marker-Assisted Backcrossing (MABC) In traditional breeding methods, backcrossing is used to transfer alleles from the donor to an elite cultivar. Backcrossing is mainly focused on the replacement of target genes from parents to the progenies that result in varieties with a large number of quality attributes. With scientific advancements, MABC has been developed to reduce the time and labor associated with the

traditional way of backcrossing. MABC has provided scientists and breeders with the advantage of recovering the phenotypes in just few backcrosses. MABC is an attractive tool to identify genomic regions of interest that are linked with various traits of importance, including yield, quality of grains, pest resistance, etc. Being a molecular marker based technique, MABC does not involve genetic transformation and so has no ethical issues as transgenic crops (Hasan et al. 2015; Vu et al. 2012).

Marker-Assisted Recurrent Selection (MARS) Marker-assisted recurrent selection has a central role in the improvement of polygenic traits, however, sometimes the efficiency and success is not up to the expectations due to environmental factors and long genotypic selection (need of a great number of crop seasons/selection). Although MARS has some limitations, it can be suggested for forward breeding of inherent genes due to its role in assimilating multiple promising genes from various resources built on a multiple-parental population. MARS is indeed a repeated selection plan using molecular markers in order to collect and detect multiple genomic regions (Massman et al. 2013; Mayor and Bernardo 2009). Such regions are known to take part in the collection of elite genotypes within single or related populations through the expression of complex characters.

Genomic-Wide Selection or Genomic Selection Genome-wide selection (GWS) or genomic selection (GS) is associated with the simultaneous selection of hundreds or more markers, thus covering the whole genome in a compact manner (Crossa et al. 2017). In GS, the selection can be made through accurate prediction of complex traits by the gene-based markers down the whole genome. Furthermore, the selection of a parent is based on a predicted value known as genomic estimated breeding value (GEBV), calculated on the basis of genomic-wide dense DNA markers. GS is not dependent on significant testing and identification of subset markers linked with a desirable trait or in other words, avoiding the QTL mapping with populations come from specific crosses. Nevertheless, the development of GS models is obligatory to predict a relationship between phenotype and genotype through various statistical programs (Massman et al. 2013; Mayor and Bernardo 2009). The precision of the GS-based method is totally dependent on the availability of efficient and appropriate computing system, high-throughput technologies and advanced statistical methods.

17.5.2 Omics Study and Traits Evaluation

For the discovery of genes and evaluation of various traits on a larger scale a robust technology is demanded in the modern age of molecular biology. Transcriptome sequencing through Illumina RNA-Seq technology is one of the best methods to sequence the transcriptome of mixed coconut samples. The method has generated about 54,931,406 short reads that contained a total of 4,943,826,540 nucleotide bases. The produced short reads were combined through de novo assembly that resulted in 57,304 unigenes having an average length of 752 bp. The ensuing unigenes were then Blast, mapped and annotated with sequence databases. The resulting output

has provided basic sequence information for global discovery of novel functional genes in *Cocos nucifera* (Fan et al. 2013). The data set were also used for SSR marker development and coconut germplasm evaluation and association studies (Xia et al. 2014; Xiao et al. 2013). Similarly, transcriptomic analysis of leaf, endosperm and embryo tissues in a gene expression study suggested that the RNA-directed DNA methylation is crucial for coconut seed development, mainly in the maturity of endosperm (Huang et al. 2014). Furthermore, transcriptomics-based analysis of phytoplasma infection leaves led to the discovery of a principle set of genes linked with the defense system of coconut in response to phytoplasma attack. The discovery of such novel genes provided valuable information to unleash both resistance-inducing genes and/or susceptibility-inducing genes that can serve as genetic tools in breeding resistant cultivars (Nejat et al. 2015). A study of aromatic coconut by de novo transcriptomics identified the gene responsible for 2AP biosynthesis. CnAMADH2 was identified as an ortholog of the rice aromatic gene and a G-to-C substitution found in exon 14 was associated with 2AP content in the aromatic green dwarf coconut accessions. The mutants result in an unstabilized dimer conformation that could lower AMADH enzyme activity (Saensuk et al. 2016).

Although coconut proteins have high nutritional value with potential health benefits, there have been limited proteomic studies available on coconuts. Quite recently, a study was carried out to generate a proteomic map to identify common coconut proteins in different species through 2-dimensional electrophoresis (2-DE) (Huang et al. 2016). Likewise, the extensive analysis and mapping of coconut milk proteome was successfully carried out at three different pH values with combinatorial peptide ligand libraries (CPLL) (D'Amato et al. 2012).

However, whole genome study of coconut is still underway. A draft coconut genome was recently released. A total of 714.67 gigabases (Gb) of raw data was acquired by the Illumina HiSeq 2000 platform, comprising approximately 285.86 × coverage of the *Cocos nucifera* genome (variety Hainan Tall). After filtering the low quality, PCR duplication and small insert size, 419.08 gigabases (Gb) of clean data was obtained, these clean reads were assembled with SOAPdenovo2. A total scaffold length of 2.20 Gb was generated, with a scaffold N50 of 418 Kb, which represents 90.91% of the estimated genome (2.42 Gb). The BUSCO evaluation demonstrated the completeness of coconut genome reached 90.8%. The coconut genome was predicted to harbor 28,039 protein-coding genes, which is less than *Phoenix dactylifera* (DPV01, 41,660) and *Elaeis guineensis* (34,802). The annotation completeness was also evaluated by BUSCO, reached 74.1%. Genome annotation results revealed that 72.75% of the coconut genome consists of transposable elements, among which long-terminal repeat elements (LTRs) make up the largest proportion (92.23%). This study provides a large amount of genomic information and will facilitate future functional genomics and molecular breeding in *Cocos nucifera* (Xiao et al. 2017).

17.5.3 Embryo Culture and Tissue Culture

Early attempts to isolate and culture zygotic embryos from coconut fruit date back to the 1950s (Cutter and Wilson 1954). However, it was another decade before in vitro plantlets could be regenerated and converted into viable plants (De Guzman and Del Rosario 1964). Today, embryo culture has become one of the safest tools in germplasm exchange. Besides, embryo culture on the one hand fulfills the requirements of phytosanitary regulations and on the other hand it has a reduced cost of transportation. The Central Plantation Crops Research Institute (CPCRI), India has developed an optimized protocol to culture coconut embryos, which has been adopted successfully during foreign expeditions to collect coconut germplasm. The well-defined protocol involves the initial step of the infield collection of coconut embryos (9–11 months old), followed by a short-term storage and finally, in vitro recovery and field establishment (Karun et al. 2008). The germplasms of both native and foreign cultivars have been collected successfully through embryo culture. This protocol has proved to be the best to initiate plantlets from soft jelly-like endosperm of some special coconut cultivars, like Mohacha Narel and Thairu Thengai, which are hard to germinate under normal conditions.

On the contrary, coconut tissue culture has a rich history, going back to the 1970s. Initials attempts at coconut somatic embryogenesis (SE) were undertaken 40 years ago at Wye College, UK (Eeuwens and Blake 1977). These and other early studies used a number of plant somatic tissues as initial explants (i.e. young leaves, stem slices from young seedlings, sections from rachillae of young inflorescences) to form embryogenic calli (Branton and Blake 1983; Gupta et al. 1984). However, a number of issues were raised worldwide during cloning of the coconut in the laboratories. Initially, considerable efforts were made to reverse floral meristem into vegetative growth, but later on, somatic embryogenesis was well focused on coconut tissue culture. Also, the in vitro plant regeneration protocol has been improved upon in recent years, where a limited number of clonal plantlets obtained mainly from zygotic tissues have been produced with a low success rate (Chan et al. 1998; Fernando et al. 2003; Verdeil et al. 1999). Although, the regeneration efficiency of coconuts through this protocol was inadequate, a small number of vegetatively-propagated coconuts were established in the field, thus classifying coconut as one of the most recalcitrant species to regenerate in vitro (George and Sherrington 1984).

As coconut is among the recalcitrant species to regeneration in vitro, more and more laboratories in 1980s got involved in coconut tissue culture. Different explants, such as inflorescence, leaf and endosperm were used as a starting material to induce callus, but plumular tissues of coconut were considered the best in micropropagation of coconuts (Rajesh et al. 2005). Some of the reasons often cited for the slow advancement in tissue culture include the heterogeneous response of diverse coconut explanted tissues, the slow growth of these explanted tissues in vitro, and their further lack of vigor when planted ex vitro (Fernando et al. 2010). Inefficient plantlet regeneration from in vitro culture systems remains a major bottleneck for many coconut research groups around the world. Novel molecular tools might become available to

further examine the regulation of the relevant genes, which can be precisely induced during the acquisition of embryogenic competence (Nguyen et al. 2015).

17.6 Seeds and Seedling Production

The seedling vigor of the coconut palm is highly dependent on the characteristics (early flowering, nut yield and copra production) of the adult palm. Coconut is normally propagated through seeds in areas where viable vegetative propagation is not in practice. Growers can face great loss concerning the time and money, when they select poor quality seednuts. As coconut is a perennial crop, much attention is needed in the selection of plant material before establishing an orchard or small-scale garden. On the other hand, due to cross fertilization, one can expect a non-true to type breed that makes the selection process more complex. According to Chattopadhyay et al. (2004a), other factors that can play a vital role in coconut farm/garden/orchard establishment are described in the following sections (Chattopadhyay et al. 2004b).

17.6.1 Selection of Planting Material

Most of the time growers can make a mistake and select an inappropriate, immature palm nut, followed by the erroneous position of the nut during planting. Such mistakes in the crucial step of coconut farming lead to poor establishment, growth, yield, quality and ultimately economic losses.

The establishment of a successful coconut plantation starts with the selection of elite class seednuts of uniform size. High quality seednuts turn out to be very productive and have an economic life span of $\geq$60 years under extremely variable conditions with the ability to provide high production per unit area. Besides, it is useful to collect fully mature nuts (11–12 month age) that endured development during the rainy season. The shape and size of the seednuts should be proper and free of damages (undamaged embryo) during harvesting. The water content inside the seed should be determined by shaking the nuts and/or getting a metallic sound on tapping.

17.6.2 Storage of Seednuts Before Planting

Storage of seednuts is another landmark in the production of quality seedling production. It is very common to store the seednuts in an open shed till the husk becomes dry, which facilitates the germination process (Fig. 17.3a). In order to stop further drying, the seednuts should be placed on the floor of a shed beneath a thick layer (7–10 cm) of clean dry sand with the stalk end pointing upward. The stalk end should

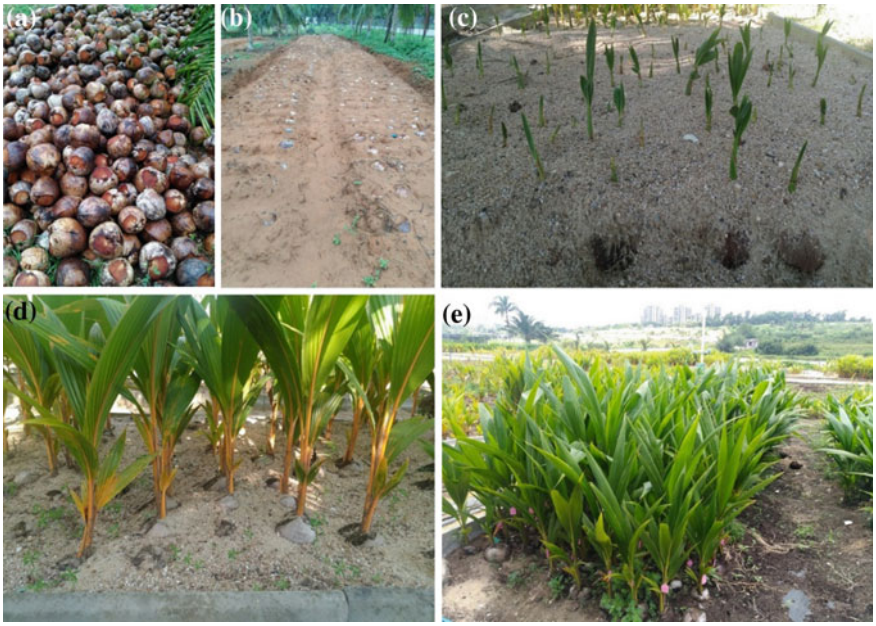

Fig. 17.3 Coconut seedlings field establishment. **a** Sorted mature seednuts, **b** Seedbed between rows of a coconut garden, **c** Seedbed with sea sand, **d** Nursery in the sand bed, **e** Nursery in coir bed

then covered with sand to avoid loss of coconut water, which is required by the nuts as an energy source during growth and germination. During summer storage, sprinkling of water over the seednuts is necessary to avert losses in coconut water.

17.6.3 Selection of Site for Nursery

A nursery site of about 3600 m^2 is required to accommodate about 12,000 seednuts. The prerequisite of a useful nursery is to be accessible, leveled with proper irrigation system and have loose-textured soil to facilitate routine nursery operations. Also, the nursery should have a clean and controlled water source to prevent flooding. The site should have a place to keep all the necessary equipment required for the maintenance of the nursery and have well-trained manpower and be easily accessible by transportation vehicles. It is necessary that the planned site should be located away from any decaying logs, sawmills, fresh animal manures, drainage systems and industrial zones to discourage insect pests and pathogens. To limit the access of humans and animals, a fence should be placed around the nursery.

17.6.4 Preparation of Seedbed

The seedbed should normally be prepared in the middle of the nursery for convenience. In order to facilitate the sowing of seednuts the sand/soil should be treated with a high-quality insecticide to avoid white grubs and termites. Besides, the soil should be clear, well plowed and harrowed to a fine tilth. The elevation of the seed bed should be 10–20 cm high to provide an efficient drainage system with a width × length of 1 × 2 m (Fig. 17.3b). Such preparation can facilitate easy inspection, management and maintenance. Also, 0.8–1.0 m pathways should be left between seedbeds to enable regular checkups, cutting, selection, maintenance activities and handling/transfer of seedlings.

17.6.5 Sowing of Seeds

Seednuts should be soaked in water for 1–2 weeks before transfer to the seedbeds to facilitate easy and early germination, as it has been observed that soaking decreased the germination time from 142.9 days to 81.1 days in Tanzania. The soaked seednuts can then be planted firmly with their germ end upright or slightly tilted at a space of 40 × 3 cm and in 20–25 cm deep trenches. To avoid termite attack, the seednuts should be covered with a thick layer of sand. For record keeping, a board should be placed in front of each seedbed, having the following information: seedbed number; name of the variety/type (for example, Hainan Tall); date and time of sowing; number of seednuts sown; number of seedbed used previously and so on.

17.6.6 Management and Maintenance of Nursery

Maintenance of the seedbed is one of the vital steps in coconut seed/seedling production. Maintenance covers daily watering except when it is raining; weeding, if necessary; partial shading when needed; and inspection for disease and pest incidence. Irrigation should be done with great care at regular intervals to avoid waterlogging or drought conditions in dry seasons (to maintain soil moisture). NPK (20:20:40 kg/ha) fertilizer should be applied in split doses to provide nourishment to the germinating seeds/growing seedlings. A proper weed control plan should be in place to eradicate weeds competing with coconut seeds for nutrients and sunlight, failure to do so can lead to severe economic losses. Likewise, coconut leaf mulch may be effective in getting early and superior germination with continuously better seedling growth and overall establishment during dry and hot phases. Regular inspection of the farm is necessary to take timely action against any threats of insect pests and pathogens. In case of scale and mealy bug attack, Dimethoate can be used according to the manufacturer's direction, whereas for mites Dicofol can be used. Furthermore, Bordeaux mixture or Indofil can be used in the area, where risk of severe fungal attack is com-

mon. The fungicide can be sprayed twice a month on both sides of the leaves and highly-infected leaves detached and burnt to avoid chances of its spread.

17.6.7 Selection of Seedlings

Germination starts in 11–12 weeks of planting and continues for 5 months or so (Fig. 17.3c). When the sprouts reach a height of 4–6 cm, seedlings that are free of pests and diseases are transferred to a field either directly in the soil or in polythene bags to allow them space to grow further (Fig. 17.3d,e). Picking of same-aged seedlings should be done on the day of transfer from the seedbed to fields or polybags and carried out on a weekly or regular intervals. After removing the germinated seedlings from seedbeds, the empty planting holes should be refilled with soil to avoid destabilization of the un-germinated seednuts. Sprouted seednuts that are thin, etiolated, spindly or albino should be discarded, whereas the un-sprouted seeds should be kept under observation for 16 weeks from the date of sowing or until a germination rate of 85% has been achieved.

17.6.8 Polybag Nursery

Polybag nurseries became popular over field nurseries due to low transplantation shock and promotion of the early establishment of the transplanted seedlings. Also, seedlings are kept in nursery for a longer time, when the conditions for planting seedlings in the field are unfavorable.

A polybag is usually black in color and restricts UV penetration, having dimensions of $45 \times 45 \times 0.015$ cm with 8–10 holes. The polybag can be filled to half with a mixture (50:50 w/w) of soil/sand and compost. The best compost use as a medium consists of decomposed sawdust, corncobs, rice hull or other organic matter. The compost has a dual function; decrease the weight of the bag and improve soil fertility. The bottom corners of the polybags should be folded inside so that the bags can stand firmly rather than falling over. Similarly, the top open edges of the bag should be folded outside to avoid tearing of the bags. The germinated sprouts are then placed upright in the center of half-filled bags and the seedlings are then fully covered with the remaining soil with great care. After filling all the polybags with seedlings they are then transferred to the polybags nursery. The layout of the polybags nursery should provide optimum uniform spacing between bags (60 cm) so that the seedlings can grow and develop normally without any extra stress.

Polybag nurseries should be well maintained and regularly checked on a priority basis for watering, weeding, and inspected for pest and disease incidence. Fertilizers should be mixed properly and applied directly to the soil around the nuts; followed by the light cultivation of the medium (to ensure faster dissolution of fertilizers and absorption by the seedlings). After about 6–8 months in the polybag, splitting of the leaves begins, which is an indication that seedlings are ready to be transferred to

Table 17.4 Recommended regime of fertilizer application for each seedling

Age after germination (months)	$(NH_4)_2SO_4$ (g)	KCl or NaCl (g)	
2	20	20	20
5	40	45	40

the field. It should be noted that simply planting good quality seednuts alone does not ensure better production. For better seedling production, one should take great care from seedbed preparation till the transfer of the seedling to the field (including adaptability of the highest yielding cultivars in local conditions, irrigation, fertilizer applications, insects, rodents and disease management) (Table. 17.4).

17.7 Conclusions and Prospects

With an increasing demand from the coconut sector, particularly for nontraditional coconut products, it is indeed crucial to identify the potential varieties that are best suited for the market. The prospective varieties, once identified and characterized, may be used in selection, replanting programs and/or for genetic improvement to generate productive coconut hybrids for the future. Replanting of coconut trees with quality planting material is highly essential for the expansion of the coconut industry.

Many coconut-producing countries have tried to improve varieties and develop hybrids for high yields; however, results were limited due to shortage of a wide range of diversity in national breeding programs (Batugal et al. 2005; Perera et al. 2009). Considering the achievements made and opportunities available, there is still room for further improvement in coconut through breeding programs, mainly focused on quantitative and qualitative traits (i.e. development of varieties with high yield coupled with value-added products, like inflorescence sap, developing stress-tolerant varieties and disease resistance to expand the coconut cultivation in non-traditional areas, development of inbred lines in coconut for production of vigorous hybrids, standardization of somatic embryogenesis protocol, application of molecular markers to aid in breeding programs, and concentrated efforts for identification of hybrid seedlings in coconut nurseries). In breeding programs, special attention should be paid to important characters other than yield, such as tolerance to disease and adverse growth conditions, slow vertical growth, resistance to high winds and other traits that brings prosperity to coconut stakeholders.

With the availability of complete genomic sequences of coconut, the opportunity for developing molecular markers will increase and will play a key role in molecular-aided breeding of coconuts. Certainly, the molecular-aided breeding will go a long way to sustain coconut production and thus management of natural resources. At present, the demands for food and non-food products from agricultural goods are increasing day by day because of soaring human population and continuous climate change. Such demands can be fulfilled through smart breeding programs to improve the genetic makeup of crop plants.

Appendix 1

A list of public and private institutes involved in research related to coconut in various countries

Country	Institution name	Address	Contact information
Bangladesh	Bangladesh Agricultural Research Institute (BARI)	GPO Box 2235, Joydepur Gazipur-1701, Bangladesh	Tel.: 880-2-9800441/9332340 Fax: 880-2-841678 Email:baridg@bttb.net.bd
Benin	Institut National Des Researches Agricoles Du Benin (INRAB)	Station de Recherché sur le Cocotier BP Cotonou, Benin	Tel.: 229-240101 Fax: 229-250266, 225-20 226985/21 248872
Brazil	Coconut Germplasm Bank EMBRAPA/CPATC	Av. Beira-Mar, 3250 CEP 49025-040 Aracaju-SE Brazil	Tel.: 55-79-2171300 Fax: 55-79-2319145 Email: tupi@cpatc.embrapa.br
China	Coconut Research Institute, Chinese Academy of Tropical Agriculture Science	Wenchang City, 571339 Hainan Province, China	Tel.: 86-898-633330684 Fax: 86-898-63330673 Email: kb0684@163.com
Cook Island	Ministry of Agriculture Government of the Cook Islands	PO Box 96 Rarotonga, Cook Islands	Tel.: 682-28711 Fax: 682-21881 Email:cimoa@oyster.net.ck
Costa Rica	Ministerio de Agricultura y Ganaderia Siquirres	Frente, Sevicentro Siquirres-Limon, Costa Rica	Tel.: 506-718 6092 Fax:506-718-7191/768-8410
Côte d'Ivoire	Station de Recherché Marc Delorme, Centre National De Recherché Agronomique (CNRA)	Port Bouet, 07 PO Box 13, Abidjan 07, Cote d'Ivoire	Tel.:225-21-248872/248067
Cuba	Ministerio de La Agricultura Instituto de Investigaciones de Citricos y Otros Frutales (IICF)	Ave. 7ma No: 3005 entre, 30 y 32, Miramar, Playa, Havana 10600, Cuba	Tel.: 537-293585/225526/246794 Fax: 537-246794/537-335217 Email: iicit@ceniai.inf.cu
Fiji	Ministry of Agriculture, Fisheries and Forests	Private Mail Bag, Raiwaqa, Suva, Fiji	Tel.: 679-477044 ext.263 Fax: 679-400262 Email: krsinfo@is.com.fj
Ghana	Oil Palm Research Institute	PO Box 74, Kade, Ghana	Tel.: 031-804-710229 (Director)/710226/710228 Fax: 031-233-46357 Email: csur@ghana.com
Guyana	National Agriculture Research Institute (NARI)	Mon Repos, East Coast Demerara, Guyana	Tel.: 592-202841/42/43 Fax: 592-204481 Email: nari@guyana.net.gu
Haiti	Ministry of Agriculture Centre de Recherché et de Documentation Agricoles (CRDA)	Damien, Republique d'Haiti	Tel.: 509 (22) 4503 Fax: 509 (45) 4034

Country	Institution name	Address	Contact information
India	Central Plantation Crops Research Institute (CPCRI), Indian Council of Agricultural Research	Kasaragod 671 124, Kerala State, India	Tel.: 91-4994-430333 Fax: 91-4994-430322 Email: cpcri@x400.nicgw.nic.in
Indonesia	Agency for Forestry and Estate Crops Research and Development, Research Institute for Coconut and Palmae	PO Box 1004, Manado 95001, Indonesia	Tel.: 62-431-812430 Fax: 62-431-812587 Email: balitka@mdo.mega.net.id
Jamaica	Coconut Industry Board	18 Waterloo Road, PO Box 204 Kingston 10, Jamaica	Tel.: 1-876-9261770 Fax: 1-876-9681360 Email: conchar@cwjamaica.com
Kenya	Regional Research Centre (RRC)	Mtwapa Box 16 Mtwapa, Kenya	Tel.: 254-11 485842/39 Fax: 254-11 486207
Kiribati	Division of Agriculture Ministry of Natural Resources and Development	PO Box 267 Bikenibeu, Tarawa, Kiribati	Tel.: 686-28-139/108 Fax: 686-28-139/21-120
Malaysia	Research Station	Jalan Air Putih PO Box 44, 24007 Kemaman Terengganu, Malaysia	Tel.: 60 09-8646361/148 Fax: 60 09-8646361 Email: abo@mardi.my
Marshall Island	Ministry of Resources and Development Agriculture Division	PO Box 1727, Majuro, Marshall Islands	Tel.: 692-625-3206/0740 Fax: 692-625-3005 Email: agridiv@ntamar.com
Mexico	Centro de Investigacion Cientifica de Yucatan, A.C. (CICY)	Apartado Postal 87, 97310 Cordemex Merida, Yucatan, Mexico	Tel.: 52-99-813923/813966 Fax: 52-99-813900/813941 Email: cos@cicy.mx
Mozambique	National Agriculture Research Institute (INIA)	Box 3658, AV. Das FPLM, INIA-Maputo, Mozambique	Tel.: 258(1)460097 Fax: 258(1)460074 Email: sancho@zebra.uem.mz
Myanmar	Department of Agriculture, Planning Ministry of Agriculture	Thiri Mingalar Lane, Off Kaba Aye Pagoda Road, Yangon, Myanmar	Tel.: 095-1-665750 Fax: 095-1-663984/651184 Email: dap.moai@mpt.maid.net.mm
Nigeria	Nigerian Institute for Oil Palm Research (NIFOR)	PMB 1030 Benin City, Nigeria	Tel.: 52-440130 Fax: 52-248549
Pakistan	Pakistan Agricultural Research Council	Plot No 20, G-5/1, Post Box 1031 45500 Islamabad, Pakistan	Tel.: 92-51-920-7402 Fax: 92-51-920-2968/920-240908 Email: hashmi@reshem.sdnpk.undp.org
Papua New Guinea	PNG Cocoa and Coconut Research Institute	PO Box 1846 Rabaul, East New Britain Province Rabaul, East New Britain, PNG	Tel.: 675 983-9108/983-9131/983-9185 Fax: 675 983-9115 Email: ccri@datec.com.pg

Country	Institution name	Address	Contact information
Philippines	Agricultural Research and Development Branch, Philippine Coconut Authority	Marcos Avenue, Diliman, Quezon City, Philippines	Tel.: 632-920-0415/632-426-1398 Fax: 632-920-0415 Email: cbcarpio@mozcom.com
Samoa	Ministry of Agriculture, Forests, Fisheries and Meteorology	PO Box 1587, Apia, Samoa	Tel.: 685 23416/20605 Fax: 685 23426/20607/23996 Email: apeters@lesamoa.net
Seychelles	Crop Development and Promotion Division, Ministry of Agriculture and Marine Resources	Grand Anse, PO Box 166, Victoria Mahe, Seychelles	Tel.: 248-378252/378312 Fax: 248-225425 Email: antmoust@seychelles.net
Solomon Islands	Dodo Creek Research Station, Ministry of Agriculture and Fisheries	PO Box G 13, Honiara, Solomon Islands	Tel.: 677-31111/31191/31037 Fax: 677-31039/21955/31037 Email: ibsram@welkam.solomon.com.sb
Sri Lanka	Coconut Research Institute	Bandirippuwa Estate, Lunuwila, Sri Lanka	Tel.: 94-31-57391/253795/55300 Fax: 94-31-57391 Email: rescri@sri.lanka.net
Tanzania	Mikocheni Agricultural Research Institute (MARI) Ministry of Agriculture and Co-operatives	PO Box 6226 Dares Salaam, Tanzania	Tel.: 255 51-700552 or 74606 Fax: 255 51-75549 or 116504 Mobile: 255-812-784031 Email: arim@africaonline.co.tz
Thailand	Department of Agriculture, Horticulture Research Institute	Chatuchak, Bangkok 10900 Thailand	Tel.: 66(2) 579-0583/579-0508/561-4666 Fax: 662-561-4667 Email: hort@doa.go.th
Tonga	Ministry of Agriculture and Forestry	Vainani Research Division, Nuku'alofa, Kingdom of Tonga	Tel.: 676-23038 Fax: 676-32132/24271/23093
Trinidad and Tobago	Ministry of Agriculture	Arima PO 52 La Florissante Garden, Dabadie, Trinidad and Tobago	Tel.: 1-809-642-8552/642-0718 Fax: 1-809-622-4246
Tuvalu	Ministry of National Resources and Environment Department of Agriculture	Private Mail Bag Vaiaku, Funafuti, Atoll, Tuvalu	Tel.: 688 20-825 or 186 Fax: 688 20-826
Vanuatu	Vanuatu Agricultural Research Centre	PO Box 231, Espiritu Santo, Vanuatu	Tel.: 678-36320/36130 Fax: 678-36355 Email: labouiss@pop.vanuatu.com.vu
Vietnam	Oil Plant Institute of Vietnam (OPI)	171-175 Ham Nghi St., District 1, Ho Chi Minh City, Vietnam	Tel.: 848-8297336/8243526 Fax: 848-8243528 Email: opi.vn@hcm.vnn.vn

Appendix 2

A list of important coconut cultivars, their important traits and cultivation location

Cultivar	Important traits	Cultivation location
Aguinaldo Tall	Good adaptability to the distinct wet and dry climatic condition; multiple ridges encircling the nut	Philippines
Andaman Giant Tall	high copra content, tolerant to drought and highly susceptible to stem bleeding	India
Andaman Ordinary Tall	Sensitive to drought; sensitive to the burrowing nematode; resistant to stem bleeding disease; high yield and high copra content	India
Antique Tall Mapatag	Husked nut shape is almost round	Philippines
Aromatic Green Dwarf	Late germinating Dwarf variety, bears fruits in 3-4 years, and with closely spaced leaf scars; young tender nuts having sweet water and meat which smells like pandan	Thailand
Ayiramkachi Tall	Susceptible to damage by rodents; susceptible to nut damage caused by the Eriophyid mite	India
Bago Oshiro Tall	Large-sized coconut; resistance to leaf spot diseases and moderate tolerance to mites *Oligonychus velascoi* Rimando	Philippines
Baguer Green Dwarf	Grow well in areas with distinct dry and wet conditions	Philippines
Ballesteros Tall	Has an enlarged base that reaches an average circumference of more than 2 m	Philippines
Bataan Tall	The crown of this variety has a spherical shape	Philippines
Baybay Tall	High copra weight per nut, thin husk; bunches with short peduncles, fruits often trapped between the petioles of the leaves, and with very robust and thick stem; resistance to leaf spot diseases; sensitive to mites *Oligonychus velascoi* Rimando	Philippines
Borneo Tall	Fruits are large and round to oval in shape; thick kernel and shell	Indonesia
Brazilian Green Dwarf	BGD is a legendary coconut variety; sweet young nuts for drinking	Brazil
Cambodia Green Dwarf	The fruits are oblong in shape and intermediate green color	Cambodia
Cambodia Tall Koh Rong	Rapid growth, and possesses a thick straight stem, long fronds and large fruits	Cambodia

Cultivar	Important traits	Cultivation location
Cameroon Kribi Tall	CKT has quite a slender stem, which begins with a discreet bole at the base; susceptible to the so-called kribi disease, a form of lethal yellowing	Cameroon
Cameroon Red Dwarf	CRD is a short-statured cultivar; susceptible to drought	Cameroon
Catigan Green Dwarf	The inflorescence has a long peduncle; large fruits and robust stature	Philippines
Chowghat Green Dwarf	Shell and kernel are thin; sensitive to drought; tolerance to root (wilt) disease; sensitive to stem bleeding; highly resistant to lethal yellowing	India
Chowghat Orange Dwarf	Thin shell and a thick kernel; sensitive to drought and water logging; susceptible to leaf spot (gray blight) and stem bleeding	India
Cochin China Tall	Tolerant to drought but slightly susceptible to nut damage caused by the eriophyid mite *Acercia guerreronis*; husked nut is round with thick kernel and strong shell	Vietnam
Comoro Moheli Tall	Widened stem, forming a broad bulb at the base	Comoro
East African Tall	Fruits are generally oval shaped with a thick husk. Nuts are oblong, thick shelled; moderately tolerant to lethal yellowing disease (LYD), sensitive to *Pseudotheraptus wayi* and mite attacks	Tanzania
Gangabondam Green Dwarf	Short inflorescence; sensitive to drought; susceptible to root wilt disease	India
Hainan Tall	Tolerant to low temperature, resistance to typhoon	China
Indian West Coast Tall	High yield under good conditions; yields good quality and quantity of coconut sap.	India
Java Tall	Fruits have a thick kernel; tolerant to drought; sensitive to root (wilt) disease; susceptible to stem bleeding; tolerant to burrowing nematodes; and sensitive to Ganoderma/Tanjavur wilt	Indonesia
Kappadam Tall	Heavy fruits with relatively low husk content; moderately tolerant to *Radopholus similis*	India
Karkar Tall	The fruits are very large with a thick strong shell and a thick layer of endosperm; susceptible to leaf spot damage caused by *Drechslera incurvata*	Papua New Guinea

Cultivar	Important traits	Cultivation location
Kinabalan Green Dwarf	Husked nut shape is either flat to almost round; grow well in areas with distinct dry and wet conditions	Philippines
Kinabuhutan Tall	Medium-sized fruit that is oblong in shape; sensitive to drought; tolerant to *Phytophthora* sp.	Indonesia
King Coconut	The fruits are small with thin husk; fruits are yellow red in color and oblong in shape; susceptible to drought; susceptible to the burrowing nematode	Sri Lanka
Laccadive Micro Tall	Large number of small and closely packed nuts; sensitive to drought, root (wilt) disease of Kerala and to stem bleeding disease; high oil content (>72%)	India
Laccadive Ordinary Tall	Fruits are oblong in shape, medium-sized, with three prominent ridges on the triangular nut; oil-rich copra; tolerant to drought but susceptible to root (wilt) disease	India
Lifou Tall	The fruits of this cultivar are big in size, greenish-yellow in color and oblong in shape; the nut inside is also oblong with a thick kernel	Lifou Island
Madang Brown Dwarf	Very susceptible to coconut foliar decay; highly susceptible to cyclones; small palm with a slender stem and no bole	Papua New Guinea
Malayan Green Dwarf	Has thin stem with no or little bole; starts to flower 2–3 years after field planting	Malaysia
Malayan Red Dwarf	The seedling sprouts, the leaf stalks, the inflorescence, and the immature fruits are not really red but some kind of bright orange; kernel is thin and gives rubbery copra; sensitive to drought and is subject to alternate bearing	Malaysia
Malayan Straight Settlement Green Tall	Kernel is thick with a solid shell; sensitive to drought; tolerant to root (wilt) diseases; sensitive to stem bleeding disease	Malaysia
Malayan Yellow Dwarf	Pale yellow is the color of the seedling sprouts, the leaf stalks, the inflorescence and the immature fruits;	Malaysia
Mamuaya Tall	Medium-sized, round-shaped fruits; thick endosperm with a thin husk	Indonesia
Mapanget Tall	Tolerant to drought and to *Phytophthora* diseases; high yield of fruit and good quality copra	Indonesia
Mapanget Tall-2	Sensitive to drought but tolerant to *Phytophthora* diseases; high ratio of endosperm and oil content	Indonesia

Cultivar	Important traits	Cultivation location
Marinsow Tall	Tolerant to drought, water-logging and low temperature and also tolerant to *Phytophthora* sp.	Indonesia
Markham Valley Tall	Susceptible to coconut root (wilt) disease and to foliar decay caused by *Mindus taffini*; MVT has very large, round and greenish-yellow fruits with nuts that have a thick endosperm	Papua New Guinea
Marshall Islands Green Dwarf	The water of the nut is particularly sweet; the shiny green fruits are elongated.	Marshall Islands
Mozambique Tall	Fruits are oblong to round-shaped, quite variable in shape and size	Mozambique
Nadora Tall	Oval-shaped fruits	India
New Caledonia Tall	NCT has a thin and slender trunk with a medium-sized bole; well adapted to dry conditions and relatively low temperatures	New Caledonia
Nigerian Tall	Nigerian Tall is highly susceptible to nut damage caused by the Eriophyid mite *Aceria guerreronis*. The quality of the tender nut water is not very good	Nigeria
Niu Kafa Tall Tonga	Very big fruit, the shell is thick and solid and shows very distinctive thick veins; the stem is very thick	Tonga
Niu Leka Dwarf	Has a bulky stem with a very marked bole for a Dwarf; high copra content	Fiji
Palu Tall	Round fruits with very low husk content; resistant to drought because of its high content of epicuticular wax and leaf oil	Indonesia
Panama Tall	The nut with a thick shell is almost round, flatter at the base and contains a thick kernel; good tender nut water; good copra yielder	Panama
Panama Tall Aguadulce	The stem, which is massive and straight, begins with a marked bole; the fruits are round to ovoid	Panama
Pandu Tall	Medium- to big-sized fruits with almost round shape; husked nuts are large and round-shaped	Indonesia
Pemba Red Dwarf Tanga	PRD bears no bole, it has a thin stem; moderately tolerant to lethal disease of coconuts	Tanzania
Philippines Lono Tall	The kernel is thick with a thin shell	Philippines
Philippines Ordinary Tall	Tolerant to drought; relatively tolerant to root (wilt) disease; moderately tolerant to the burrowing nematode; thick kernel	Philippines

Cultivar	Important traits	Cultivation location
Pilipog Green Dwarf	The pinkish color of its female flowers, root tips, and the base of shoots of newly germinated fruits; moderate tolerance to leaf spot diseases and tolerance to the attack of mites *Oligonychus velascoi* Rimando	Philippines
Pungkol Tall	Tolerant to *Phytophthora*	Indonesia
Raja Brown Dwarf Ternate	Small palm with a slender stem	Indonesia
Rangiroa Tall	RGT show susceptibility to *Helminthosporium* leaf spot	Rangiroa
Rangoon Kobbari Tall	Oil content in copra is 70%	India
Rennell Island Tall	Has a bulky stem that starts with a very big bole; leaf is quite short given its huge stem development	Solomon Islands
Rotuman Tall	Large fruits, with a good composition	Fiji
Saint Vincent Tall	The fruits are greenish yellow in color and oblong in shape; the husked nut is somewhat angular with thick kernel and thin shell; resistant to root (wilt) disease	Trinidad and Tobago
Sakhi Gopal Tall	Nut is oval in shape; kernel is thick	India
Samoan Tall Spicata	Bearing inflorescences with numerous female flowers	Samoa
Samoan Yellow Dwarf	The stem is thicker than those of the Malayan Yellow Dwarf; the stem starts producing a little bole under good conditions	Samoa
San Ramon Tall	The husk is thin while the kernel is thick; high copra and oil yields	Philippines
Santongbolang Tall	Small to medium in size with a round shape; has a stem that is rather thin for a tall coconut	Indonesia
Sea Tall	Sensitive to drought	Indonesia
Seychelles Tall	The nut is small and angular. The kernel is thick; high husk content	Seychelles
Solomon Island Tall	The fruits of the Solomon Island Tall are predominantly brown, slightly coppery and rarely green; flowering takes place 5 years after planting	Solomon Islands

Cultivar	Important traits	Cultivation location
Sri Lanka Green Dwarf	Resistant to the lethal yellowing; under good conditions, this Dwarf starts bearing 3 years after planting	Sri Lanka
Sri Lanka Tall	The husk content is slightly high; the kernel and shell are thick; tolerant to root (wilt) disease	Sri Lanka
Sri Lanka Tall Ambakelle	Fruit with more meat and less husk; ripe fruits do not have the equatorial belt	Sri Lanka
Surinam Brown Dwarf	Very short-statured palm; no bole and the crown is circular; the leaves are short with long, thin petioles; susceptible to fatal wilt/heart-rot disease	Surinam
Tacunan Green Dwarf	Has a thinner stem and fruits	Philippines
Tagnanan Tall	Good tolerance of nut fall and bud rot caused by *Phytophthora* fungi	Philippines
Tahitian Red Dwarf	Palm with red fruits, whose flowers, young fruits and root tips reveal a pink coloration of internal tissues	Tahiti
Tahitian Tall	Some fruits, of average size and excellent composition, have a surprisingly thin husk, less than 1 cm thick in some places; susceptible to *Helminthosporium* leaf spot	Tahiti
Thailand Green Dwarf	Round small fruits; young tender nuts having sweet water and meat without smell like pandan	Thailand
Thailand Tall Sawi	Fruits begin to germinate high in the palms before falling naturally to the ground	Thailand
Tiptur Tall	Performs well in the low-rainfall areas; water of the tender coconut is tasty and sweet	India
Tonga Tall	An early yielder compare to the other talls	Tonga
Vanuatu Red Dwarf	Thin stem and no bole; the leaves are yellowish; small size of the fruit	Vanuatu
Vanuatu Tall	Stem is slender but broadens at the base; bearing numerous small fruits	Vanuatu
West African Tall	The stem of WAT is rather thin for a tall cultivar and it has a thin but sometimes curved bole; solid shell and thick kernel; drought susceptible	Côte d'Ivoire
West African Tall Ouidah	Fruit production generally begins 6–7 years after field planting	Benin

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Chapter 18
Advances in Date Palm (*Phoenix dactylifera* L.) Breeding

Jameel M. Al-Khayri, Poornananda M. Naik, Shri Mohan Jain and Dennis V. Johnson

Abstract Date palm is one of the oldest cultivated plants, grown in the arid and semi-arid regions of the world. The date fruit serves as a vital worldwide component of the human diet and a staple food for millions of people. Unfortunately, various abiotic and biotic stresses along with agronomic constraints are hindering date productivity. Those date cultivars adapted to stress conditions have low fruit production. Conventional breeding, depending on crosses and backcrosses, is a time-consuming process. The applied research carried out on date palm is limited, still there is enormous potential to improve date palm breeding methods. Advanced biotechnology creates unparalleled opportunities to develop new varieties with quality fruit, increased fruit yield and resistance to pests and pathogens. It also minimizes the application of potentially-harmful fungicides and pesticides and increases crop productivity. This chapter provides current and innovative information about date palm progress in terms of distribution, production, marketing strategy, current achievements, limitations and challenges facing date palm breeding. It also focuses on recent advances in tissue culture, genetic transformation and molecular breeding to improve the productivity and quality of the date.

J. M. Al-Khayri (✉) · P. M. Naik
Department of Agricultural Biotechnology, College of Agriculture and Food Sciences, King Faisal University, P.O. Box 420, Al-Hassa 31982, Saudi Arabia
e-mail: jkhayri@kfu.edu.sa

P. M. Naik
e-mail: poornanandnaik@gmail.com

S. M. Jain
Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland
e-mail: mohan.jain@helsinki.fi

D. V. Johnson
Consultant, 3726 Middlebrook Ave, Cincinnati, OH 45208, USA
e-mail: djohn37@aol.com

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18.1 Introduction

Date palm (*Phoenix dactylifera* L.) belongs to the family Arecaceae (Palmae) and is one of mankind's oldest cultivated plants. Based on archeological evidence, Iraq is considered as the center of origin (Johnson et al. 2013). The majority of the date palm groves are distributed in the region of the Middle Eastern countries where the date is an important commercial crop with high cultural and religious value. Date fruits harbor nutritional sources such as essential minerals, antioxidants, vitamins, amino acids and phenolic compounds (Kamal-Eldin and Ghnimi 2018; Vayalil 2012). The production and utilization of date fruits vary from one country to another due to the influence of climatic conditions, cultivation practices and farm management. The date fruit has a significant international market from which any country can build a strong economy (El Hadrami et al. 2011a).

Most of the date palm growing countries practice conventional breeding methods, and they are facing problems related to germplasm identification, sex determination, agronomic traits, abiotic stress and biotic stress (Jain et al. 2011). The major date palm growing countries are located in the desert, and experience harsh environmental conditions or abiotic stresses, such as high temperature, drought and salinity (Shabani et al. 2012). These abiotic stresses affect the physiology and biochemical process of the date tree and ultimately productivity. Bayoud disease caused by *Fusarium oxysporum* f. sp. *albedinis* and red palm weevil (*Rhynchophorus ferrugineus* Oliv.) pest are the two deadliest threats to the date palm. The application of fungicides and pesticides is ineffective in controlling both maladies. To overcome these constraints, along with the conventional breeding, advanced biotechnological tools need to be applied.

Recent plant biotechnological tools such as plant tissue culture, protoplast culture, embryo rescue, in vitro mutagenesis, cryopreservation, genetic engineering, marker-assisted selection and genomics represent new means to create date fruits with desired traits. The present chapter deals with the distribution, production, marketing strategy, current achievements and challenges facing date palm breeding. It also reports on the latest applications of tissue culture, genetic engineering and molecular breeding towards the improvement of productivity and quality as well as tolerance to abiotic stresses and disease/pest resistance.

18.1.1 Botanical Overview

The scientific name *Phoenix dactylifera* is derived from the word *phoenix*, a long-lived bird from Greek mythology and *dactylos* meaning *finger* which refers to the

shape of the fruit. The date palm is dioecious in nature, where separate trees bear male and female organs. The mature date palm reaches up to 30 m height, branches are absent on the trunk and it is the tallest among the Phoenix species. The plant possesses a well-developed root system and also one terminal shoot apex responsible for linear growth. The leaves are arranged alternatively, pinnate and erect with a number of stiff leaflets. An adult date tree contains about 100–120 leaves of about 3–6 m in length and forms the terminal rosette crown. In a year a plant produces 12 leaves with a life span of each leaf of 3–7 years. The leaf is very tough and needle-like, adaptations which may be to protect the growing tip against grazing animals during its early years of development and under drastic environmental conditions. Auxiliary buds are produced from the leaf axil that may be vegetative, floral or intermediate (Bouguedoura 1991; Bouguedoura et al. 1990). During the early years of growth, auxiliary buds can form shoots, called *suckers* or *offshoots*, and produce inflorescences. The appearance of fruiting is from auxiliary buds within the terminal rosette in the form of branched clusters. Male and female flowers are found on separate palms and only female trees bear fruits. The flowers are white in color and small in size, richly branched, the spadix surrounded by a large single spathe. The calyx and petals are three-toothed and cup-shaped. (El Hadrami and Al-Khayri 2012). Flowers consist of three ovaries out of which only one develops into a fruit. Six stamens have a linear dorsifixed anther arrangement. Pollination is anemophilous and artificial pollination is recommended to achieve high productivity. A fully productive date palm tree can yield more than 100 kg of fruit annually, productivity depending on the cultivar, environmental conditions and cultivation practices. There are four stages of fruit development viz. Kimri, Khalal, Rutab and Tamar stages (the names of Arabic origin) containing average moisture contents of about 80, 60, 40 and 20%, respectively (Al-Shahib and Marshall 2002; Fayadh and Al-Showiman 1990). The date fruit is usually cylindrical, with a single elongated seed, variable in size, 2.5–7.5 cm long × 4 cm wide with a fleshy sweet mesocarp covered with a thin epicarp, yellowish to reddish brown in color (Purseglove 1972; Saker 2011). A date palm plantation has an economic life of about 50 years, subsequently yield decreases (Johnson et al. 2015).

18.1.2 Distribution and Biodiversity

Date palm is believed to be the oldest cultivated tree. The earliest records go back 7000 years in Iran, Egypt and Pakistan, while early cultivation was found around 3700 B.C. (Munier 1973) in the area between the Euphrates and the Nile rivers. From these regions date palm spread to the rest of the world mainly between 9–39° north latitude of arid and semiarid regions with suitable climatic conditions. The geographical distribution of date palm was driven by a number of factors such as zoochory, ornithochory and anthropochory. The center of origin of date palm is still debatable although available evidence suggests it originated from the region of Mesopotamia, modern Iraq (Johnson et al. 2013; Wrigley 1995). In Dalma, an Abu

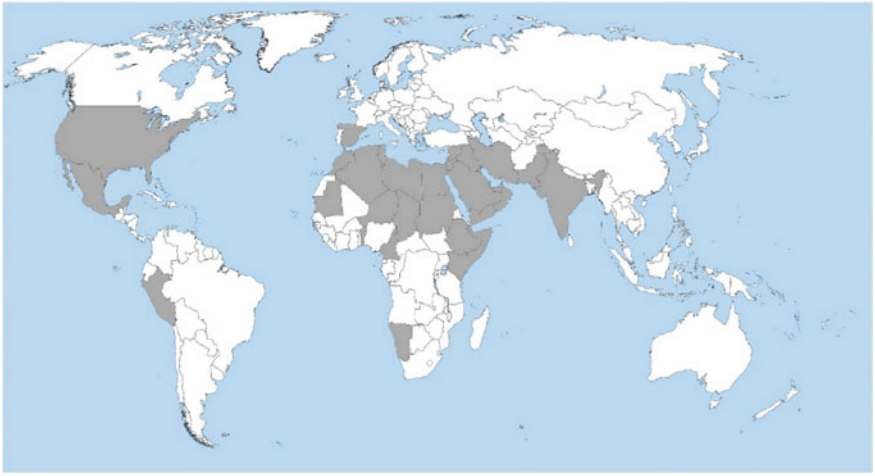

Fig. 18.1 Date palm distribution in the world. *Source of blank map* http://onlinemaps.blogspot.com/2011_11_01_archive.html

Dhabi island, the oldest ^{14}C-dated seeds were found. The two oldest seeds, dated to 5110 B.C. and 4670 B.C., were found in 1998 (El Hadrami et al. 2011a). It has been reported that ancient date seeds over 2000 years old were found in the Judean Dead Sea region during an excavation, and the seeds were tested for viability, germinated and produced a date palm similar to that of modern date seedlings (Ben-Yehoshua and Ben-Yehoshua 2012; Sallon et al. 2008).

At present, date palms are abundant in many Old World arid regions, and to a lesser extent in temperate zones. The major date production is in Middle Eastern countries such as Iraq, Iran, Saudi Arabia, United Arab Emirates, and also in African countries where the plant grows in drier regions (Fig. 18.1). There are hundreds of different date cultivars grown worldwide with different colors, tastes, texture and sizes due to varietal diversity and growth conditions. Date culture apparently spread throughout the Arabian Peninsula, North Africa and the Middle East from its center of origin. The expansion of Islam extended the cultivation of date palm and it reached to Spain and Pakistan (Chao and Krueger 2007). Based on rich historical evidence and documents it has been revealed that that the introduction of date seeds to America was from Spain. The Spanish were the first to introduce date palms outside the Arabian Peninsula and North African countries; beginning in the late nineteenth century offshoots carried them directly to the USA (Nixon 1951; Rivera et al. 2013). In the past century or so, a number of elite cultivars have been traded throughout the world for modern date palm plantations (Johnson et al. 2015).

18.1.3 World Production and Economics

The date palm is an important crop, not only because of its history as one of the oldest crops of human civilization; it is an economically-important crop grown widely. World annual production of dates amounted to 6.4 million mt in 2003 (El Hadrami and El Hadrami 2009), over 6.8 million mt in 2010 (FAOSTAT 2010) and according to the FAOSTAT (2012) the world date fruit production reached over 7.5 million mt in 2012. The production of each country is listed in Table 18.1 in descending order of production magnitude. According to this, Egypt, Saudi Arabia and Iran are the top three date producing countries. About 100 million date palms are present in the world's estimated area of 1 million ha, out of these 60% is present in North Africa and the Middle East, where the date is of cultural and religious importance to the people in these regions (Wakil et al. 2015). Date production offers employment and it is estimated around 50 million people are benefitted, out of which 35% are located in the southern Mediterranean countries (El Hadrami and El Hadrami 2009). Date fruit is not only a staple food of the local population of the many countries, it also yields significant contributions towards the economic status of those countries (Chao and Krueger 2007). The production of dates and their annual trade market value is estimated at almost 2 billion USD (El Hadrami et al. 2011a). India is the world's largest importer of dates with over 250,000 mt and an expenditure of 70 million USD per year. Iran became the leading exporter with over 240,000 mt, generating annual revenues of over 80 million USD. Medjool is the most popular and appreciated cultivar and Deglet Noor is the most exported cultivar (Al-Khayri et al. 2015a, b).

18.1.4 Nutritional and Medicinal Benefits

Since time immemorial, date fruits have comprised a vital component of the human diet and a staple food for millions of people around the world. As a part of regular diet, dates are also consumed by people as a religious practice and as nutritional therapy in different traditions around the world. Date fruit is ascribed to have a number of medicinal properties (Vayalil 2012). Dates are a high energy source in the form of carbohydrates (fructose and glucose) constituting about 70–80%. These carbohydrates are quickly assimilating by the human body (Al-Farsi et al. 2005a; Saafi et al. 2008). Dates are also a good source of essential minerals such as calcium, iron, magnesium, phosphorus, potassium, zinc, selenium and manganese which enhance their nutritional values (Al-Farsi et al. 2005a; Al-Shahib and Marshall 2002, 2003; Elleuch et al. 2008). The nutritional value of date cvs. Deglet Noor and Medjool are listed in the Table 18.2. Studies have revealed that aqueous extracts of date have a potential antioxidant and antimutagenic activity (Al-Farsi et al. 2005b, 2007; Biglari et al. 2008; Saafi et al. 2009; Vayalil 2002). The antioxidant activity is due to the presence of wide range of phenolic compounds present in the fruits, viz. gallic, protocatechuic, *p* hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic and

Table 18.1 World production of date fruit

Ranking	Country	Production (mt)
1	Egypt	1,470,000
2	Iran	1,066,000
3	Saudi Arabia	1,050,000
4	Algeria	789,357
5	Iraq	650,000
6	Pakistan	600,000
7	Sudan (former)	433,500
8	Oman	270,000
9	United Arab Emirates	250,000
10	Tunisia	190,000
11	Libya	170,000
12	Morocco	113,397
13	Yemen	55,181
14	Israel	42,866
15	Kuwait	34,600
16	USA	28,213
17	Mauritania	22,000
18	Qatar	21,843
19	Chad	20,000
20	Niger	17,000
21	Bahrain	15,000
22	Somalia	13,000
23	Jordan	10,417
24	Mexico	6012
25	Spain	4000
26	Syria	3986
27	Palestine	3600
28	Benin	1300
29	Kenya	1100
30	Cameroon	600
31	Namibia	400
32	Peru	400
33	Djibouti	86

Source FAOSTAT (2012)

o-coumaric acid as well as sinapic acids, flavonoids and procyanidins (Al-Farsi et al. 2005b; Biglari et al. 2008; El Hadrami et al. 2011b; Hong et al. 2006; Jaiti et al. 2009; Mansouri et al. 2005). Flavonoids such as luteolin, quercetin and kaempferol, as well as more or less polymerized proanthocyanidins are also found in abundant quantities in fresh dates (El Hadrami and Al-Khayri 2012). Dates also comprise a good source of vitamins, especially β-carotene (vitamin A) thiamine (B1), riboflavin (B2), niacin, ascorbic acid (C) and folic acid (folacin) (Al-Shahib and Marshall 2002; El Hadrami and El Hadrami 2009; El Hadrami et al. 2011b). Dates provide essential amino acids which cannot be synthesized by the human body. Assirey (2015) reported the presence of amino acids in date extract viz. aspartic acid, proline, glycine, histidine, valine, leucine, arginine, threonine, serine, methionine, isoleucine, tyrosine, phenylalanine, lysine and alanine. Dates are also the source of a number of secondary metabolites such as carotenoids, polyphenols especially phenolic acids isoflavons, lignans, and flavonoids, tannins and sterols Dates are also a rich source of low glycemic index sugar fructose (Al-Farsi and Lee 2008; Miller et al. 2003; Vayalil 2012).

Indian and Middle Eastern traditional medicine, Ayurveda and folklore, respectively, consider the date fruits to have a number of medicinal properties (Puri 2002). Dates are purported to act as a demulcent, aphrodisiac, nutrient, emetic, laxative, expectorant and are widely used to treat tuberculosis, gastroenteritis, coughs, respiratory diseases, asthma, diabetes, hypertension, various infectious diseases and cancer (Puri 2002; Tahraoui et al. 2007). Dates are claimed to have potential use to treat cardiovascular, cerebrovascular diseases and bacterial and fungal infections (Vayalil 2012). Mineral compounds such as potassium, calcium and phosphorus are essential for metabolic activity in the human body, for the healthy development of bones and energy metabolism. Calcium and magnesium play an important role in the production of red blood cell iron; to treat hypertension, high content of potassium and low sodium, all of the above compounds are available in date fruits (Assirey 2015). Low glycemic index diet improves the level of blood glucose level, reduces insulin demand and reduction in the level of blood lipids which manage and play important roles in the control of a number of chronic diseases (Augustin et al. 2002; Brand-Miller 2003; Jenkins et al. 2002; Ludwig 2002).

Phenolic compounds such as phenolic acids, hydroxycinnamates and flavonoids, including tannins present in dates, are known for their beneficial effects on human health as well as acting against oxidation-linked chronic or degenerative illnesses such as cancer and cardiovascular diseases. Phenolics are well-known for inhibiting pathogens and parasites. The antioxidant metabolites contained in dates seem to be linked to the role of these phytochemicals in maintaining specific cellular homeostasis and contributing in a preventive manner to beneficial effects across diverse biological systems and cell types (Vayalil 2012). Phenolics such as caffeic and ferulic acids, highly present in dates, are known to react with nitrite and inhibit the in vivo formation of nitrosamine, hence inhibiting skin tumors (Kaul and Khanduja 1998). Phenolics contained in dates may also be able to reduce blood pressure and have antithrombotic and anti-inflammatory effects (Gerritsen et al. 1995; Muldoon and Kritchvesky 1996). In addition, phenolics are known to inhibit α-amylase and α-glucosidase activities

Table 18.2 Nutritional value of dates cvs. Deglet Noor and Medjool (nutrient values and weights are for edible portion)

Nutrient	Unit	Value per 100 g	
		Deglet Noor cv.	Medjool cv.
Proximates			
Water	g	20.53	21.32
Energy	kcal	282	277
Protein	g	2.45	1.81
Total lipid (fat)	g	0.39	0.15
Carbohydrate	g	75.03	74.97
Fiber, total dietary	g	8.0	6.7
Sugars, total	g	63.35	66.47
Minerals			
Calcium, Ca	mg	39	64
Iron, Fe	mg	1.02	0.90
Magnesium, Mg	mg	43	54
Phosphorus, P	mg	62	62
Potassium, K	mg	656	696
Sodium, Na	mg	2	1
Zinc, Zn	mg	0.29	0.44
Vitamins			
Vitamin. C, ascorbic acid	mg	0.4	0.0
Thiamin	mg	0.052	0.050
Riboflavin	mg	0.066	0.060
Niacin	mg	1.274	1.610
Vitamin B_6	mg	0.165	0.249
Folate, DFE	µg	19	15
Vitamin A, RAE	µg	0	7
Vitamin A, IU	IU	10	149
Vitamin E	mg	0.05	–
Vitamin K	µg	2.7	2.7
Lipids			
Fatty acids, total saturated	g	0.032	–
Fatty acids, total monounsaturated	g	0.036	–
Fatty acids, total polyunsaturated	g	0.019	–
Fatty acids, total trans	g	0	0

Source United States Department of Agriculture (2016)

causing the postprandial increase in blood glucose level, often manifested in type-II diabetes (Andlauer and Furst 2003; McCue and Shetty 2004; Vayalil 2012).

Phytosterols are beneficial to human health and are thought to be involved in lowering the blood levels of low-density lipoprotein LDL, the so-called *bad* cholesterol. Phytosterols are also involved in blocking the absorption of dietary cholesterol into the bloodstream and in inhibiting the reabsorption of cholesterol from bile acids in the digestive process, thus reducing the amount of cholesterol returned to the bloodstream. Other health benefits may include positive effects for patients with autoimmune diseases. For instance, phytosterols were shown to reduce inflammation and suppress over-reactive immune systems in patients suffering from rheumatoid arthritis. In addition, the use of concentrated extracts of phytosterols has been shown to be effective against certain forms of cancer (i.e. prostate) and to enhance the insulin production of pancreatic cells (El Hadrami et al. 2011b).

Alpha-tocopherol (vitamin E) is often used orally to treat deficiencies, and in preventing cardiovascular diseases, diabetes and its complications, and benign prostatic hyperplasia. This vitamin is also administered against angina, thrombophlebitis, intermittent claudication, hypertension, and to prevent ischemia-reperfusion injury after coronary artery bypass surgery. Alpha-tocopherol is claimed to reduce the risks of various cancers, Alzheimer's and Parkinson's diseases and other dementias. Vitamin E is also used against allergies, asthma and other respiratory problems, as well as digestive or circulatory diseases. Additionally, vitamin E is used topically against dermatitis, aging skin, granuloma annulare and in preventing skin ulceration as a consequence of hemotherapeutic drug use (El Hadrami et al. 2011b).

18.1.5 Uses of Different Date Palm Tree Parts

Date palm is a socioeconomically and traditionally important crop; apart from the nutritious fruit, the rest of the tree parts have also been used for centuries and are a resource of the rural economy. In its entirety, the tree provides good habitat, food, shade and shelter from the extreme desert conditions. The canopy provides abundant shade and one can successfully grow vegetables, and other field crops beneath it. Other parts of the palms such as seeds, leaves, trunk and sap are used for hundreds of different purposes. In some countries, roasted and ground seed powder of the dates is used as an additive to coffee and flour (El Hadrami and Al-Khayri 2012). Soaked seeds are used as a feed for camels, horses, sheep, goats and dried powdered seeds mixed with chicken feed. The leaf and leaflets are used for making handicrafts such as beds, brooms, furniture, mats, hats, trays and baskets (Al-Khayri et al. 2015b). Thus it promotes cottage industries, and provides many employment opportunities in rural areas. The high fiber leaf material is used to make rope and twine; also, the woody midrib is used to make traditional fishing cages. The trunk or stem of the date tree is used to construct roof coverings for houses, wooden boats and timber for wood industry (Al-Khayri et al. 2015a). As well, the various tissues of leaves serve as raw material for making paper and fibers. Both trunk and leaves also serve as a

good source of fuel. Tapping of the date tree provides sap, which is a good source for palm syrup, palm sugar and *jaggery*. Tapping is practiced in many countries in North and West Africa and also in India. The sweet sap is an excellent source for making sweet and alcoholic beverages; in India date palm wine is called *neera* and is commonly consumed in rural areas.

18.2 Current Challenges

World date palm cultivation is concentrated in the Middle East, North and East African countries. Egypt, Iran, Saudi Arabia, Algeria and Iraq are the top five date-producing countries of the world. In recent years there has been a decline in the diversity of date palm groves in most of the date palm growing countries due to the extensive exploitation for both economic and social factors. The production and utilization of the date fruits also varies from country to country due to the influence of current environmental conditions and agricultural priority of the countries. There are a number of constraints to the production of date palm, which can be summarized as follows: poor cultivation practices (monoculture), major pests and diseases (red palm weevil and bayoud disease), quality of soil and water (salinity and drought), poor harvest and postharvest practices, low quality of processing facilities, scarcity in technical support and extension services, an insufficient number of agricultural laborers, poor marketing strategies on a national and international level, inadequate rapid modes of transportation, limited research funds, lack of adequate advanced research infrastructure and lack of efficiency experts (Al-Khayri et al. 2015a, b; El-Juhany 2010).

18.2.1 Cultivation

Date palm cultivation has been recorded from 7000 years ago. At Mohenjo Daro in the Indus River Valley and at the site of the temple of the Moon god in Ur of lower Mesopotamia 5000- and 4500-year-old-date seeds were collected, respectively. Cultivation practices vary from one region to another. Egyptian farmers have very long experience in irrigation methods and have a traditional irrigation system in the date fields of dividing them into 10×10 m basins. The basin supplies uniform water amounts to the date field. To improve yield and fruit quality, fertilizer application is most important; the application of chemical fertilizer is most common (Osman 2009). Farmers are showing interest in the application of organic manure to increase date palm yield and fruit color, either alone or in combination with chemical fertilizer (Kassem and Marzouk 2010). To control pests and diseases a number of steps are taken, including destruction of infected plants along with the use of chemicals and biocontrol methods.

In Iran, farmers are following traditional practices with dates cultivated along with the other crops like citrus. Growers artificially pollinate the flowers; the days of pollination depend on the elite cultivars used (Al-Khayri et al. 2015b). Flood irrigation is the primary practice in Saudi Arabia; modern irrigation methods like drip and sprinkler methods are also used by the most farmers (Fig. 18.2). The time of application of fertilizer varies among growers in Saudi Arabia. Farmers consider manure to be superior to inorganic fertilizers. For pollination, male seedling date palms are used. To control red palm weevil, both insecticide treatment by stem injection and pheromone technology in date plantations are used (Al-Khayri et al. 2015b). Currently there is a remarkable potential to increase fruit yield and quality by adopting best-practices technology in cultivation.

18.2.2 Marketing

The global production of date fruit exceeds 7.5 million mt. Dried date fruits have a unique marketing nature for they can be stored in normal environmental conditions at low cost for a long duration. Dates are considered to be the most suitable fruit to export, with the annual export in the world market of about 420,000 mt; Egypt, Iran, Saudi Arabia, Algeria and Iraq are the leading exporters (FAOSTAT 2005). In Egypt, the market strategy is not well developed as a long chain of intermediaries exist between producer and consumer. There are three kinds of markets in Egypt viz. farmers' markets, assembly markets and wholesale markets. Depending on the quality of the date, the export price in 2004 was fixed at USD 500–1500 per mt (FAOSTAT 2005). Hanyany and Siwy cvs. are the most important Egyptian exports. Iran has annual production of 1 million mt of date fruits, of which around 55–60% is consumed domestically. The exporting percentage is 12–16% of the total production and 24–33% are wasted. In the market, quality stands first; the select cultivars should possess good taste, size, color, texture, freshness, be undamaged and free of insects.

The major cultivars of Saudi Arabia are Ajwa, Khalas, Anbara, Barhee, Sheshy and Ruzeiz and the country is giving more attention to cultivation and field production to increase their export quantity (Aleid et al. 2015). Saudi Arabian dates are exported to India, France and Germany. The comparison of export percentage of the domestic production and global exports is 6.8 and 8.7%, respectively, and the average unit price of the export is USD 1065 per mt (El-Habba and Al-Mulhim 2013). Saudi Arabia has quality dates for export to the international market, thanks to the presence of advanced date palm plantations which control the quality of dates, the presence of elite cultivars which meet the international market needs, companies with advanced technologies to launch innovative products, upgraded packing systems to the international market and finally the date is consider as a holy fruit among Muslim populations worldwide (Al-Shreed et al. 2012). Some of the important technical aspects should be taken into account in exporting dates viz. types of the dates: dates with strong physical and chemical properties are suitable for export, the size and quality of the date package, specifications required by the importing countries and mode of transport.

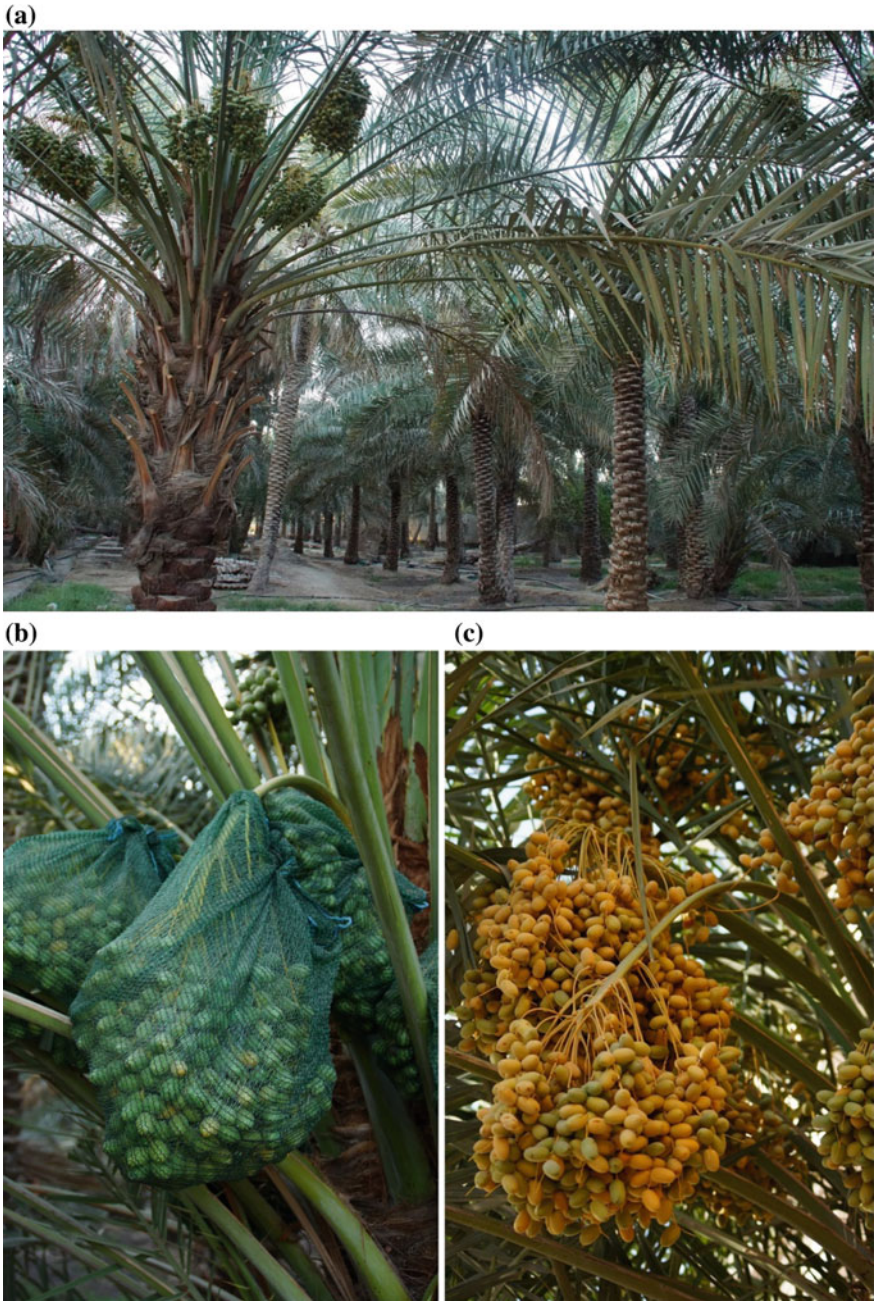

Fig. 18.2 Date palm grove. **a** Date palm plantation at Al-Hassa region of Saudi Arabia, **b** Bagged fruits at Khalal stage, **c** Rutab stage. *Photos* Courtesy of Hussain A. Alwael

18.2.3 Processing

On national as well as international markets, dates processed and properly packed have their own potential demand. Processing procedures include fumigating, sorting, washing, grading, glazing, weighing and packaging (Al-Khayri et al. 2015b). Fumigation is the primary step to disinfect the fruits. In the date industry huge losses occur due to insect attack insect and fumigation is the one method to avoid insect-caused losses. Chemicals such as phosphine and carbon dioxide are used as effective fumigants; in some cases heat treatment is also used as an alternative to date disinfection. Sorting of dates starts as soon as the harvest is over and is done manually by removing the damaged fruits and other impurities. Washing is the general procedure to remove dust, dirt and debris from the fruit. Washing with a sanitizer is important to reduce the microbial count and to avoid cross-contamination, to maintain the quality as well as shelf-life of the fruit. Generally, two types of washing systems are used in a hygienic environment, sprinklers and circular washers combined with a drier using the hot air blower system. Temperatures ranging from 55–65 °C are used to dry the date, which is considered a partial pasteurization to control the activity of enzymes and microorganisms. The final step is packing and it is a crucial process because it must meet national and international standards. Nowadays vacuum packaging technologies are applied for lengthy storage of dates using plastic coverings or polyethylene-polyamide bags (Mohsen et al. 2003). In the date industry, the processing factories produce a number of value-added products such as jelly, paste, jam and syrup. Date pastes are used in the baking industry, in pastries, pickles, chutney, candy, biscuits, cake, bread and chocolate; it is also used in the preparation of animal feed, alcohol, vinegar and citric acid production (Al-Khayri et al. 2015a, b). Date syrup serves as a sweetener in traditional foodstuffs. In addition, date extracts are used in pharmaceutical, cosmetic and medicinal products.

18.3 Conventional Breeding

Plant breeding is the branch of agriculture wherein desired characters are generated by altering the plant traits. The practice of agriculture originated 8000–10,000 years ago, when farmers simply selected the best-looking crop plant with desired characters; over generations, valuable traits were accumulated. Plant breeding focuses on improvement of present cultivated crops by developing new varieties that are higher-yielding, possess highly-valued agronomical traits, such as fruit quality, disease resistance and can tolerate extreme environmental conditions. Plant breeding success depends on the collection and selection of germplasm, evaluation of the crop for desired characters, multiplication of germplasm and release and distribution of new varieties. Date palm is one of the oldest cultivated crop and up to now most countries have followed conventional breeding methods. Date palm breeding objec-

tives mostly depend on regional problems such as abiotic and biotic stresses (Jain et al. 2011).

18.3.1 Breeding Achievements

The combination of conventional breeding and applied biotechnological tools will introduce genetic variations and produce fast-propagating new genotypes with superior fruit quality, and resistance to biotic and abiotic stresses. In date-palm breeding, one of the major objective is reduction of plant height. Use of tissue culture techniques and conventional breeding in Kuwait have produced a cross between tall female cvs. and a dwarf palm (*Phoenix pusilla*), which has set seedless fruit (Sudhersan et al. 2009). From these, embryos were rescued and cultured in vitro to regenerate a plant. The plants are acclimatized and transferred into the field and progeny selected and tested for their inheritance of dwarfism. The effective identification of the genotype is becoming possible by using biochemical and molecular markers (Al-Faifi et al. 2016; Bendiab et al. 1993; Qacif et al. 2007; Yusuf et al. 2015;). The identification of date-palm gender is not possible before the onset of fruiting, which takes 5–7 years; scientists have made a series of attempts to identify the sex of date palm using sex specific DNA markers and in some cultivars they have succeeded (Heikrujam et al. 2015; Maryam et al. 2016; Milewicz and Sawicki 2013).

Bayoud is the one of the deadliest diseases of date palm; Morocco and Algeria have experienced great losses due to this disease (Sedra and Lazrek 2011). The disease is caused by the soil fungus *Fusarium oxysporum* f. sp. *albedinis*. Studies suggest that the fungus penetrates through the roots and then enters the vascular system, causing leaf dryness and bleaching; the name bayoud means *white*. When the fungus reaches the terminal bud the plant dies (Matheron and Benbadis 1985). Various control measures have been undertaken against bayoud disease such as agriculture practices, biological and chemical applications, but use of resistant cultivars has emerged as the most promising and effective. Several breeding programs and research groups are working to identify suitable resistant cultivars to bayoud which also produce high-quality fruits, in regions of Morocco and Algeria (Fernandez et al. 1998; Louvet and Toutain 1973; Saaidi 1992). Taqerbucht cv. was found to be the most effective against bayoud in the oases of Tidikelt and Salah, Algeria (Al-Khayri et al. 2015a). Conventional breeding and application of advanced biotechnological tools will provide future solutions to the problems of date palm cultivation (Sedra 2011a).

18.3.2 Breeding Limitations

Date-palm breeding has various limitations due to environmental conditions of the desert, where the plant has to survive under extreme drought and salt stress. Date

cultivars adapted to these stresses, however, have low-quality fruit production. Conventional breeding of date palm which depends on crosses and backcrosses, is time-consuming, requiring 30 years to complete 3 backcrosses due the palm's long life-cycle. Another constraint is gender identification, which is not possible before the onset of fruiting because date palm reaches sexual maturity 5–7 years after planting. Bayoud is one of the most devastating biotic constraint initially reported in Morocco, and has spread throughout the North African countries with the destruction so far of an estimated 12 million date palms. The rise of a new disease *maladie des feuilles cassantes* or *brittle leaf disease* in Tunisia (Triki et al. 2003) where in a short time span 40,000 date trees were destroyed due to wilting; the cause of this disease remains unknown. The large number of senescent date groves and replacement of these trees through natural offshoots propagation is becoming one of the major challenges because of an insufficient supply of offshoots of good quality and at a low price (Al-Sakran and Muneer 2006). Date palm farmers are using traditional and undeveloped agricultural practices from generation to generation. There is an almost total lack of operational field stations to guide the farmers with proper cultivation methods to improve date production. A substandard level of crop and farm management, scarcity of water, lack of knowledge about advanced breeding techniques, low mechanization, traditional irrigation system, overuse of water resulting in the falling of the water table, the shortage of well-qualified and highly-trained national staff and laborers and the lack of national programs in rural areas regarding the promotion of date palm culture are some of the limitations in the date palm breeding (Al-Khayri et al. 2015a).

18.4 Tissue Culture Approaches

Tissue culture refers to the regeneration of a whole plant from cells, tissues or organs under an aseptic culture medium supplemented with macroelements, microelements as nutrients, sucrose as a source of energy and vitamins, carried out in a controlled environment at an optimal level of temperature, humidity and illumination. Tissue culture produces large numbers of homogenous plantlets in a short time, which are genetically identical and true-to-type, as well as virus, pest and disease free. With tissue culture, the plant source is not seasonal and the exchange of these plant materials is easy and safe (Jain et al. 2011). Plant tissue culture techniques such as somatic hybridization, embryo rescue, in vitro fertilization and in vitro flowering provide a solid platform for plant breeders to understand underlying genetic principles. Traditional means of date palm propagation include sexually through seeds and vegetatively by offshoots. Tisserat (1979) first reported the propagation of date palm by somatic embryogenesis from offshoots; later, several research groups and scientists worked on the standardization of date palm regeneration of different elite cultivars using different tissue culture techniques. Rapid multiplication of date palm through tissue culture techniques has greatly contributed to meeting world demand for planting material (Jain et al. 2011).

18.4.1 Advances in Micropropagation

Over the last four decades, Murashige and Skoog (1962) medium has been used as the standard culture medium to carry out tissue-culture experiments in date palm (Bhansali 2010). Date palm micropropagation has been achieved from shoot tips, zygotic embryos, lateral buds, leaves and inflorescences (Abul-Soad et al. 2008; Al-Khayri 2005, 2007; Mazri et al. 2017). Somatic embryogenesis and direct organogenesis are the two main methods of date palm micropropagation adopted by most researchers (Al-Khayri and Naik 2017; Bhansali 2010).

18.4.1.1 Somatic Embryogenesis

Somatic embryogenesis is considered to be one of the most efficient regeneration process for date palm micropropagation (Fig. 18.3). A number of research studies were carried out to standardize the protocol for somatic embryogenesis of date palm through altering the culture medium ingredients using amino acids (Abdel-Rahim et al. 1998; El-Shiaty et al. 2004; Zouine and El Hadrami 2007), auxins and cytokinins (Al-Khayri 2003; Eshraghi et al. 2005; Roshanfekrrad et al. 2017; Zouine and El Hadrami 2007), abscisic acid (Alwael et al. 2017; Sghaier et al. 2009, 2010; Zouine et al. 2005), N-phenyl N′-1,2,3-thidiazol-5-ylurea (TDZ) for direct somatic embryo (Sidky and Zaid 2011), physical status (Fki et al. 2003; Taha et al. 2001), sucrose (Sghaier et al. 2010; Taha et al. 2001), silver nitrate as an inhibitor of ethylene (Al-Khayri and Al-Bahrany 2001, 2004a, b), biotin and thiamine (Al-Khayri 2001; El-Shiaty, et al. 2004), basal salts formulation and strength (Al-Khayri 2003, 2011; Taha et al. 2001) and organic additives (Al-Khayri 2010; El-Dawayati et al. 2018; Khierallah and Hussein 2013). In most of the reports, activated charcoal is used as an agent to absorb the phenolic compounds from the embryogenic culture media (Al-Khayri 2010, 2012; El Hadrami 1995; Naik and Al-Khayri 2016). Othmani et al. (2009a, b) reported significant improvement of plant regeneration via partial desiccation of somatic embryos and showed improved differentiation of embryogenic callus via embryogenic suspension culture of elite date cv. Deglet Bey.

Callus culture (somatic embryogenesis) combined with cell suspension culture provides a new pathway for genetic improvement of date palm based on in vitro selection studies and mutagenesis (Fig. 18.4) (Abohatem et al. 2011; Al-Khayri 2012; Jain 2007; Sidky and Gadalla 2013). Recently, Naik and Al-Khayri (2016) reported the protocol procedure of induction of callus from date palm shoot-tip explants, cell-suspension culture, somatic-embryo development and plant regeneration. Fki et al. (2011) and Al-Khayri (2013) reviewed the effects of different factors with respect to date palm somatic embryogenesis. These studies help in understanding the effects of different medium components and culture conditions leading to enhanced plant regeneration. On the other hand, the standardization of callus induction and somatic embryogenesis still continues because there are a number of factors which control callus induction.

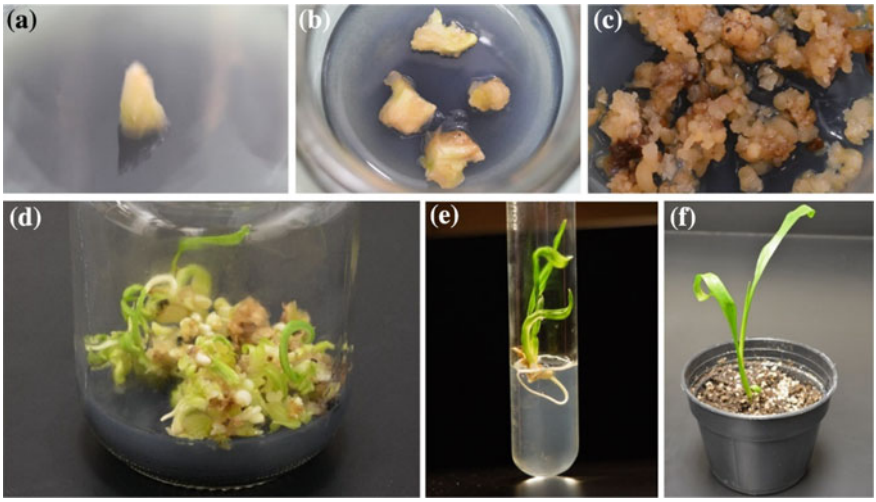

Fig. 18.3 Stages of date palm tissue culture. **a** Culture initiation, **b** Callus induction, **c** Callus proliferation, **d** Embryo formation, **e** Rooting, **f** Transplanted plant

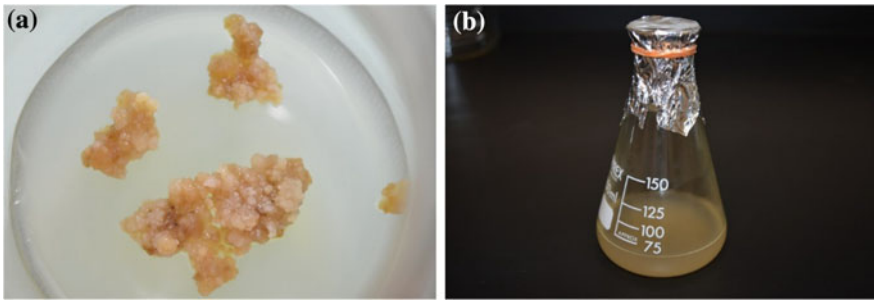

Fig. 18.4 Date palm suspension culture. **a** Actively growing callus, **b** Suspension culture

18.4.1.2 Organogenesis

In plant tissue culture, organogenesis refers to the initiation and development of organ tissue and finally leads to the regeneration of a plant, avoiding the callus stage from the cultured explants. Organogenesis maintains the consistency in the in vitro cultured plants and also minimizes the risk of genetic variation. The application of direct organogenesis is widely adopted in elite genetic material for rapid clonal propagation (Khierallah and Bader 2007). There are four steps in the organogenesis technique: vegetative buds, bud multiplication, shoot elongation and rooting. The initial step is crucial and determines the outcome of the culture. A number of researchers have performed experiments on organogenesis of date palm (Bekheet 2013; Jazinizadeh et al. 2015; Khan and Bi 2012; Khierallah and Bader 2007). Aaouine (2003) reported direct-shoot organogenesis and plant regeneration from 30 genotypes of date palm.

Direct organogenesis shoot tips of Zaghloul cv. were achieved by Bekheet (2013). Jazinizadeh et al. (2015) investigated the direct organogenesis of cv. Barhee by using different media, sucrose levels and hormone concentrations. Currently Morocco, Saudi Arabia and United Arab Emirates are employing micropropagation techniques as a tool to produce commercially-important date varieties.

18.4.1.3 Protoplasts

Protoplast consists of cytoplasm surrounded by the plasma membrane, but without a cell wall. To the present, more than 400 plant species have been regenerated through protoplast culture (Davey et al. 2005). The protoplast-culture technique in date palm is still in its juvenile stage. Initial reports of protoplast isolation and cultures were reported by Chabane et al. (2007) and Rizkalla et al. (2007). Chabane et al. (2007) reported the isolation of protoplast from young leaves of offshoots and embryogenic calli and subsequently achieved callus formation in cvs. Deglet Noor and Takerboucht. Rizkalla et al. (2007) isolated the protoplast from young leaves of offshoots in cvs. Barhee and Zaghloul and succeeded in initiating callus growth. Chabane et al. (2010), as part of extended research, succeeded in producing embryos from protoplast culture. Yatta et al. (2013) isolated the protoplast from cell suspension cultures of Algerian date palm cvs. Takerbucht and Tegaza. Recently Titouh et al. (2015) isolated the protoplast from embryogenic callus of date cvs. Deglet Noor, Akerbouch, and Degla Beida, and also induced callus growth. However, efforts are still ongoing to regenerate the plant from protoplast callus. The protoplast technique has the potential for a number of applications including somatic hybridization, introduction of desired genes through genetic transformation of date palm (disease resistance, plant height and improved fruit quality).

18.4.2 In Vitro Embryo Rescue

Embryo rescue is the one of the oldest and most successful of in vitro culture techniques, with the objective to encourage development of an immature or weak embryo into a complete plant. This technique has a number of key applications in the field of crop improvement. Embryo rescue, also referred to as embryo culture, initially began with the crucifers (Hanning 1904). Rescued embryos converted directly into seedlings resulted in the reduction of the time-span for the next generation (Lulsdorf et al. 2014). It also contributed to the introduction of novel genetic variability (Mujeeb-Kazi 2003). This technique has been successfully applied in several crops to produce interspecific hybrids such as *Helianthus annuus* × *H. argophyllus* (Sauca and Lazar 2011), *Allium cepa* × *A. roylei* (Chuda and Adamus 2012), *Triticum durum* × *Aegilops tauschii* (Trethowan 2014) and *Eruca sativa* × *Brassica campestris* (Agnihotri et al. 1990).

Before the 1970s, it was very difficult to obtain genetically superior high-yielding date palms for large-scale plantation to expand the date industry. Using in vitro culture technology solved this problem. The recent development in the date palm mass clonal propagation is based on the embryo rescue technique (Sudhersan and Al-Shayji 2011). Date palm zygotic embryo culture studies were started about four decades ago and in vitro germination of zygotic embryos plantlets was achieved (Tisserat 1979; Zaid 1987). The medium supplemented with 2,4-D and hormone-free medium where the callus regeneration followed by asexual somatic embryogenesis from zygotic embryos and finally date palm plantlet regeneration was reported by Tisserat (1979). By using the embryo rescue technique, the successful cross between the dwarf palm species *Phoenix pusilla* and cultivated selected *P. dactylifera* cultivars resulting in the reduction of date palm height (Sudhersan et al. 2009). This is the first ever report to produce a shorter date palm using embryo rescue. This opens a new avenue to improve the date palm genetically in a short time span.

18.4.3 Somaclonal Variation

Somaclonal variation is the variation observed in the phenotype and genotype of a plant regenerated from tissue culture. The variation is due to the changes in the chloroplast, mitochondrial or nuclear genomes of the regenerated plants. These variations may carry some desirable characters of agronomic and commercial interest and ultimately lead to new varieties (El Hadrami et al. 2011c). The concept of somaclonal variation became known in the early 1980s, with cell culture described as having variation (Larkin and Scowcroft 1981). In the micropropagated plants, somaclonal variations is quite common and is observed in many plant species; through the optimal in vitro culture techniques it can be controlled. On the other hand, somatic variation can be induced by manipulating the factors which control the cell cycle during micropropagation, which includes the source of the explant, age of explant, culture age, subculture number and growth supplement. Some other sources which have the capability to induce variations include mutagens are microbial or synthetic toxins, sulfonate, X-rays and gamma rays (Co-60). An efficient in vitro selection is possible through induction of mutation which creates a wide range of variation and also a broad genetic base (El Hadrami et al. 2011c). Somaclonal variation also provides opportunities such as production of synthetic seeds, mass propagation and cryopreservation systems. Date palm biotechnology and breeding are in need of somatic variation, in which they produce the somatic cells capable of introducing new genotypes with tolerance to biotic and abiotic stresses, disease resistant and improved fruit quality (Ahloowalia and Maluszynski 2001; El Hadrami and El Hadrami 2009; Jain 2001). Saker et al. (2000) reported that the frequency of somaclonal variations was age-dependent in date palm, and also analyzed the somaclonal variation in plant tissue cultured derived plants through DNA fingerprinting. Somaclonal variations are also observed in tissue cultured derived date cvs. Barhee and Khalas which exhibit

abnormal flower development and dwarfism (Al-Kaabi et al. 2007; Zaid and Al-Kaabi 2003).

18.4.4 In Vitro Mutagenesis

Induced mutation is achieved by treating plant parts or seeds with chemical or physical mutagens and then selecting for desirable changes to mimic spontaneous mutations and artificially change the genetic architecture. The application of physical and chemical mutagens for crop improvement over the past 80 years has increased crop biodiversity and productivity in different parts of the world. Mutation induction has become a proven way to create variation in a crop cultivar and induce desired traits not found in nature or those lost during evolution. The created variability is limited and may add a change that was almost found in the species, unlike transgenic variability that inserts foreign DNA material and faces a major limiting factor of silencing of the transgene (Mondal et al. 1997). Starting in the 1970s, the International Atomic Energy Agency (IAEA) and Food and Agriculture Organization (FAO) jointly sponsored research on mutation induction to enhance genetic improvement of food and industrial crops to breed new improved varieties. The number of officially released crop mutant varieties exceeds 2252 (Maluszynski et al. 2000). China (26.8%) contributed the most mutant varieties, followed by India (11.5%), USSR/Russia (9.3%), the Netherlands (7.8%), USA (5.7%) and Japan (5.3%). Tissue culture induced variations are most effective and are successfully associated with cellular level selection and easy handling of large populations for screening (Suprasanna et al. 2009). The use of chemical mutagens as compared to physical mutagens is less practical and radiation-induced mutation alone accounts for up to 90% of in vitro mutant varieties (Ahloowalia and Maluszynski 2001).

In date palm, little work has been done on mutation induction, apart from that of the FAO/IAEA Coordinated Research Project on the development of bayoud-disease resistant date palm mutant cultivars in North Africa (Jain 2005, 2006). Gamma radiation has been used to induce mutants resistant to the bayoud disease based on in vitro selection in the presence of toxin extracted for *Fusarium oxysporum* f. sp. *albedinis* culture (Sedra and Lazerk 2011). Mutation induction in date palm is feasible now due to a reliable plant regeneration system via somatic embryogenesis and organogenesis. The somatic embryogenesis system is the most preferable approach due to the single cell origin of somatic embryos, which prevents or reduces the occurrence of chimeras. Moreover, mutant somatic embryos are germinated into direct plantlets in a single step, avoiding the laborious rooting step. The irradiation of multicellular structures, e.g. seed, meristem tissue or offshoots, may result in chimeras in regenerated plants requiring significant extra work to dissociate chimeras by plant multiplication up to M1V4 generation (Jain 2007). The effect of X-ray on physiological and biochemical aspects such as DNA content, ions, photosynthetic pigments, proline content, growth and water content were estimated in date palm

seedlings by Al-Enezi and Al-Khayri (2012a, b). This information will facilitate the developing strategy for date palm genetic improvement through mutation techniques.

18.4.5 Tolerance to Abiotic Stress

Date palm growth and productivity is affected adversely by extreme environmental factors, such as high temperature, high water or soil salinity and drought (Al Busaidi and Farag 2015). Tissue culture is one of the best tools to understand the effect of these abiotic stresses on physiological aspects of in vitro cultures. Presently, further research relevant to in vitro section is necessary to harness the full benefit of this approach. Deriving reliable abiotic stress-resistant date palm is of particular importance to combat the effects of global climate changes (El Hadrami et al. 2011d; Shabani et al. 2012). Several researchers have attempted to understand the behavior of date palm in vitro culture by creating artificial stress conditions in the culture media (Abass 2016; Al-Bahrany and Al-Khayri 2012; Al-Khayri and Al-Bahrany 2004a, b; Al-Khayri and Ibraheem 2014; Al-Khayri et al. 2017; Al Mansoori and Eldeen 2007; Bekheet 2015; Jasim et al. 2010). Al-Khayri and Al-Bahrany (2004a, b) selected polyethylene glycol (PEG) as a drought agent, and examined the effect of PEG on callus culture of cvs. Barhee and Hilali; they found a reduction in the relative growth rate, callus fresh mass, water content, index of tolerance and elevation in the endogenous free proline content as the PEG concentration increased. Similar results were observed by Bekheet (2015) where the growth parameters such as survival, fresh mass and osmotic tolerance ratio decreased with increased mannitol or PEG concentration in the culture medium.

In a salinity test Al Mansoori and Eldeen (2007) found that when the callus culture was treated with NaCl it retarded growth of callus and zygotic immature embryos, and complete reduction of growth was observed at 3.0% NaCl. Jasim et al. (2010) also observed similar results where the treatment of NaCl leads to a negative pathway in the growth of date palm callus and somatic embryos. The experiment carried out by Al-Bahrany and Al-Khayri (2012) revealed that the exposure of $CaCl_2$, KCl and NaCl to the cell suspension media leads in the negative growth of callus, callus water content and Na^+/K^+ ratio. The supplementation of NaCl to the media resulted in a significant reduction on the percentages of somatic embryo germination, and also affects germination time (Al-Zubaydi et al. 2013; Ibraheem et al. 2012). Al-Mulla et al. (2013) examined salinity stress on tissue-cultured date palms maintained under greenhouse conditions. Al-Ahamad, Nabusaif, Barhee, Khalas and Kasab cvs. were treated with different salinity levels; after 1 year they found cvs. Khalas, Kasab and Barhee tolerated salinity to the level of 10 dS/m; whereas after 2 years they observed Khalas tolerated up to 20 dS/m. They also noted average heights of the palms and the number of leaves, which were inversely related to increasing salinity levels.

18.4.6 Date Palm Germplasm Conservation

The primary goal of the date palm breeder should be to construct a germplasm collection, in vitro conservation and preservation of the germplasm for the long-term without any alteration. Due to human activities and urbanization, the genetic diversity of the date palm is being reduced; other important reasons are a lack of suitable arable land, extreme climatic conditions, monoculture and diseases. To maintain date palm germplasm biodiversity and successful utilization of genetic resources requires suitable conservation methods. There are two major germplasm conservation methods: in situ and ex situ. From the experience gained so far it is concluded that germplasm diversity can be maintained by applying diverse conservation methods.

18.4.6.1 In Situ/Ex Situ Conservation

In situ conservation refers to the maintenance of genetic integrity of germplasm samples within their natural environment. Farmers play an important role in the conservation of the date-palm germplasm by cultivating different varieties in their traditional groves. Little attention has been paid to in situ conservation of wild relatives of the date palm, and it is not included in the movement of in situ conservation of wild crop relatives or in any global review of their status (Meilleur and Hodgkin 2004). Due to the loss of genetic diversity and an accelerating rate of species extinction, wild crop relatives were identified in the 1980s as a target for conservation (IPGRI 2005). The conservation of germplasm is not feasible using only the in situ method; ex situ conservation is also necessary to maintain the integrity of the genetic resources (Singh et al. 2012). Ex situ conservation is concerned with the maintenance of genetic integrity of the germplasm samples outside their natural habitat. Ex situ collections serve researchers, allowing access to disease-free germplasm. It is also necessary for characterization and evaluation of germplasm, and their utilization in breeding programs and other research activities. Although ex situ conservation of date palm germplasm has increased, it is comparably less than in other crops, due to the relatively narrow base of genetic diversity. Ex situ accessions in the gene banks of five plants (rice, barley, wheat, maize and beans) represent one third of the total collections worldwide (Lanteri and Barcaccia 2005). There are only few date palm gene banks known; the major ones are in Algeria, India, Iraq, Nigeria and the United States (Krueger 2011).

18.4.6.2 Cold Storage

Cold storage is the one of the most appropriate and cost-effective, as well as having the highest potential of in vitro culture methods of germplasm conservation, which includes date palm, vegetatively-propagated crops and other horticultural crops (Al-Khayri et al. 2015a; Bekheet et al. 2001; Jain 2010; Othmani et al. 2009c). Cold stor-

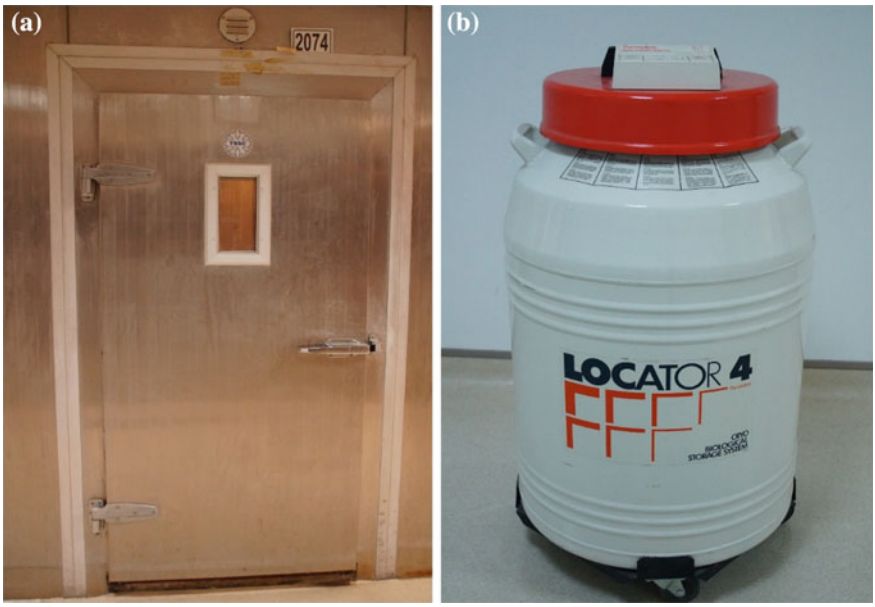

Fig. 18.5 Date palm germplasm conservation. **a** Cold storage room, **b** Cryogenic container

age temperatures are set according to the nature and origin of the stored germplasm (Fig. 18.5a). Tropical origin germplasm needs to be stored at 15–20 °C, while temperate region species require 4 °C. Due to cold storage, subculture periods in many species are reported to extend for 1–4 years (Bekheet 2011). Various studies have reported that the plants recovered from cold storage aim to minimize cell division and growth to increase longevity without any genetic alteration. (Hao et al. 2004; Renau-Morata et al. 2006). This method provides prolonged storage facility and also reduces maintenance costs by lengthening the time between transfers. The Egyptian cold storage facility of the National Gene Bank (NGB) contains more than 30,000 accessions (NGB 2007).

18.4.6.3 Cryopreservation

Cryopreservation is the process where in vitro cultured genetic materials such as shoot tips, callus, cell suspension, microspore and somatic embryos are stored long-term under ultra-low temperatures, usually at –196 °C in liquid nitrogen (Fig. 18.5b). This method is aimed at the preservation of contamination-free genetic material and to avoid somaclonal variation. A reliable plant regeneration method from the in vitro explants and a suitable large-scale disease-free plant multiplication is the basic requirement of cryopreservation of genetic material and in vitro conservation. Several research groups have standardized the date palm in vitro culture for plant regeneration and initiated the cryopreservation of date palm tissues viz. shoot tips,

callus, nodular cultures and somatic embryos (Bekheet et al. 2007). The first attempt at date palm cryopreservation was made in the late 1970s (Finkle et al. 1979; Ulrich et al. 1979) and later Tisserat et al. (1985) reported cryopreserved pollen did not alter the fruit yield of 10-year-old Deglet Noor cv. date palms. To avoid ice-crystal formation and damage to cells or tissue from the liquid nitrogen, cryoprotectant treatment is given to cells or tissue to perform their normal functions after thawing. Some of the cryoprotectants used are polyethylene glycol (PEG), glucose, sucrose, glycerol and dimethylsulfoxide (DMSO).

Date palm embryogenic calli were subjected to cryogenic treatments and stored in liquid nitrogen at −196 °C for several months. Regenerated date plantlets were recorded after the calli was revived from 4–8 weeks of latency (Tisserat 1982). Mater (1987) achieved the successful cryopreservation of date palm callus after 4 months at –25 °C by treating it with a mixture of PEG, glucose and DMSO. When the callus was thawed, it resulted in the initial slowdown of callus growth for 2 months, but did not affect the callus becoming embryogenic. MyCock et al. (1997) observed the normal growth and development when late globular and early torpedo stage date palm embryos were pre-treated with a glycerol and sucrose cryoprotectant mixture, and maintained the water content of 0.4–0.7 g/g. Kristina and Towill (1993) reported the cryopreservation protocol for handling pollen. Bekheet et al. (2002) developed the method to preserve the tissue cultures for long-term use in vitro shoot bud and callus cultures. A relatively high percent of cultures remained viable even after the 12 months treatment at 5 °C. Al-Bahrany and Al-Khayri (2012) developed the suitable cryoprotectant solution for date palm cell suspension of Khalas, a popular commercial cultivar in Saudi Arabia. The findings summarized above provide the basic knowledge to conserve the date palm through a cryopreservation gene bank.

18.5 Genetic Engineering

Advanced biotechnology provides a number of tools to ameliorate the effects of salinity, drought, extreme heat, insect attacks, diseases and productivity. These technologies can minimize the use of potentially-harmful chemicals currently used to enhance the agriculture productivity (Saker 2011). Genetic engineering, or genetic transformation, is one such tool which has the capability to solve most breeding problems. The simple meaning of transformation is change, but in plant biotechnology the definition connotes the process of introduction of a desired gene into a target plant cell, bringing about a permanent alteration in the genetic constituency of the cell and its derivatives. Some crops are hard to transform and there is great need for the application of optimal procedures to those crops (Finer 2010). Horsch et al. (1985) first reported the transformation procedure from plant cell to the whole plant. Later, this finding transformed plant science by creating genetically-modified crops. In the history of transformation several different methods have been widely employed, among them *Agrobacterium* and biolistic-mediated genetic transformation. A number of transformation protocols have been reported for both dicots and

monocots including oil palm via embryogenic and organogenic regeneration processes (Bhore and Shah 2012; Chavarri et al. 2010; Gong and Liu 2013; Kadir 2008; Mahatre 2013; Tripathi et al. 2012)

18.5.1 Current Status

The history of date palm biotechnological research and its progress can be divided into four stages. First is plant tissue culture where embryogenesis and organogenesis are the two major steps in micropropagation. The application of DNA markers and fingerprinting in date palm is the second stage where the analysis of germplasm diversity, phylogenetic relationships, disease resistance genes, genetic analysis of tissue culture-derived date palm offshoots and sex identification were carried out (Saker 2011). Stage three is genetic engineering or transformation, where only scant published data are available regarding the *Agrobacterium* or biolistic-mediated transformation of date palm (Aslam et al. 2015; Habashi et al. 2008; Mousavi et al. 2009, 2014; Saker et al. 2007, 2009). The evidence available regarding transformation is still at a rudimentary stage; there is the need of a rapid advance to promote transformation. The fourth stage is genomic analysis; many research groups have worked on the sequencing of the genome and genetic mapping and achieved success to a small extent (Al-Dous et al. 2011; Al-Mssallem et al. 2013; Hazzouri et al. 2015; Mathew et al. 2014). Research in genomic analysis of the date palm is in a rapid mode as compared to transformation studies. Habashi et al. (2008) reported DNA delivery by microprojectile bombardment to date palm embryogenic calli and somatic embryos. The study was to optimize the different parameters such as osmotic conditioning of the explants before and after bombardment, explant types and acceleration pressure, bombardment distance and gold particle size. Similarly, Mousavi et al. (2009) established an efficient genetic transformation system in embryogenic callus of date palm through particle bombardment and physical, biological and DNA parameters. A successful gene delivery in the somatic embryos of Estamaran cv. was performed by Mousavi et al. (2014) using the particle bombardment technique. Saker et al. (2009) successfully established the *Agrobacterium*-mediated transformation of the GUS gene in embryogenic callus of date palm. Recently Aslam et al. (2015) reported the efficient *Agrobacterium*-mediated genetic transformation system in cv. Khalasa, using matured somatic embryos; the protocol also involves the rapid regeneration of somatic embryos. These available data are highly valuable and will help in future improvement of date palm productivity.

18.5.2 Opportunities for Trait Improvements

Generally, date palm is grown under adverse climatic conditions and the plants experience extreme heat shock, prolonged periods of drought and high soil and/or water

salinity (Shabani et al. 2012) which adversely affect physiological and biochemical processes. High salt concentration in soil creates osmotic stress and makes it harder for roots to absorb water and nutrients from the soil, and ultimately leads to stunted growth (Munns and Tester 2008). It also affects cell growth and toxicity resulting from extreme salinity leading to cellular damage (Abass 2016). Finally, the productivity of the date is affected, so there is a need to develop new date cultivars with resistance to abiotic stresses. Bayoud is a fungal disease caused by *Fusarium oxysporum* f. sp. *albedinis* one of the deadliest diseases in date palm; this disease has made the cv. Medjool nearly extinct in North Africa (Al-Khayri et al. 2015a). The fungus is soil-borne, so chemical fungicides are ineffective to control the disease. The red palm weevil, *Rhynchophorus ferrugineus* Oliv., is one the most destructive pests attacking the date palm. Application of pesticide likewise is ineffective to manage the pests which inhabit the trunk of the tree (Saker 2011). Production of disease resistance to bayoud and red palm weevil pest resistance through conventional breeding is a time-consuming and tedious process. There is enormous scope in the transformation technology to introduce disease-resistant traits in the date plant. Date fruits face a storage problem, as the shelf-life of the date declines at normal environmental conditions, affecting the date-marketing potential internationally (Al-Khayri et al. 2015b). There is need to improve date cultivars to give them moderate shelf-life. Date fruits can be stored in freezers for up to one year, but that alternative is impractical in many date-growing areas, especially those which are remote and lack a reliable electricity supply. There is a remarkable opportunity for the improvement of date palm productivity through advanced plant biotechnological applications, and the genetic engineering is the most important one.

18.6 Molecular Breeding

Molecular breeding is the application of molecular biology and DNA-based analysis of genome polymorphism in plant-breeding programs. Molecular breeding includes quantitative trait loci (QTL) mapping, marker-assisted selection, genomics selection and genetic engineering. Currently, molecular markers are gaining in popularity as major resources in all type of crops. DNA markers are playing an important role in the identification of plant cultivars, germplasm diversity, phylogenetic relationships, disease resistance genes and other breeding activities (Elsafy et al. 2016; Mirbahar et al. 2014; Pintaud et al. 2010; Soliman et al. 2003; Trifi et al. 2000).

18.6.1 Genetic Diversity Analysis with Molecular Markers

Currently a number of molecular markers are available for cultivar identification in addition to markers to identify specific traits including resistance to bayoud disease (Sedra 2011b). The most widely used and important DNA markers are random

amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), inter simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP) and microsatellite markers containing simple sequence repeats (SSRs). Date palm has a wide range of genetic diversity, this fact opens an avenue to explore the identification of cultivars and their genetic relationships.

A number of research teams using molecular markers have made important contributions to the identification of date palm cultivars and their genetic diversity. Cao and Chao (2002) carried out a study to identify the date cultivars grown in California using AFLP analysis with near infrared fluorescence labelled primers. They collected 21 date cultivars and 4 sets of primers were used to detect polymorphism. An unweighted pair group method with arithmetic mean (UPGMA) cluster analysis showed 328 polymorphic bands, by which a majority of the cultivars were divided into two main groups. Diaz et al. (2003) identified date cultivars using the AFLP technique, also with fluorescence labelled primers. An automated DNA sequencer with the analysis fragment option was used to detect amplified fragments on capillary gel electrophoresis. A total of 310 AFLP fragments were derived from 5 primer combinations. Adawy et al. (2005) used 16 AFLP primer combinations to study the genomic diversity of 14 date palm accessions from 6 Egyptian cultivars (Bertmoda, Fraihy, Gandila, Malkaby, Sakkoty and Siwi) collected from different locations. The result showed 657 amplicons with 45.8% of polymorphism. AFLP results exhibited a high sum effective number of alleles (205.7) compared to those obtained by RAPD (45.1) and ISSR (17.8). Heterozygosity in AFLP was 0.39 when compared to 0.36 and 0.35 in RAPD and ISSR, respectively. Also, the marker index in AFLP was greater than that of RAPD and ISSR. These indicate that AFLP is more suitable in determining polymorphism than RAPD and ISSR. Haider et al. (2012) assessed the genetic polymorphism of 23 date palm cultivars in Syria (18 female and 5 male) using RAPD and ISSR markers. RAPD and ISSR markers exhibited average polymorphism of 58.5 and 50.6%, respectively.

Marsafari and Mehrabi (2013) collected 15 native cultivars of date palm from south and southwest Iran to study the molecular identification and genetic diversity by using RAPD and ISSR. Five genotypes were selected from each cultivar, 10 RAPD and 14 ISSR primers were used in the experiment. RAPD primers generated 132 amplicons with 92.4% polymorphism, on the other hand, ISSR exhibited 162 amplicons with 95.67% polymorphism. Dice coefficients were used to analyze the genetic similarity among cultivars and 94.1 and 99.4% genetic similarity was observed in RAPD and ISSR, respectively. Srivashtav et al. (2013) analyzed the genetic diversity of 8 date palm genotypes grown in the Kutch region of India. The 8 RAPD primers tested yielded 88 bands, out of which 35 were polymorphic, with an average of 2.69 polymorphic fragments per primer. From the 2 ISSR primers 13 bands were produced from which 3 were polymorphic. UPGMA cluster analysis revealed the dendrogram data obtained from the RAPD and RAPD in combination with ISSR were the same. Khierallah et al. (2014) reported the genetic characterization of 17 date cultivars (10 female and 7 male) from Iraq using 30 RAPD universal primers and 12 ISSR primers. From RAPD analysis 86 polymorphic bands were detected and 85 bands from ISSR. Combined data of both the markers yielded a total of 2530

scorable bands with an average of 72.29 fragments per primer. UPGMA analysis of the same divided the date palm cultivars into two major clusters depending of their origin and sex. Sabir et al. (2014a) explored the genetic relationship among 10 Saudi Arabian cultivars using AFLP and ISSR markers. A total of 6 AFLP and 13 ISSR primers were tested, out of which an average polymorphism of 76% was observed in AFLPs and 85% from ISSRs.

A collaborative Italian and Libyan research group completed the genetic characterization of 377 female date palm trees representing 18 Libyan cultivars having common genotypes from the Al Jufrah Oasis, using 16 highly polymorphic microsatellite markers (SSR). They scored the total number of 110 alleles with an average of 6.88 alleles per locus, which indicated the existence of a high level of polymorphism (Racchi et al. 2014). The date palms of Nigeria and neighboring countries exhibit gene recombinations due to introduced cultivars, resulting in the creation of considerable genetic variation. To identify the Nigerian germplasm and their genetic relationship they were studied by using SSR as a tool kit. A group of researcher conducted a study of the genetic diversity among 10 Nigerian and 4 Saudi Arabian cultivars using SSR markers. Of the 14 cultivars, 6 SSR markers were tested against these cultivars and high genetic diversity was observed with 83.3% polymorphism (Yusuf et al. 2015). Al-Faifi et al. (2016) collected 32 Saudi Arabian date palm cultivars from different geographical regions of the country, and screened 93 SSR markers to assess the genetic relationship and polymorphism. Results revealed 71% of genomic SSRs were dinucleotides, of 93 SSR markers a total of 91 alleles were generated from 22 primers with 4.14 alleles per locus and 100% polymorphism. Principal coordinate analysis (PCoA) displayed the cultivars into different groups according to their region of cultivation, class of maturity and fruit color. Recently Elsafy et al. (2016) determined the genetic diversity of 89 female date palms, representing 18 cultivar groups from Sudan using 10 loci of SSR primers. The SSR primers showed high polymorphism with 12.6 alleles per locus. From these reports SSR markers can be considered as superior to RAPDs, ISSRs and AFLPs because of their co-dominant nature, high polymorphism and high reproducibility.

18.6.2 Sex Determination

The date palm is dioecious, making it impossible for farmers to identify male and female trees at the seedling or early growth stage. If it were possible to identify the sex at an early stage, the farmer could cultivate large numbers of female plants with but a few superior male plants for pollination. To reach maturity a date palm requires 5–7 years (Shaheen 1990), which is the significant limiting factor of traditional genetic improvement programs. The palm's heterozygous nature and long life span make it difficult to select superior characters over existed improved cultivars. The genetics of sex determination is not yet fully understood. The initiation and development of sex organs are key incidents in plant morphogenesis. Environmental factors such as illumination, temperature, soil nutrients and as well as the application

of hormones may influence or alter the sex expression of plants (Heikrujam et al. 2015).

18.6.2.1 Morphological Markers

Morphological features of plants are the root, stem, leaf, flower, height, fruit traits like size, shape, color, taste, and other visible parts. Traditionally, morphological characters have been used to analyze the diversity of various crop species. In general, traits used for identification are affected by environmental conditions and are laborious to study and the method is applicable only at plant maturity and not at a juvenile stage (Khosla and Kumari 2015). Date palm genotype identification normally is based on fruit characters and vegetative morphology of female cultivars. Other characters to identify a date genotype include the phenotypic expression of leaves and spines. Al-Khalifah et al. (2012) characterized 14 elite date cultivars from Saudi Arabia using morphological characters. Recently Soliman et al. (2013) evaluated male date palms originating from various elite cultivars at the Research and Agriculture Experiment Station, Dirab, King Saud University, Riyadh, based on the leaf morphology characters. There are a number of reports that the various qualitative and quantitative morphological the leaf and leaflet traits selected to identify the elite cultivars are stable and did not exhibit any deviation in response to any environmental factors. One can select those morphological traits to describe and identify date cultivars at any growth stage (Ahmed et al. 2011; Hammadi et al. 2009).

18.6.2.2 Biochemical Markers

Biochemical markers exist to differentiate between male and female date palms. Peroxidase can be used as a biochemical marker, which expresses more in the reproductive organs; based on the diversity of the peroxidases in male and female inflorescences one can develop a gender-specific marker. Qacif et al. (2007) reported the presence of low peroxidase activity in male flowers and a single major isoperoxidase band with Rf 0.35 but it showed two major isoperoxidase in rachillae with Rf 0.32 and 0.35 in the female inflorescence. Furthermore, Bekheet et al. (2008) observed a higher level of peroxidase activity in adult and offshoot females than in male date palms. Similarly, glutamate oxaloacetate activity was found at a maximum in females (249 units/g fresh weight) when compared to males (190 units/g fresh weight). But in contrast, acid phosphatase activity was shown to be lower in females but observed as optimum in males. Sonia et al. (2013) extracted the leaf protein of Deglet Noor cv. and used it to identify gender. Only one distinguished spot was observed in the male gender, which indicates the protein that is specifically accumulated in the male tree. Later on the validity of the polypeptide as a gender biomarker was confirmed in other cultivars such as Aligue, Kenta, Kentichi and Khouet Aligue. These biomarkers are helpful in quick identification of date palm gender in immature trees.

18.6.2.3 Molecular Markers

Sex identification leads to physical separation of male and female gametes into two different individuals of a species. In recent years, biotechnology has emerged as a prime tool in plant breeding and can be useful in the early detection of sex and to help breeders and producers to improve the qualities of the date palm. Over the past decade, a serious effort has been made to understand the genetic basis of sex determination in date palms and to develop methods to identify sex at an early stage of development by using molecular markers. Molecular biology techniques such as RAPD, RFLP, ISSR, and microsatellite markers containing SSRs can be used to identify sex-specific DNA markers (Al-Khalifah et al. 2006; Bekheet and Hanafy 2011; Bekheet et al. 2008; Elmeer and Mattat 2012; Heikrujam et al. 2015; Maryam et al. 2016; Milewicz and Sawicki 2013; Soliman et al. 2003; Younis et al. 2008).

A series of attempts have been made to identify the genetic difference between male and female date palms. Ben-Abdallah et al. (2000) extracted genomic DNA from the leaves of Deglet Noor, Allig, Kentich, Menakher female cultivars, a male genotype pollinator T23 and an F1 hybrid. Out of 53 RAPD primers, 11 primers gave reproducible polymorphic bands. Therefore, RAPD primers can successfully be used to differentiate female and male cultivars, along with F1 hybrids (Fig. 18.6). The genetic material of 4 female date palms (Zaghloul, Amhat, Samany and Siw) and 4 unknown male Egyptian trees were tested with RAPD. The banding profile revealed that the unknown male trees are genetically closely related (87.5–98.9%) to the 4 female date palm (Soliman et al. 2003). RAPD analysis of genomic DNA and RNA extract of 4 known females and 3 unknown males of Egyptian date palm provided the rapid and effective detection of genetic relationship and similarities between the tested date palms (Ahmed et al. 2006). Al-Khalifah et al. (2006) reported the RAPD analysis of early detection of cultivars and genetic diversity among male and female palms (Barhee and Sukkary cvs.), date palm genotypes of different origin, offshoot-derived, seed-derived plants and 2 in vitro cultures of Barhee and Sukkary. The results expressed 73.6% of genetic similarity between the offshoot-derived male and female plants of Barhee, with 43.1% similarity between male and female plants of Sukkary. In seedlings, 87.2% male and female plants of Barhee are alike and 62.3% Sukkary were genetically similar. Early identification of sex in the in vitro differentiated cultures of date palm embryos were also analyzed by using RAPD markers (Bekheet et al. 2008). Younis et al. (2008) developed a gender specific ISSR marker with a different fragment size specific to male individuals. Recently Al-Ameri et al. (2016a) screened the ISSR primer as a putative sex-marker to identify the male and female date palms. Where they found two primers, primer IS A02 with a band size 390 bp are clearly visible in all female plants and are absent in all males, in contrast to another primer IS A71 with a band size 380 bp were observed in all male and was absent in all the female plants. From this evidence, these markers can be used for early detection of the sex in the date palms. Dhawan et al. (2013) screened 100 RAPD primers and developed sequence characterized amplified region (SCAR) markers specific to identify the male date palm (Fig. 18.7). Al-Ameri et al. (2016b) developed the SCAR marker and validated it independently on the male and female

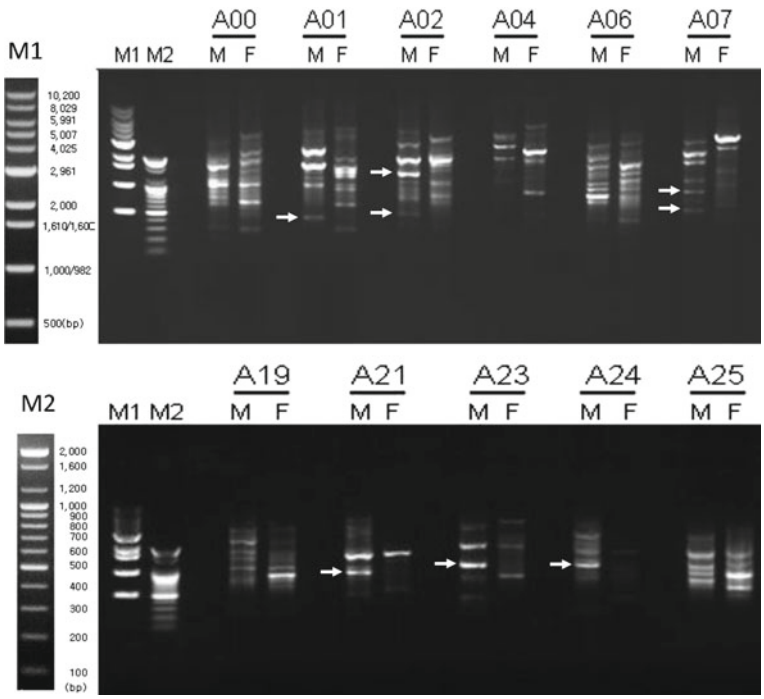

Fig. 18.6 Sex determination through RAPD analysis: M1 is 1 KB ladder, M2 is 100 bp ladder. DNA polymorphisms of (M) male and (F) female date cultivars amplified with Bex RAPD primers A00, A01, A 02, A 04, A06, A07, A19, A21, A23, A24 and A25. Arrows indicating the selected fragments for further analysis *Source* Ageez and Madboly (2011)

date trees at flowering stage. The specific and unique band was amplified in the male but was absent in the female samples.

Elmeer and Mattat (2012) used microsatellites (SSR) to determine the sex of immature date palms. Among the tested SSR markers, primer mPdCIR048 reoccurred 4 times in the 12 individual male samples but was absent in the 117 female samples, and hence a promising candidate marker to detect male sex in date palm was discovered. Maryam et al. (2016) reported the analysis of SSR markers to detect the sex in 1-year-old date palm seedlings. In the results the primer mpdCIR48, with a specific locus 250/250 bp occurred in all male samples but was absent in the female, another primer DP-168, a locus of 300/310 bp observed in 5 male date palm samples, which indicates the promising candidate markers involved in the early detection of sex in date palm seedlings.

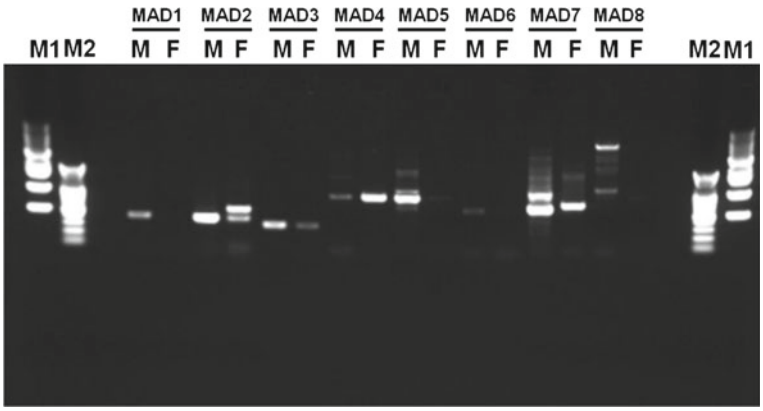

Fig. 18.7 SCAR analysis with the male-specific primers: M1 is 1 KB ladder, M2 is 100 bp ladder. DNA from ten male and ten female date plants were used. *Source* Ageez and Madboly (2011)

18.6.3 Genomics

Genomics deals with the sequencing and analysis of the genome of an organism, or is defined as the sum of total DNA content of all genes of an organism. Genomics has several applications in determining genetic diversity of plant/animal species, disease research, evolutionary processes and synthetic biology. Sequencing of the whole genome of any plant species helps in the development of new cultivars with desirable traits. Recently, the date palm genome was sequenced and its genetic map developed (Al-Dous et al. 2011; Al-Mssallem et al. 2013; Hazzouri et al. 2015; Mathew et al. 2014; Sabir et al. 2014b) but these studies are limited and there is still enormous possibility to improve date palm genomics.

To expand the knowledge about date palm genomics, a number of projects have been conducted worldwide. In Qatar, a research team from Weil Cornell Medical College, genomics laboratory sequenced the whole date palm genome using the next-generation sequencing approach, the Solexa (illumine) sequencer based on a shotgun sequencing method. The date palm genome was made available in 2009 (https://qatar-weill.cornell.edu/research-labs-and-programs/date-palm-research-program/date-palm-genome-data). The genome size is 500 Mbp. King Abdulaziz City for Science and Technology (KACST), Riyadh, Saudi Arabia in 2008 conducted the Date Palm Genome Project (DPGP) in association with Beijing Institute of Genomics, Chinese Academy of Science (BIG/CAS). The main objectives of the project included date palm genome sequencing and sequencing of chloroplast, mitochondrial and nuclear genomes (Zhang et al. 2011). From the DPGP they developed a genome diversity map for 30 cultivars using the shotgun-sequencing method. The transcriptome study resulted in the more than 30,000 unigenes, full-length cDNA and the expression profile for nearly 50 tissue samples of roots, leaves and flowers.

Yang et al. (2010) sequenced and assembled the date palm chloroplast genome using pyrosequencing technology. The size of the chloroplast genome was found to be 158,462 bp double-stranded circular DNA molecules. The mitochondrial genome of date palm, cv. Khalas, was assembled by Fang et al. (2012) using pyrosequencing and ligation-based sequencing. They found the date palm mitochondrial genome size of 715,001 bp with circular DNA molecules. Mitochondrial gene expression levels were studied in the female flower, male flower, bud and root based on the transcriptomic data and were compared to tissue of the leaf, seed and fruit. In Saudi Arabia, Al-Mssallem et al. (2013) reported the successful sequencing of the nuclear genome of date palm cv. Khalas using pyrosequencing. Based on the results the genomic size is 605.4 Mb which consists of about 90% of the genome and 96% of its genes. Furthermore, Sabir et al. (2014b) sequenced the mitochondrial and plastid genomes of nine Saudi Arabian cultivars using Illumina HiSeq 2000 platform. The published date palm mitochondrial and plastid reference genomes are compared with the sequences of each cultivar, and SNPs were identified. Out of nine cultivars, eight were identified as cultivar-specific SNPs. The first genetic map for date palm and identification of a putative sex chromosome was reported by Mathew et al. (2014). They developed the genetic map from the date palm cv. Khalas, with the draft genome. From this draft genome, for the first time, 19% of sequence scaffolds were placed onto linkage groups. The analysis results gave ~1.9 cM/Mb on the map. The comparison of linkage groups in the date palm showed remarkable long-range synteny to oil palm.

Date palms growing under adverse climatic conditions have evolved in response to stress conditions. The date palm is not a halophytic species, but it has the capacity to withstand extreme drought, heat and salinity-stress conditions. The salinity tolerance of the date palm varies from one cultivar to another (Alrasbi et al. 2010; Ramoliya and Pandey 2003). This nature of the date palm yielded a valuable genome for identifying tolerance genes. Little research information is available on the expression analysis of date palm in response to abiotic stress (Bourgis et al. 2011; Zhang et al. 2012). To understand the mechanisms underlying date palm adaptation to salinity, more knowledge about physiological and molecular studies are required. The combination of genomics and transcriptomics can fulfill the above need. Radwan et al. (2015) subjected the young roots of Deglet Beida cv. to salt-stress treatment, applying the RNA-Seq expression profiling and transmission electron microscopy (TEM) analysis. There they found that regulatory genes belong to DNA/RNA, protein, membrane and signaling functional categories play functional roles in tolerance to salt stress. It also revealed certain key genes are involved in sodium uptake and transport are down-regulated, which means slowing down the up-take mechanism and salt solute transport within plant tissues. This information can be used to improve abiotic stress in sensitive cultivars of date palm. Recently, Yaish et al. (2015) conducted experiments on date palm cv. Khalas and revealed some miRNAs in date palm expressed differential regulation under salinity, and identified the miRNAs and their targets which have the ability play a critical role in adverse environmental conditions such as extreme temperature, drought and salinity.

18.7 Conclusions and Prospects

Date palm is one of the most important tree crop species in the world, especially in the Middle Eastern countries which practice conventional breeding methods that are time-consuming and laborious. The combined application of conventional breeding and applied biotechnological tools can generate genetic variations and produce fast-propagating new genotypes with superior fruit quality, and resistance to biotic and abiotic stresses. Well-established plant tissue culture systems can be used for the rapid propagation of elite cultivars, to establish germplasm banks and for mutagenic studies in vitro to isolate useful mutants. The successful application of protoplast techniques can be utilized in somatic hybridization and introduction of desired genes through genetic transformation of date palm. Available evidence regarding genetic transformation is still at a rudimentary stage and there is need of a rapid pace to promote transformation studies. DNA markers are playing an important role in the identification of plant cultivars, germplasm diversity, phylogenetic relationships and other breeding activities. The dioecious nature of date palm makes it difficult for farmers to identify male and female palms at the seedling or early growth stage. If it is possible to identify the sex at an early stage, farmers can cultivate a large number of female plants with a small number of superior male plants for pollination. Researchers are continuing their efforts to determine the sex at the seedling stage by using sex-specific DNA markers. To understand the mechanisms underlying date palm adaptation to abiotic and biotic stresses, more knowledge about physiological and molecular studies is required. The identification and sequencing of the genomic region related to disease resistance, quality and productivity are necessary for breeding and improvement programs. The application of molecular breeding, genomics, proteomics and transcriptomic studies are needed to meet the above objectives.

Appendix 1

Research institutes concerned with date palm.

Appendix 2

Genetic resources of date palm.

This information is available in the 2-volume book Date Palm Genetic Resources and Utilization (Al-Khayri et al. 2015a, b).

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Chapter 19
Breeding of *Garcinia* spp.

Hosakatte Niranjana Murthy, Vijayalaxmi S. Dandin, Dayanand Dalawai, So-Young Park and Kee-Yoeup Paek

Abstract Garcinias are trees known for their delicious fruits and medicinal value. *Garcinia mangostana* L. (mangosteen or purple mangosteen); *G. gummi-gutta* (L.) N. Bobson. (Malabar tamarind) and *G. indica* Choisy (kokum) are the major species cultivated in tropical countries. Most *Garcinia* species are apomictic and hence, have narrow genetic diversity. However, germplasm exploration and analysis of genetic diversity using morphological and molecular markers depict variability in architecture of the tree, fruit shape, color, size, yield, nutrients, phytochemical levels, tolerance to drought and salinity, and other useful characteristics. Therefore, selection is the first criterion for the improvement of *Garcinia* species. Superior genotypes have been selected with respect to size and shape of the fruit, seed number, shelf life, fruiting precocity and external coloration, in all three *Garcinia* species from natural populations and released for cultivation. Efforts have been made to improve these crops through mutation breeding as well. Selection of rootstock and grafting has been carried out for the improvement of drought tolerance, salinity tolerance, tree architecture and early flowering. Micropropagation of mangosteen and kokum was achieved using seed and seedling explants. Recent breeding programs to improve the Garcinias are highlighted in this review.

H. N. Murthy (✉) · V. S. Dandin · D. Dalawai
Department of Botany, Karnatak University, Dharwad 580003, India
e-mail: nmurthy60@yahoo.co.in

V. S. Dandin
e-mail: vijayalaxmidandin@gmail.com

D. Dalawai
e-mail: dayananddalawai@gmail.com

S.-Y. Park · K.-Y. Paek
Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju 261-763, Republic of Korea
e-mail: soypark7@chungbuk.ac.kr

K.-Y. Paek
e-mail: cbnbio@hotmail.com

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19.1 Introduction

Garcinias are trees or shrubs belonging to the family Clusiaceae and are native to Asia, Australia, tropical and southern Africa, and Polynesia. Around 250 species are reported from lowland tropical forests (Sweeney 2008). *Garcinia* plants bear fruits which are edible and are popularly called *Garcinias* or *monkey fruit*. The fruits are small, yellow, white, green or reddish in color, with a sweet to savory in taste. Fruit juice or syrup is used as a coolant. Fruit rinds are processed as a condiment in various vegetarian and non-vegetarian preparations to impart flavor, taste and to improve keeping quality. The seeds yield a protein and a butter or oil. Garcinias contain phytochemicals which have pharmaceutical and therapeutic value (Hemshekhar et al. 2011). Linnaeus named the genus in honor of the French naturalist Laurent Garcin for his botanical contributions in the eighteenth century and it was Garcin who provided the detailed description of *Garcinia* fruits.

According to Lim (2012) *Garcinia* species originate from the Malay Archipelago; he listed some of the wild as well as semi-domesticated major fruit yielding species as follows: *G. atroviridis* Griffith ex. T. Anderson (asam gelugor); *G. cowa* Roxburg ex DC. (cowa or cowa mangosteen); *G. dulcis* (Roxb.) Kurz (egg tree); *G. forbesii* King. (red mangosteen); *G. gummi-gutta* (L.) N. Bobson. (Malabar tamarind); *G. hombroniana* Pierre (seashore mangosteen); *G. humilis* (Vahl) C. D. Adams (Bolivian mangosteen); *G. indica* Choisy (kokum); *G. intermedia* (Pitter) Hammel (lemon drop mangosteen); *G. livingstonei* T. Anderson (African mangosteen); *G. macrophylla* Mart. (big leaf garcinia); *G. madruno* (Kunth) Hammel. (Costa Rica garcinia); *G. malaccensis* Hook. f. (forest mangosteen); *G. mangostana* L. (mangosteen or purple mangosteen); *G. nervosa* Miq. (pear mangosteen); *G. nitida* Pierre. (Brunei cherry); *G. parvifolia* (Miq.) Miq. (cherry mangosteen); *G. prainiana* King. (button mangosteen); *G. schomburgkiana* Pierre (ma dan); *G. spicata* (Wight & Arn.) Hook. f. (gamboge tree); and *G. xanthochymus* Hook. f. ex T. Anderson (Mysore gamboge or yellow mangosteen). Among these, *G. mangostana*; *G. gummi-gutta* and *G. indica* are prominent cultivated species in various countries (Lim 2012).

19.1.1 Distribution and Importance

19.1.1.1 *Garcinia mangostana*

Garcinia mangostana L. (mangosteen or purple mangosteen) is naturally distributed in Southeast Asia specially Indonesia and Malaysia and it has been cultivated in other tropical regions such as Australia, Cuba, Dominica, Ecuador, Gabon, Ghana,

Guatemala, Honduras, India, Jamaica, Liberia, Myanmar, The Philippines, Puerto Rico, Singapore, Sri Lanka, Thailand, Trinidad and Tobago, USA, Vietnam and Zanzibar (Lim 2012; Orwa et al. 2009; Fig. 19.1a). Mangosteen is native to the Malay Archipelago and it has been suggested that it originated through hybridization between *G. malaciensis* and *G. hombroniana* (Richards 1990b). Mangosteen fruits are soft, juicy with round, globular, red, dark red and purplish color, sweet flavor and pleasant aroma. Mangosteen fruits are popularly referred to as the *Queen of fruits* (Jung et al. 2006; Fig. 19.2a). The rind or pericarp of the fruit is pigmented with abundant anthocyanins (Fig. 19.2b), and there are 4–8 wedge shaped seeds covered with arils that are white, juicy, sweet and slightly acidic, with a pleasant flavor (Fu et al. 2007; Ji et al. 2007; Fig. 19.2c). Seeds are eaten roasted.

19.1.1.2 *Garcinia gummi-gutta*

Garcinia gummi-gutta (L.) Roxb. [Syn. *G. cambogia* (Gaertn.) Desr.] is native to Southern India and is found distributed in Sri Lanka and Nepal. It is popularly used as a culinary agent (in place of tamarind) in food preparation. In India, it is found in the Western Ghats as a semi-domesticated tree, which grows at an elevation range of 400–900 m (Lim 2012). This plant is introduced to other tropical and subtropical regions of Asia including China, Malaysia and the Philippines (Orwa et al. 2009, Semwal et al. 2015; Fig. 19.1b). Fruits are small, edible, and acidic and are about 5 cm in diameter with 6–8 grooves. They are green in color before ripening and turn yellow to yellow or red after ripening (Fig. 19.2d). Seeds are kidney-shaped with whitish to yellow arils (Fig. 19.2e, f). Sun-dried fruit rind is used as the condiment for flavoring the curries in place of lime or tamarind in Malabar regions of the Western Ghats in India. Therefore, this plant is popularly called *Malabar tamarind*. The fruit is commonly used as a preservative and flavoring agent. The dried fruit rind of Malabar tamarind is rich in α, β-dihydroxy tricarboxylic acid or hydroxy citric acid (HCA) responsible for its anti-obesity property (Jena et al. 2002). Various commercial anti-obesity products made of Malabar tamarind fruits are available in the market.

19.1.1.3 *Garcinia indica*

Garcinia indica Choisy (kokum) is a fruit-yielding species naturally found in the peninsular coastal regions i.e., Western Ghats, Eastern Ghats and North-eastern regions of India (Baliga et al. 2011; Fig. 19.1c). Recently, it has been cultivated in different parts of peninsular India. The tender fruits are oval, round or oblong with pointed tips, light green to dark in color and turn yellow to purple when fully ripe (Fig. 19.2g). The fruits are small and weigh around 21–85 g. The fruit has a red to purple rind which is juicy, sour and 4–8 seeds covered by white to yellow arils (Fig. 19.2h, i). The fruit juice is used as a coolant. Kokum rind is dried and used as a condiment and flavoring agent in food preparations. The seed amount is a quarter

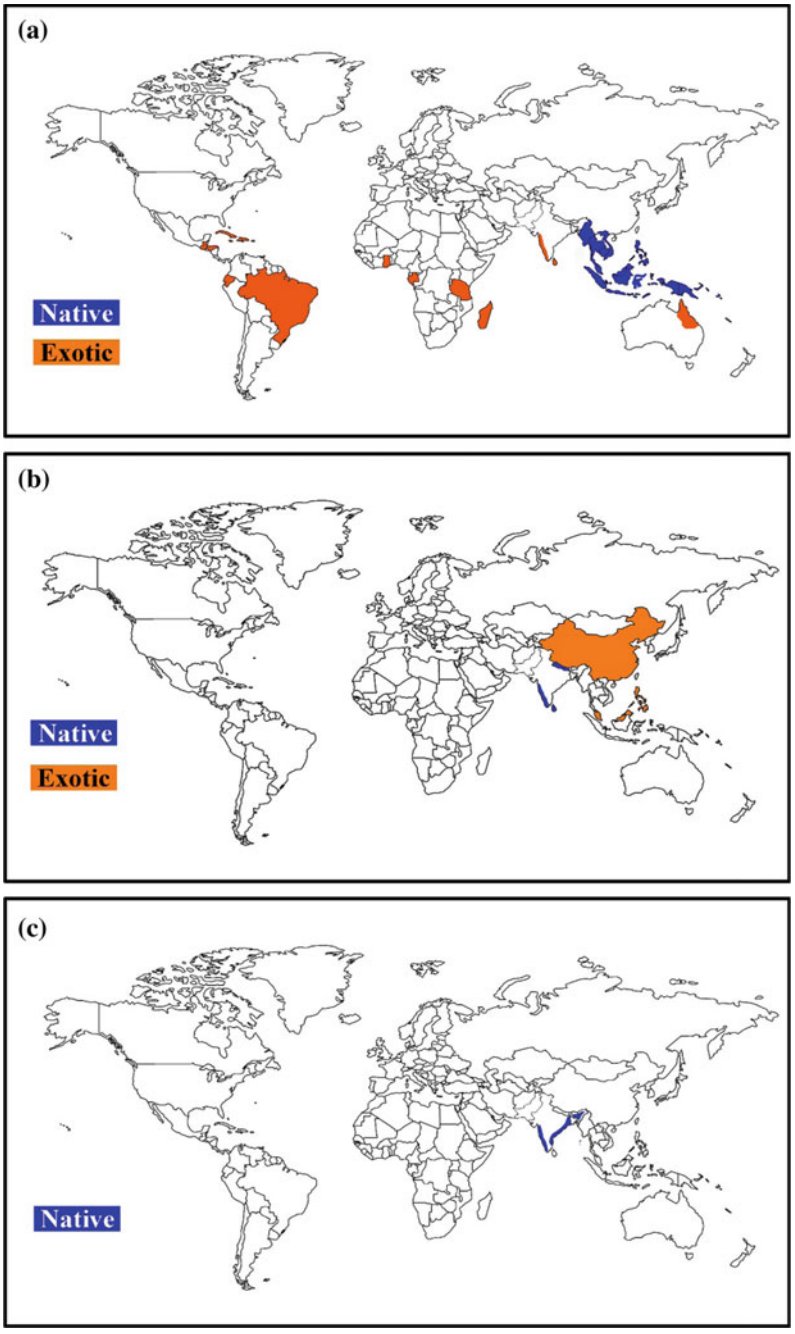

Fig. 19.1 World distribution of *Garcinia* species (native and exotic). **a** *G. mangostana*. **b** *G. gummi-gutta*. **c** *G. indica*

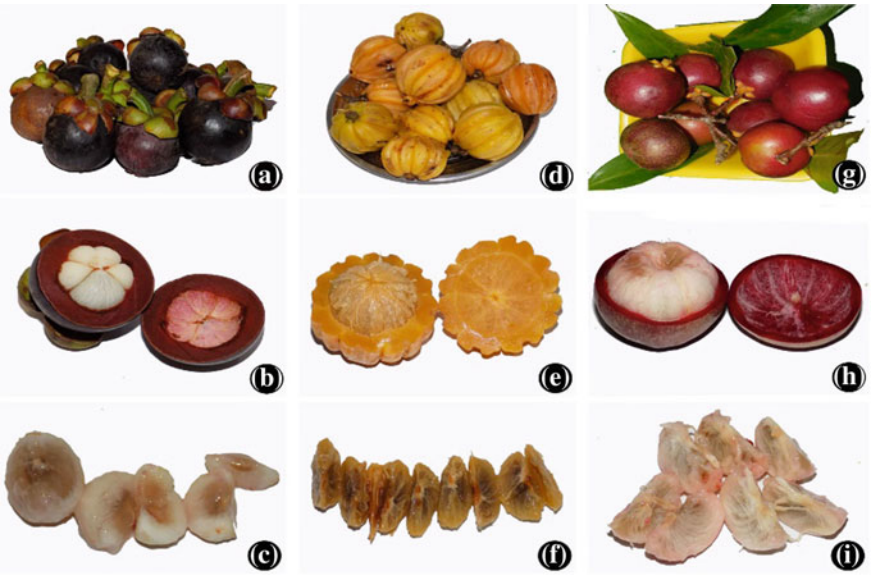

Fig. 19.2 Morphology of *Garcinia* fruits. **a**–**c** *G. mangostana*. **d**–**f** *G. gummi-gutta*. **g**–**i** *G. indica*. **a**, **d**, **g** Complete fruits. **b**, **e**, **h** Cross-section of fruit. **c**, **f**, **i** Arils containing seeds

of the total fruit weight and is rich in oil or fat. The seed oil remains solid at room temperature and it is popular as *kokum butter*. Kokum butter is yellow or light gray in color, greasy, and bland in taste (Nayak et al. 2010).

19.1.2 Uses and Nutritional Composition

19.1.2.1 *Garcinia mangostana*

Mangosteen fruits are eaten fresh; the fleshy white arils are delicious, sweet, and slightly acidic and have a mild flavor. Fleshy segments are sometimes canned. Syrup and jam are also prepared (Ketsa and Paull 2011). The rind is astringent and it is made into jelly by treatment with 6% sodium chloride. The seeds are eaten roasted or boiled. The aril portion of the fruit possesses 18.4 g carbohydrate, 0.5 g protein, 0.2 g fat, 1.7 g fiber, thus a modest source of essential nutrients (Table 19.1; Ketsa and Paull 2011). Mangosteen juice products sometimes possess polyphenols extracted from the pericarp to add polyphenol value (Ketsa and Paull 2011). The arils also contain volatiles namely hexyl acetate, hexanol and α-copaene which are responsible for the mangosteen fragrance (MacLeod and Pieris 1982). Mangosteen seeds are rich in oil (21.68%; Ajayi et al. 2007) and seed flour is a good source of minerals specially potassium (7071 mg/kg), magnesium (865 mg/kg) and calcium (454 mg/kg). Seed

Table 19.1 Nutritive value of *Garcinia mangostana* L. (mangosteen) fruit

Constituents	Content
Moisture	80.9 g
Energy	76.0 k cal
Protein	0.5 g
Fat	0.2 g
Carbohydrate	18.4 g
Dietary fiber	1.7 g
Ash	0.2 g
Calcium	9.0 mg
Phosphorous	14.0 mg
Iron	0.5 mg
Copper	0.11 mg
Zinc	0.1 mg
Vitamin B1 (Thiamine)	0.09 mg
Vitamin B2 (Riboflavin)	0.06 mg
Niacin	0.1 mg
Vitamin C	2.0 mg

Source Anonymous (2004). Fruits of Thailand, Department of Agricultural Extension, Ministry of Agriculture and Cooperatives, Bangkok, Thailand

oil is golden-orange in color, with a specific gravity of 0.98 and remains liquid at room temperature (25 °C). The chemical properties of mangosteen seed oil suggests that it could be used as an edible oil (Ajayi et al. 2007).

Mangosteen fruit is used as a medicine to treat skin infections, wounds, (Mahabusarakam et al. 1987) and dysentery (Chopra et al. 1956). The fruit pericarp has been used for treatment of inflammation and diarrhea (Balasubramanian and Rajagopalan 1988), and cholera and dysentery (Sen et al. 1980) in Ayurvedic system of medicine. Secondary metabolites such as prenylated and oxygenated xanthones are the major components of the pericarp and whole fruit. According to Obolskiy et al. (2009) and Pedraza-Chaverri et al. (2008) α-, β- and γ-mangostin, garcinone E, 8-deoxygartanin and gartanin are major xanthones obtained from fruit of mangosteen. Antibacterial, antifungal, antiviral, antimalarial, antioxidant, anti-inflammatory, anti-allergy and anti-tumoral properties are attributed to the toxanthones obtained from mangosteen fruits (see: Obolskiy et al. 2009; Pedraza-Chaverri et al. 2008 and references therein).

19.1.2.2 *Garcinia gummi-gutta* (L.) Roxb. [Syn. *G. cambogia* (Gaertn.) Desr.] (Malabar Tamarind)

Malabar tamarind fruits are edible, whereas dried rind is used as a condiment and an acidulant in place of tamarind or lime and hence the name has been attributed to the plant (Lim 2012). Fruit juice and syrup are used as a coolant and to reduce body fat (Orwa et al. 2009). Raw as well as dried fruits are excessively acidic in nature. Nutritional analysis of fruits shows 8.6% total sugars 3.1% fiber, and 2.1 mg sodium and 193.5 mg potassium (Table 19.2; Nazarudeen 2010). Other than these components, the fruits are rich in organic acids such as (-)-hydroxycitric acid (HCA), tartaric acid, and malic acids and the amount of HCA varies from 16–18% (Jayaprakasha and Sakariah 2000). The seed of Malabar tamarind yields protein (7.72%; Patil et al. 2016) and fat-rich butter (11.21%; Patil et al. 2016), popularly called *uppagetuppa* (Orwa et al. 2009).

Malabar tamarind fruit extract is used in the treatment of rheumatism and bowel complaints as a purgative, hydragogue, anthelmintic and emetic (Abraham et al. 2006). It is also used in veterinary medicine to treat mouth diseases in cattle. The fruit rind is used to polish gold and silver ornaments and as a substitute for acetic acid for coagulation of rubber latex (Abraham et al. 2006). The dried fruit rind is a unique source of HCA, which has a sour taste and has been safely used for centuries in Southeast Asian countries to make meals more filling (Lim 2012). Polyisoprenylated benzophenones such as garcinol, guttiferone I, guttiferone J, guttiferone K, guttiferone M, guttiferone N, and oxidized derivate of guttiferone K were isolated from the fruits of Malabar tamarind (Masullo et al. 2008). HCA is the major organic acid occurring in the fruits. Fruit extracts as well as pure compounds obtained from

Table 19.2 Nutritive value of *Garcinia gummi-gutta* (L.) Roxb. (Malabar tamarind) fruit

Constituents	Content
Moisture	86.91%
Protein	0.28%
Fat	0.21%
Reducing sugar	5.92%
Non-reducing sugar	2.67%
Total sugars	8.6%
Fiber	3.1%
Mineral Matter	0.49%
Vitamin C	Traces
Iron	Traces
Sodium	2.1 mg
Potassium	169 mg
Energy	38.7 k Cal

Source Nazarudeen (2010)

Malabar tamarind have been shown to express biological activity in both in vitro and in vivo models (Marquez et al. 2012).

19.1.2.3 *Garcinia indica* Choisy (Komum)

Kokum fruits are used to prepare a syrup which is taken as a health drink and the kokum rind is dried and used as a culinary agent in both vegetarian and non-vegetarian food preparations. Kokum rind powder is available in market as a culinary agent (Menezes 2000, 2002; Padhye et al. 2009; Shenoy 1989). Kokum juice is also fermented to make wine (Nayak et al. 2010). Kokum butter extracted from the seeds is used in confectionary, medicine and the cosmetic industries (Baliga et al. 2011). The fatty acid and triacylgycerol composition of kokum butter is similar to cocoa butter and is helpful in overcoming heat-induced softening and loss of consistency of chocolates and is therefore an ideal ingredient in chocolate candies (Maheswari and Reddy 2005; Reddy and Prabhakar 1994). Kokum butter is also used in the preparation of soaps and candles (Nayak et al. 2010).

Kokum fruits are sour, astringent, thermogenic, constipating and digestive; hence, they have been used in Indian traditional system of medicine such as Ayurveda (Baliga et al. 2011). Kokum is a natural antacid and used in treating gastric ulcerations and burning sensations; the fruits are also considered as antihelmintic and cardiotonic. Herbal preparations made out of kokum rind are used in the treatment of inflammatory aliments, for rheumatic pains and for bowel complaints. The fruit juice is used in the treatment of hemorrhoids, diarrhea, dysentery and ear problems (Chemexcil 1992). Nutritional analysis of kokum fruit have shown that rind possesses 80% moisture, 1% protein, 1.7% tannin, 0.9% pectin, 4.1% total sugar and 1.4% fat (Table 19.3; Krishnamurthy et al. 1982). Fruits are also rich in cyanidin-3-glucoside and cynidin-3-sambubioside type of anthocyanins (2.4 g/100 g; Nayak et al. 2010), garcinol and isogarcinol type of polyisoprenlyated–phenolics (Rastagi and Mehrotra 1990), hydroxycitric acid as the major organic acid (10.3–12.7%; Jayaprakasha and Sakariah 2002). Due to the richness of these phytochemicals, kokum fruits exhibit antibacterial, antifungal, anti-lipid peroxidation, anti-carbonyl, antiaging, neuroprotective, gastroprotective, anti-obesity, antidiabetic, cardioprotective, antineoplastic and anti-oxidant effects (see Baliga et al. 2011 and references therein).

19.2 Botany

19.2.1 Taxonomy

Garcinia species are small to large trees, usually with yellow latex. Flowers are polygamous i.e., with a variety of sexes bearing some flowers with stamens only, some with pistils only, and some with both, on the same or different plants. Flowers are

Table 19.3 Nutritive value of *Garcinia indica* Choisy (kokum) fruit

Constituents	Content
Moisture (g/100 g)	80.0
Protein (N × 6.25%)	1.0
Total Ash (%)	2.6
Tannins (%)	1.7
Pectin (%)	0.9
Total sugars (%)	4.1
Crude fat (%)	1.4
Organic acid (as hydroxyl citric acid)	5.9
Pigments (%)	2.4

Source Krishnamurthy et al. (1982)

either solitary or many arranged in fascicles or panicles, axillary or terminal. Sepals 4, petals 4 with imbricate arrangement. Male flowers contain many stamens, free or united into an entire or 4-lobed mass, arranged around a rudimentary style; anthers are sessile or with short filaments, 4 celled and dehiscing by a circular slit. Female or hermaphrodite flowers consist of staminodes free or variously united. Ovary 2–12 celled, ovule solitary, erect or laterally affixed; stigma sessile or subsessile, broadly peltate. Fruit a berry with a coriaceous rind. Seeds with a pulpy aril, embryo a solid homogeneous mass without distinct cotyledons (Cooke 1903).

19.2.1.1 Mangosteen

Mangosteen is a small, evergreen tree grows up to 25 m, erect with a pyramidal crown, bark dark-brown with latex (Fig. 19.3a). Leaves opposite, short-stalked, ovate-oblong, leathery and thick, dark-green, yellowish-green beneath, 9–25 cm long and 5–10 cm wide, with a conspicuous midrib (Fig. 19.3b). Flowers, male or hermaphrodite on the same tree, fleshy. Four sepals green in color, petals 4 green with red spots. Stamens many, fertile or sterile. Fruit a berry, sub-globose in shape, persistent calyx at the stem end and flat remnants of the stigma in a rosette at the apex (Fig. 19.3c), dark-purple to red in color, smooth externally, 4–8 cm in diameter. Rind 6–10 mm thick, red in cross section, purplish white inside (Fig. 19.3d) with snow white, juicy, soft fleshy arils. Fruits have 0–5 seeds which are ovoid-oblong, somewhat flattened, 2.5 cm long and 1.5 cm wide (Lim 2012).

19.2.1.2 Malabar Tamarind

Malabar tamarind is an evergreen, medium-sized tree reaching 20 m tall with a rounded crown and horizontal branches (Fig. 19.4a). Dioecious in nature i.e., separate

Fig. 19.3 *Garcinia mangostana.* **a** Habit. **b** Twig showing leaves. **c** Fruit. **d** Cross-section of fruit

males and female trees. The bark is dark and smooth. Leaves opposite, petiolate, dark green, shining, 13–18 cm long and 4–8 cm in width, elliptic to obovate, glabrous (Fig. 19.4b). Flowers solitary or clusters of 4–20, usually red, but some trees have yellow ones. Petals 4, each about 12 mm wide and 11 mm long, anthers attached to a pistillode with a non-functional stigma. Female flowers occur singly or in clusters of up to 4 pistillate flowers have rudimentary and non-functional staminodes. Fruits are green when tender (Fig. 19.4c), ovoid berry with 6–8 grooves, 5 cm in diameter, yellow or red when ripe (Fig. 19.4d). Seeds 6–8, smooth, large, about 5 cm long and 2 cm wide surrounded by succulent aril (Lim 2012).

19.2.1.3 Kokum

Kokum is medium-sized tree growing to a height up to 18 m and with drooping branches (Fig. 19.5a). Leaves possess a small petiole, red when young, oblong lanceolate, 13–18 cm long and 4–8 cm in width (Fig. 19.5b). The plant is dioecious. The flowers are axillary or terminal, solitary or spreading in fascicles. Sepals 4, thick, fleshy, petals 4, thick, larger than sepals. Male flowers 3–8, in axillary and terminal

Fig. 19.4 *Garcinia gummi-gutta*. **a** Habit. **b** Twig showing leaves. **c** Fruits. **d** Cross-section of fruit

fascicle, stamens numerous, on a short column, anther 2 celled, filaments very short. Female flowers sessile or vary short pedicelled, staminodes in four bundles. Ovary 4–8 celled, stigma sessile. Fruit globose (Fig. 19.5c), with smooth surface, raw fruits green in color, and matured fruits purple in color. Seeds 5–8, compressed embedded in aril (Fig. 19.5d; Cooke 1903; Nayak et al. 2010).

19.3 Diseases and Pests

Common diseases of mangosteen are similar to those of other tropical tree species; however, there are no reports on the diseases and pests of Malarbar tamarind and kokum (Lim 2012). Pestalotiopsis leaf blight caused by *Pestalotiopsis flagisettula* is a leaf disease of mangosteen reported from Thailand. Furthermore, *P. flagisettula* is also responsible for fruit rot (Lim and Sangchote 2003). From Thailand, Malaysia and North Queensland stem canker is also reported. Another major disease of mangosteen is white thread blight disease caused by *Marasmielluss candens* which damages the

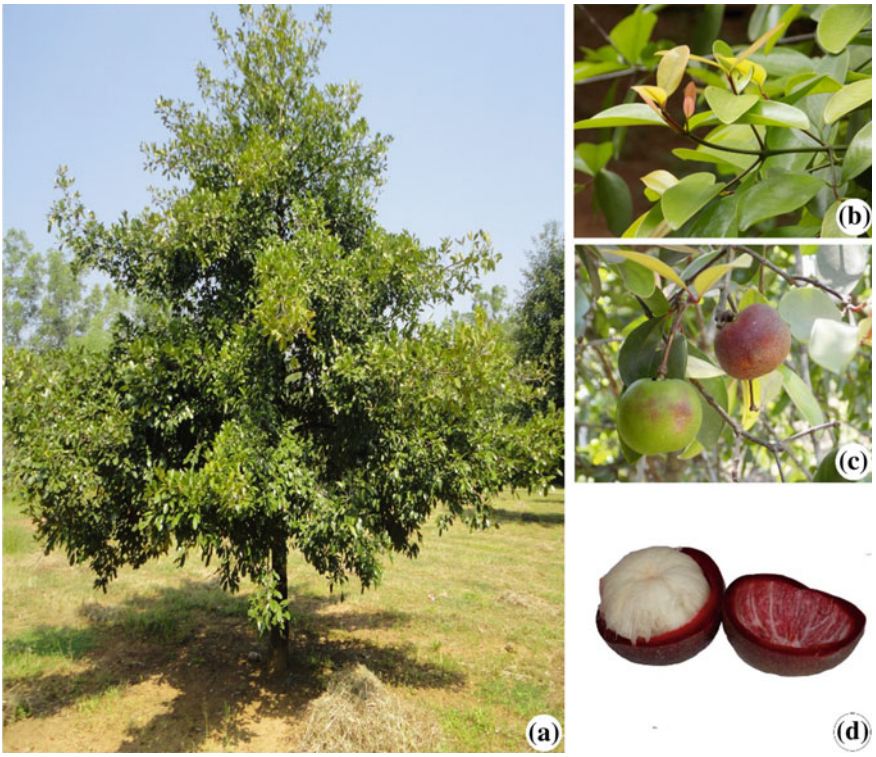

Fig. 19.5 *Garcinia indica*. **a** Habit. **b** Twig showing leaves. **c** Fruits. **d** Cross-section of fruit

leaves, twigs and branches (Lim and Sangchote 2003). One more important disease is Diplodia fruit rot caused by *Diplodia theobromae*. Brown rot disease due to *Phellinus noxius* infects the trunk and roots. Some pests affecting mangosteen are *Stictopetera* sp. (leaf and fruit eater), *Phyllocnistis citrella* (leaf miner), *Curculio* sp. (fruit borer). Use of fungicides and insecticides are suggested for the control of fungi and insects, respectively (Lim and Sangchote 2003).

19.4 Germplasm Collection, Conservation and Utilization

Extensive research work has been carried out by various research groups in collection, conservation and utilization of Garcinia germplasm, from its center of diversity (Abraham et al. 2010a, b, c; Keatmetha et al. 2006; Mansyah et al. 2010a). The International Plant Genetic Resources Institute (IPGRI) was also involved in large-scale collection and conservation of Mangosteen from Southeast Asian countries including Myanmar, Laos, Thailand, Cambodia, Vietnam, Malaysia, Brunei, Singapore,

The Philippines, Indonesia and Papua New Guinea (Coronel 1995). Scientists from the National Bureau of Plant Genetic Resources (NBPGR) and various agricultural universities in India have been involved in extensive collection of Malabar tamarind and kokum in the Western Ghats, Andaman and Nicobar Islands and Northeastern India (Abraham et al. 2010a). Much emphasis in Garcinia germplasm collection in the past has been aimed at identifying the trees with outstanding fruit characters including fruit shape, size, rind thickness, attractiveness, number of arils per fruit, seed shape, size and color and yield. However, in recent years the focus has been on selection of germplasm for early maturity, early and late blooming, drought and disease tolerance (Abraham et al. 2010a, b, c; Mansyah et al. 2010a).

19.4.1 Strategies of Conservation of Garcinia Germplasm

Various approaches have been proposed to conserve Garcinia genetic resources; among them the in situ based conservation strategies are very prominent because Garcinia seeds are recalcitrant. It was reported that mangosteen, Malabar tamarind, and kokum seeds are high in moisture content, possess no dormancy, exhibit low seed viability and short lived (Chacko and Pillai 1997; Malik et al. 2005a, b; Morton 1987). Mangosteen, Malabar tamarind, and kokum seeds are described as agamospermic without a differentiated embryo, endosperm or embryonic axis (Malik et al. 2005a, b; Normah et al. 1992). Therefore, seed storage is hampered. Freshly harvested seeds of these species can be stored at ambient temperatures for up to 30 days and seed longevity could be extended up to 60 days by storage at 15 °C. However, chilling treatment at 5 °C, leads to considerable loss of viability of up to 60% (Malik et al. 2005b). Nevertheless, ex situ conservation methods are also followed for conservation of Garcinia germplasm and the gene banks are established at the National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India, wherein Malabar tamarind and kokum seeds are maintained in cold storage at 5–20 °C.

19.4.2 In Situ Conservation of Garcinia Germplasm

In situ conservation methods maintain plant populations in their distribution range, thus providing optimum conditions for manipulating the genetic variability of the species. This approach involves two methods: the first is the genetic reserve, which is defined as the location, management and monitoring of genetically-diversity wild populations within the defined areas for active and long-term conservation. The second is on-farm conservation, which involves the maintenance of traditional crop cultivars or landraces and farming systems by farmers within traditional agricultural systems (Jarvis 1999). This latter conservation method has been gaining importance in recent years, although farmers have been using it for centuries.

Table 19.4 On-farm conservation of Malabar tamarind in India

Place	Number of sites
Alappuzha, Kerala	20
Ernakulum, Kerala	16
Kannur, Kerala	10
Kasaragod, Kerala	2
Kollam, Kerala	11
Kottayam, Kerala	9
Kozhikode, Kerala	10
Malappuram, Kerala	8
Plakkad, Kerala	1
Pathanamthitta, Kerala	10
Thiruvanathapuram, Kerala	1
Thrissur, Kerala	11
Wayanad, Kerala	1

Source Abraham et al. (2010a)

Table 19.5 In situ germplasm conservation sites of kokum in India

Places	Number of accessions
Shimoga, Karnataka, India	6
Uttar Kannada, Karnataka, India	14
Udupi, Karnataka, India	4
Dakshin Kannada, Karnataka, India	6
Kodagu, Karnataka, India	5
North Goa, Goa, India	2
South Goa, Goa, India	2
Kasargod, Kerala, India	3
Sindhudurg, Maharashtra, India	8
Ratnagiri, Maharashtra, India	9

Source Abraham et al. (2010a)

Exploration and collection missions of the NBPGR regional station, Thrissur, India have identified 110 sites in 13 districts of Kerala for on-farm conservation of Malabar tamarind (Table 19.4). Similarly, 59 in situ conservation sites were explored from the Western Ghats regions of India involving 5 districts of Karnataka, 2 each of Goa and Maharashtra and 1 of Kerala for conservation of kokum germplasm (Table 19.5).

19.4.3 Ex Situ Conservation of Garcinia Germplasm

Ex situ conservation of Garcinia genetic resources and germplasm involves field gene banks, seed banks and in vitro banks (Table 19.6). Mangosteen germplasm is maintained at the National Biological Institute, Bogor Agricultural University, Bogor, Indonesia; PNG National Agricultural Research Station, Keravat, Papua New Guinea; Institute of Plant Breeding, Laguna, The Philippines and Plew Horticultural Research Experimental Station, Chantaburi, Thailand (Coronel 1995). In India, scientists of the NBPGR, Regional station, Thrissur, Kerala have maintained 124 accessions of Malabar tamarind and 76 accessions of kokum in the field gene bank; these accessions were collected from the Western Ghats regions of Karnataka, Maharashtra, Goa and Kerala (Abraham et al. 2010a).

In vitro conservation involves maintenance of explants especially meristems/shoots/embryos or plantlets in a sterile, pathogen-free environment and are therefore preferentially applied to clonal crop germplasm and multiplication of

Table 19.6 Mangosteen, Malabar tamarind and kokum germplasm maintained in India and Southeast Asian countries

Germplasm preserved	Institution and country
Mangosteen	National Biological Institute, Bogor Agricultural University, Indonesia
	PNG National Agricultural Research Institute, Islands Regional Centre, Keravat, PO Box 204, Kokopo, East New Britain
	Institute of Plant Breeding, UPLB College of Agriculture and Food Sciences, University of the Philippines Los Baños, Laguna, Philippines
	Horticultural Research Station, Sukhumvit Road, Tonton, Khlung, Chanthaburi 22110, Thailand
Malabar tamarind	National Bureau of Plant Genetic Resources, Pusa campus, New Delhi, India
	National Bureau of Plant Genetic Resources Regional Station, Kerala Agricultural University, Thrissur, Vellinikkara, India
	College of Forestry, University of Agricultural Sciences, Sirsi, India
Kokum	National Bureau of Plant Genetic Resources, Pusa campus, New Delhi, India
	National Bureau of Plant Genetic Resources Regional Station, Kerala Agricultural University, Thrissur, Vellinikkara, India
	College of Forestry, University of Agricultural Sciences, Sirsi, India
	Indian Council of Agricultural Research Complex for Goa, Ela Old Goa, India
	ICAR Central Horticultural Research Station, Indian Institute of Horticultural Research, Chettalli, Karnataka, India
	Department of Horticulture, College of Agriculture, Dapoli, Maharashtra, India

Source Abraham et al. (2010a), Coronel (1995)

species that produce recalcitrant seeds. It also supports safe germplasm transfers under regulated phytosanitary control (Englemann 1991). Cultures in the active gene bank are maintained by successive sub-culturing allowing culture renewal and distribution. For medium-term storage, sub-culture intervals are extended, reducing processing costs by arresting growth using cold treatments, adapted light conditions, culture medium modifications (osmotic active compounds, growth retardants). This increases efficient use of resources and staff time and offsets selection risks and contamination. The in vitro method was developed for maintenance of mangosteen germplasm in the form of shoot tips by Keatmetha et al. (2006), using growth retardants in the culture medium. They tested the effect of abscisic acid (ABA), pacloburazol at 0, 0.5, 1.0, 1.5 and 2.0 mg/l supplemented to Murashige and Skoog (MS) nutrient medium. ABA at 2 mg/l suppressed shoot growth of mangosteen better than paclobutrazol. Shoots can be maintained in the form of slow-growing cultures for up to 12 months. Regrowth was achieved by such stored shoots upon transfer to shoot-proliferation medium. Similarly, an in vitro protocol has been developed for conservation of kokum germplasm (Malik et al. 2005b). Nodal segments of kokum accessions (IC-136685-1 and IC-136682-2) were cultured on MS nutrient medium supplemented with 5 μM benzylamino purine and different concentrations of sucrose (1, 2 and 3%) and the cultures kept in diffused light (5 $\mu mol^{-2}\ s^{-1}$) as well as in normal light (30 $\mu mol^{-2}\ s^{-1}$) to study the effect of light intensity on in vitro conservation. It was realized that the cultures involved in slow growth under diffused light conditions and such cultures could be maintained up to 10–11 months. Shoots upon transferring to recovery medium under normal conditions exhibited 95% survival. These findings imply that slow-growth techniques [presence of growth retardants (abscisic acid), reduction of sucrose concentrations, treatment cultures with low light intensity] could be successfully used for in vitro conservation of garcinia species.

Garcinia germplasm is freely available through the International Plant Genetic Resource Institute (IPGRI), Rome, Italy; NBPGR, New Delhi and the various other institutes listed in the Table 19.9, Appendix 1 and 2.

19.5 Breeding

Most *Garcinia* species are apomictic (Corner 1988) and fruit and seed development in these species is through agamospermy i.e., production of seed without the fusion of gametes (Thomas 1997). The mode of embryo and seed development has been studied only in *G. mangostana* and it was reported that embryo development in mangosteen is from integuments rather from the egg (Lan 1989). Therefore, *Garcinia* species are considered as exclusively apomictic (Horn 1940; Richards 1990a) and plants produced by seed or vegetative propagation are homogeneous and genetically identical (Horn 1940). Consequently, variability observed in mangosteen, Malabar tamarind and kokum in the field/wild is likely due to difference of environmental conditions. Nevertheless, several studies have revealed that a population from apomictic reproduction does not always carrying the same genetic properties, even in obligate

apomixes (Asker and Jerling 1992); variability in progenies and populations has been reported in obligate apomictic species *Taraxacum* (Ford and Richards 1985). Hence, improvement of *Garcinia* species is mostly exploitation of existing genetic variability and improvement of these species is only through selection rather than conventional breeding. The major breeding objectives for improvement of *Garcinia* species are as follows:

(a) Fruit quality: Great variability exists among natural population of mangosteen, Malabar tamarind and kokum with respect to fruit size, shape, content of fruit pulp, number of arils and seeds, and shelf life. Accordingly, selection of plants with respect to the above characteristics along with color, flavor, texture and nutritional value of fresh fruits is the main priority in the improvement *Garcinia* species.
(b) Tree characteristics: Mangosteen, Malabar tamarind and kokum trees raised from seeds have a long juvenile period (7–12 years for fruiting) and depict constraints of less fruit-bearing capacity. Therefore, selection of early maturing, early and late blooming (in a year), and high yielding/fruit bearing plants/population is highly desirable.
(c) Drought tolerance: *Garcinia* species are sensitive to drought during the early growth period (up to 5 years) and shade should be provided during that period. Hence, selection of drought tolerant plants/populations is very much essential.
(d) Rootstock improvement: Information of rootstocks, stock-scion relationships is fragmentary in *Garcinia* species; thus, selection of root stock and scion with respect to wide geographic adaptability, resistance to biotic and abiotic stresses, and high productivity with good quality fruits is another area to be explored.

19.5.1 Genetic Resources and Improvement

19.5.1.1 Mangosteen

Mangosteen is a polyploid (2n = ≈90) hybrid between *G. homroniana* (2n = 48) and *G. malacensis* (2n = ≈42) (Richards 1990b) and exhibits much variability in its native lands of Indonesia and Malaysia (Sobir et al. 2008). Mansyah (2010a, b), Prabowo (2002), Purwanti (2002) and Suhaeri (2003) carried out extensive studies on morphological variability in four mangosteen populations in Java Island, Indonesia, including sites at Leuwilang, Wanayasa, Watulimo and Kaligesing, and found homogeneity of variance in morphological characters such as leaf weight, individual leaf area, leaf length/width ratio. They also attributed such variation to environmental factors. They also reported variability in fruit characteristics namely fruit weight, length, diameter, length/diameter ratio, rind thickness, peduncle length, total soluble solids and presence of fruit latex in populations collected from the four named sites. They achieved a good positive correlation with fruit diameter, fruit weight, fruit length, rind thickness and number of seed/fruit. Mansyah (2010a, b), Prabowo (2002),

Purwanti (2002) and Suhaeri (2003) also suggested that the fruits from Kaligesing were superior for larger size and seed number/fruit, and the fruits from Watulimo for sweetness and lower yellow latex content. Such studies suggest the usefulness of population evaluation for morphological variability and selection of plants/clones for superior characteristics.

Selections were made by a different group of scientists in different South-eastern countries; Horn (1940) reported a cultivar from The Philippines called *Julu*, which possessed larger fruits than normal, the seeds were larger and the pulp was more acidic. The Malaysian Department of Agriculture has identified several accessions which show variation in fruit size and shape, seed number, shelf life, fruiting precocity and external coloration, and has released the clones GA1 and GA2. The commercially-available Malaysian cultivar Mesta is a seedless variety with a smaller fruit size than the typical mangosteen. Bogor Agricultural University has released new Indonesian mangosteen varieties Mangosteen Wanayasa, Mangosteen Puspahiang and Mangosteen Malinau for cultivation.

19.5.1.2 Malabar Tamarind

Malabar tamarind is naturally distributed in India, Sri Lanka and Nepal and it exhibits much natural diversity in semi-evergreen to evergreen forests of Southwest India, especially western Ghat regions (Abraham et al. 2006; Lim 2012). Abraham et al. (2006) and Lim (2012) collected a total of 56 accessions from Kerala and Karnataka provinces of India and assessed variability of fruit and seed characteristics (Table 19.7). They reported variation in fruit length (43.0–82.0 mm), fruit girth (122–217 mm), nipple thickness (4.0–29.0 mm), nipple length (0.6–18.0 mm), number of ridges and furrows (5–10), number of secondary furrows (1–8), stalk length (2.0–7.0 mm), rind weight (7.5–56.0 g) and fruit weight (8.2–16.0 g). They also observed variation in the seed characters such as seed number per fruit (2–8), seed length (15.6–35.0 mm), seed width (7.9–33.0 mm), seed thickness (4.3–9.0 mm) and seed weight (0.6–3.0 g). They made selections from among the above accessions for fruit weight (161 g; IC354028) and for mean rind thickness (15 mm) and mean fruit weight (125 g; IC354019), for pinkish red color of fruits (IC 3544047 and 354063) and for half smooth surface (IC 3540070). Variability with respect to number of flowers per inflorescence/flowering twig (solitary, two, three or many; Fig. 19.6), flower color (red, orange, reddish-brown, reddish-yellow, pinkish orange and yellow; Fig. 19.7), variation of sex organs in the male and female flowers (male flowers without and with pistillode; female flowers without or with staminode), fruit shape, size, color and number of furrows (Fig. 19.8) was also apparent in our collections from Tamilnadu, Kerala, Karnataka and Goa, India. The Indian Council of Agricultural Research, Regional Research Centre, Thrissur released Malabar tamarind IC244100-2 (INGR No.04061) and IC244111-1(INGR No. 04062) which are maturing varieties (6–7 years) for the farmers.

Table 19.7 Variability of fruit and seed characters of Malabar tamarind

S. no.	Characters	Mean	Range	Standard deviation	Standard error	Co-efficient of variation
Fruit characters						
1	Fruit length (mm)	62.5	43–82	10.0	1.4	16
2	Fruit girth (mm)	166.8	122–217	21.4	3.0	13
3	Fruit size (g)	72	8.2–161	25.1	3.5	35
4	Nipple thickness (mm)	8	4–29	4.0	0.6	47
5	Nipple length (mm)	5	0.6–18	3.4	0.5	68
6	No. of ridges	7	5–0	1.0	0.1	14
7	No. of furrows	7	5–10	1.0	0.1	14
8	No. of secondary ridges	9	2–15	3.7	0.5	43
9	No. of secondary furrows	5	1–8	1.9	0.3	40
10	Stalk length (mm)	4	2–7	1.3	0.2	28
11	Rind weight (g)	53	7.2–125	20.2	1.9	38
12	Rind thickness (mm)	7	4.3–15	1.9	0.3	26
13	Pulp weight (g)	18	7.5–56	7.5	1.1	40
Seed characters						
14	Seed number	5	2–8	1.3	0.2	26
15	Seed length (mm)	25	15.6–35	4.1	0.6	16
16	Seed width (mm)	11	7.9–33	3.6	0.5	31
17	Seed thickness (mm)	6	4.3–9	1.0	0.1	17
18	Seed weight (g)	1.2	0.6–3	0.5	0.1	43

Source Abraham et al. (2006)

Fig. 19.6 *Garcinia gummi-gutta*: Flower variability. **a** Solitary flower. **b** Two flowers. **c** Three flowers. **d** Many flowers

19.5.1.3 Kokum

Kore et al. (2005) analyzed the variability of fruit characters in 22 kokum genotypes collected from Maharashtra and Karnataka, which were maintained in the Dapoli Research Station, Maharashtra, India. They have reported much variation in length, breadth, weight, circumference, fresh weight, fresh and dry rind weight, pulp weight and seed number (Table 19.8; Fig. 19.9). The genotype and phenotype coefficients of variation for fruit characters were all similar in magnitude for all the fruit characters they studied, whereas, environmental coefficient variation was very low. Based on their analysis they selected varied collections namely K-29, K-32, K-146, KN-1, KN-3, KN-4 and KN-8 as superior genotypes. Scientists of the Balasaheb Sawant Agricultural University, Dapoli, India have conserved around 300 accessions of kokum and have released two important varieties, Konkan Amruta and Konkan Hatis, which are early- and late-maturing varieties with superior fruit characteristics (Table 19.9; Sawant et al. 1999). Similarly, the Indian Council of Agricultural Research, Regional

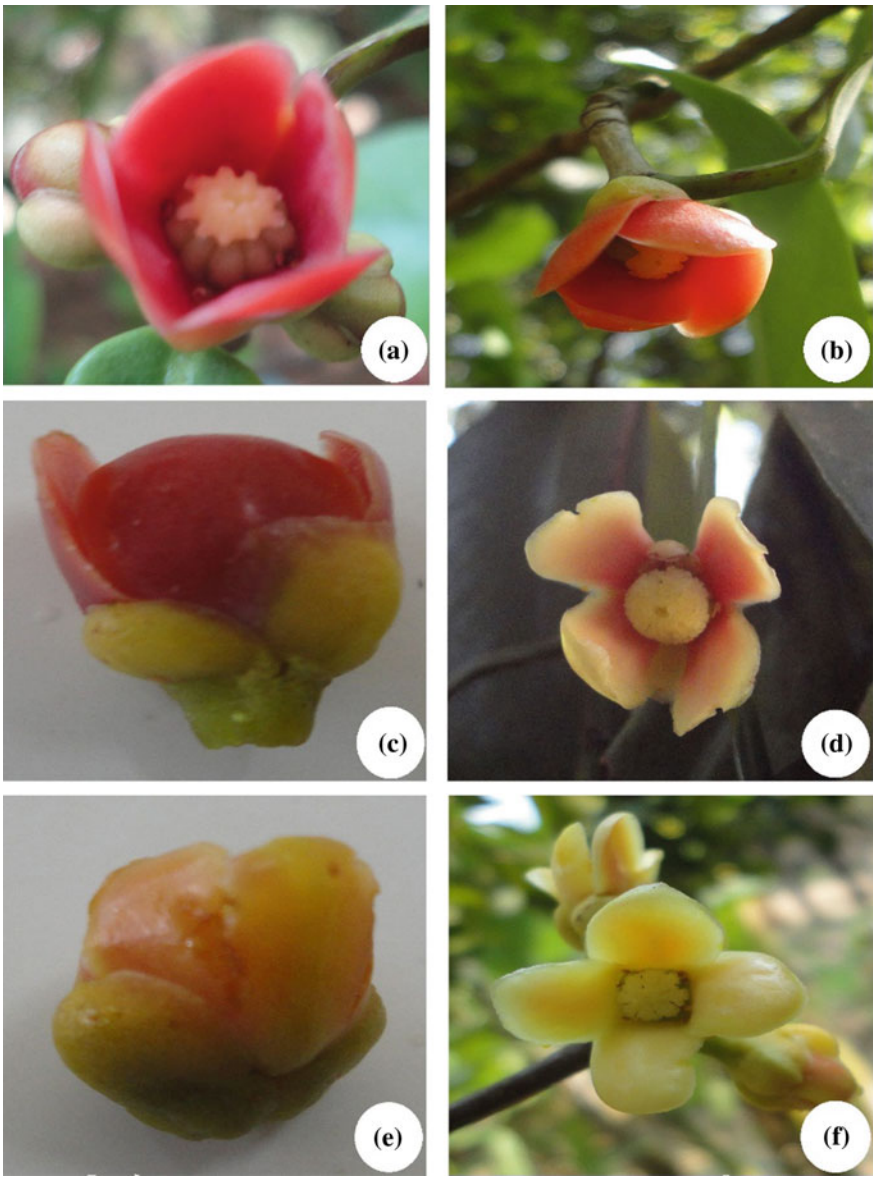

Fig. 19.7 *Garcinia gummi-gutta* variability in flower color. **a** Red flower. **b** Orange flower, **c** Reddish-brown flower. **d** Reddish-yellow flower. **e** Pinkish flower. **f** Yellow flower

Research Centre, Thrissur has released kokum IC136687-3 (INGR No. 04063) which is a high-yielding variety.

Table 19.8 Variation in fruit characteristics of different genotypes in kokum

Genotype	Weight (g)	Length (cm)	Breadth (cm)	Circumference (cm)	Fresh rind weight (g)	Dry rind weight (g)	Pulp weight (g)	Seed number per fruit
K-4	14.60	2.92	2.98	9.56	6.80	1.18	2.75	6.10
K-10	22.85	3.38	3.48	11.19	11.90	1.40	6.25	6.20
K-29	33.45	3.45	4.08	13.23	17.20	1.99	7.70	7.05
K-32	30.95	3.70	3.85	12.68	12.45	2.00	9.80	5.65
K-42	24.75	3.14	3.63	11.97	12.10	1.95	6.45	6.85
K-70	21.05	3.11	3.47	11.19	9.75	1.62	5.90	6.85
K-133	16.30	2.62	3.22	11.36	8.35	1.45	3.50	6.00
K-141	27.15	3.27	3.74	11.97	10.95	1.74	7.75	6.75
K-143	24.80	2.89	3.83	12.21	10.90	1.71	6.60	7.30
K-146	32.45	3.61	3.55	12.91	12.70	1.99	11.65	7.35
K-173	20.00	3.45	3.32	10.11	11.35	1.82	4.45	6.00
K-211	25.00	3.21	3.67	11.71	11.40	1.70	6.80	6.80
K-212	22.70	2.79	3.69	11.71	10.55	1.75	6.25	6.75
K-216	22.95	3.00	3.62	11.68	9.65	1.75	5.63	7.70
K-217	25.00	3.24	3.72	11.88	11.90	1.82	6.65	6.65
K-219	27.90	3.14	3.73	12.24	12.85	1.78	8.85	6.80
KN-1	41.25	3.09	4.54	14.60	14.90	2.70	18.30	7.75
KN-2	22.96	3.48	3.40	10.98	10.45	1.60	7.06	5.85

(continued)

Table 19.8 (continued)

Genotype	Weight (g)	Length (cm)	Breadth (cm)	Circumference (cm)	Fresh rind weight (g)	Dry rind weight (g)	Pulp weight (g)	Seed number per fruit
KN-3	35.98	3.79	4.09	13.03	15.45	2.04	13.78	6.00
KN-4	32.22	3.78	4.00	12.79	16.55	2.51	8.27	7.85
KN-5	32.52	4.31	3.90	12.25	16.05	2.14	11.70	7.35
KN-6	26.06	3.54	3.57	11.54	12.30	1.86	6.76	6.80
Mean	26.49	3.31	3.68	11.94	12.11	1.84	7.86	6.75
Range	14.60–41.25	2.62–4.31	2.98–4.54	9.56–14.60	6.80–17.20	1.18–2.70	2.75–18.30	5.65–7.85
SE ± m	1.60	0.06	0.12	0.34	0.70	0.09	0.82	0.20
SD	6.41	0.39	0.33	1.07	2.62	0.34	3.50	0.64
CV (%)	24.19	11.68	9.01	8.98	21.64	18.23	44.50	9.54
CD (P = 0.05)	4.69	0.16	0.36	1.00	2.04	0.27	2.40	0.60

Source Kore et al. (2005)

Fig. 19.8 *Garcinia gummi-gutta* variability in fruit shape, size, color and number of furrows

Fig. 19.9 *Garcinia indica* variability in fruit shape, size and color

19.5.2 Use of Molecular Markers in Assessing Genetic Diversity

Molecular markers are useful in detecting genetic variability among plant populations and they also corroborate the variability assessed by using phenological and morpho-

Table 19.9 Yield and fruit characters of kokum varieties Konkan Amruta and Konkan Hatis

S. no.	Characters	Kokum varieties	
		Konkan Amruta	Konkan Hatis
1	Average yield/kg	138	250
2	Average number of fruits/kg	29	11
3	Average height of the fruits (cm)	3.74	4.22
4	Average width of the fruits (cm)	4.15	6.29
5	Average circumference of fruits (cm)	13.15	20.10
6	Average weight of the fruit (g)	34.45	91.50
7	Average weight of rind (g)	17.55	48.34
8	Average number of seeds/fruit	6.40	5.60
9	Shape of the fruit	Apple shape	Apple shape
10	Shelf life (days)	15	18
11	Diameter of fruits (cm)	35.50	112.8
12	Thickness of rind (cm)	4.45	5.58
13	TSS	9.08	9.20
14	Total sugar (%)	4.52	4.10
15	Reducing sugar (%)	2.41	2.40
16	Acidity (%)	5.12	5.10
17	Flower initiation	2nd week of November	2nd week of December
18	Harvesting period	March–April	April–May

Source Sawant et al. (1999)

logical characteristics (Kumar 1999). Prabowo (2002) and Mansyah (2010b) assessed genetic variability of *Garcinia mangostana* (mangosteen) populations (collected from Java Island, Indonesia) using RAPD markers. Ramage et al. (2004) also evaluated the genetic relationship between 37 accessions of mangosteen (including the collections from Indonesia, Malaysia, Thailand, Borneo, Singapore, Australia) and 11 accessions of eight other *Garcinia* species by randomly amplified DNA fingerprinting (RAF). The results showed genetic diversity within *G. mangostana* and among other *Garcinia* species. For 26 (70%) of the accessions, no marker variation was detected in over 530 loci, eight (22%) accession exhibited very low variation (0.2–1%), and

the three accessions (8%) showed extensive variation (22–31%) compared to majority accessions. Compared to other *Garcinia* species, the three groups of mangosteen differed at 63–70% of dissimilarity level. Ravishankar et al. (2017) developed simple-sequence repeats (SSRs) markers for assessing variability among *G. gummi-gutta* (Malabar tamarind) genotypes. The polymorphic information content (PIC) ranged from 0.867 to 0.95, with a mean value of 0.917. The observed heterozygosity ranged from 0.00 to 0.63 and 0.896 to 0.974, respectively. RAPD and ISSR (inter simple sequence repeats) markers were used to establish such genetic variability among *G. indica* (kokum) populations (Thatte et al. 2012). The genetic variability observed in these three apomictic species might be due to natural mutations.

19.5.3 Use of Molecular Markers for Sex Typing

Mangosteen, Malarbar tamarind and kokum are dioecious with male and female individuals (Abraham et al. 2006; Richards 1990a). These species are apomictic and the existence of male or male function is debated (Richards 1990a). Flowering starts in seed-grown plants after 10–12 years and will attain the stage of full bearing at the age of 12–15 years (Orwa et al. 2009). Sex determination in these species at an early juvenile stage would be useful for planning the male and female tree ratio in the orchards and enabling tree improvement programs. Recently, molecular tools were employed in dioecious taxa for early identification of sex and understanding the developmental and evolutionary pathways of sexual dimorphism. Specific molecular markers can be deduced from unique, single-copy segments of the genome and can be considered codominant and used in sex determination. Sequence characterized markers (SCAR) which are based on randomly amplified polymorphic DNA (RAPD) analysis are locus-specific, more reliable and more reproducible for molecular identification (Paran and Michelmore 1993). Joseph et al. (2014) identified a sex-linked DNA fragment in *Garcinia gummi-gutta* (Malabar tamarind) by screening 150 RAPD primers. One of the RAPD primers (OPBD20) showed differential amplification associated male specific DNA. They sequenced the male specific DNA fragment (556 bp) and developed a sequence-characterized amplified region (SCAR) marker, CAM-566; based on this male specific marker they could identify the sex of plants at seedling stage itself. Similarly, Joseph et al. (2015) developed male specific SCAR marker (1501 bp) in *G. indica* (kokum) for identification of male individuals from females at a very early developmental stage (seedling stage). *Garcinia* species take 7–12 years to reach to reproductive stage (flowering stage) and thus SCAR markers are quite useful in these dioecious plants for sex typing at an early developmental stage.

19.5.4 Transcriptome Study in Mangosteen

Matra et al. (2016) performed de novo transcriptome analysis in *Garcinia mangostana* L. (mangosteen) through RNA-Seq technology. They obtained the data from 12 libraries through the Ion Proton System and acquired 267,851 transcripts as well as 155,850 unigenes, having N50 value of 555 and 433 bp, respectively. Transcript/unigene length ranged from 201 to 5916 bp. These transcriptomic data will be beneficial for studying transcriptome of mangosteen.

19.5.5 Induced Mutation

Several studies have been carried out to generate variability in mangosteen by induced mutations. Chasanah (2005) irradiated mangosteen seeds by gamma rays at 0, 10, 20 and 30 Gy and raised plants, 18 months old seedlings depicted morphological and molecular variations. Similar experiments were carried out in mangosteen by Harahap (2005) and obtained variants and variability was confirmed by morphological, isoenzyme and RAPD analysis. Qosim (2006) irradiated nodular callus of mangosteen with gamma rays (0, 5, 10, 15, 20, 25, 30, 35 and 40 Gy) and obtained regenerants representing variability. RAPD analysis of regenerated plants showed genetic distance of 0.40 based on a dissimilarity index.

19.5.6 Improvement of Rootstocks and Grafting

Selection of rootstock has been studied in *Garcinia* species for the improvement of drought tolerance, salinity tolerance, tree architecture and early flowering. *Garcinia lateriflora*, *G. hombroniana*, *G. livingstonei*, *G. morella* and *G. xanthochymus* were used as useful rootstocks for the improvement of mangosteen (Osman and Milan 2006). Similarly, *G. kydia* and *G. venulosa* were also useful rootstocks also partially compatible with mangosteen. These species have been shown to provide desirable characteristics such as precocity, dwarfness and plant architecture that promote easy picking of fruits and pruning (Osman and Milan 2006). Cleft and softwood grating methods were used for grafting of mangosteen using different species of *Garcinia* rootstocks. Healthy rootstocks of about 30–35 cm in height are preferred and the size of scion should be around 6–12 cm long to achieve successful grafting (Osman and Milan 2006). Softwood grafting has been followed for the improvement of kokum especially for imparting drought resistance and adaption to marshy soil (Haldankar et al. 1993). Studies have shown that rootstocks of *G. gummi-gutta*, *G. cowa* and *G. hombronina* were quite useful in transmitting drought resistance and to establish kokum plantation in rainfed areas. Seedlings of 0.25 cm thickness and more than 22 weeks of age were used as rootstock material for the purpose. Similarly, shoots

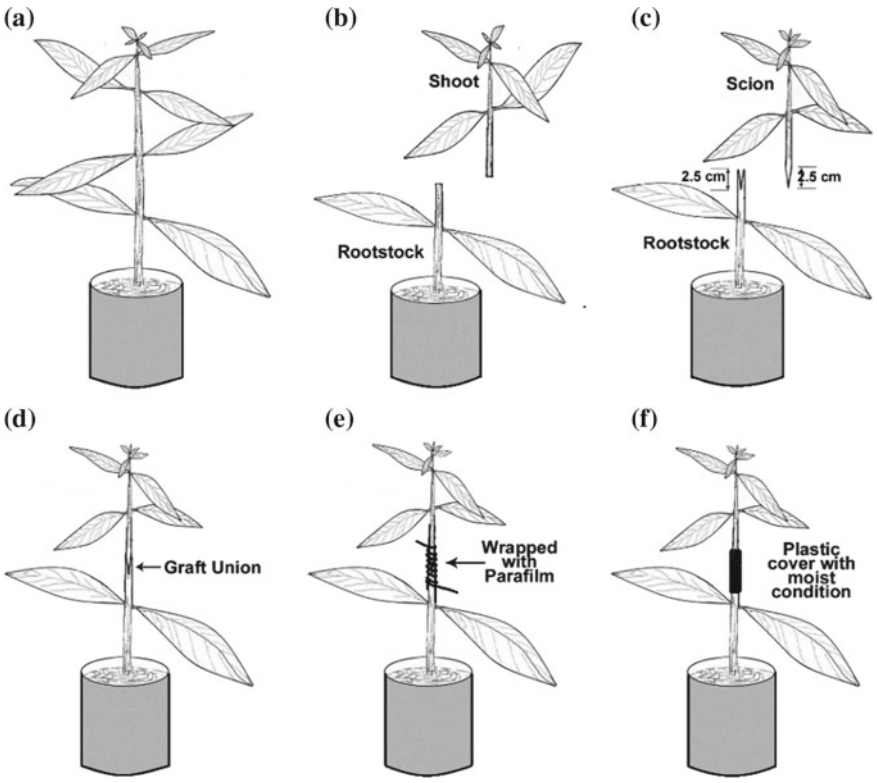

Fig. 19.10 Softwood grafting techniques adopted for improvement of *Garcinia indica*

of 0.5–0.6 cm thickness and 10–12 cm length were used for scions (Haldankar et al. 1993; Fig. 19.10).

Poor root initiation is the main hindrance for in vitro propagation of kokum. Chabukswar and Deodhar (2006a) developed an in vitro grafting technique for restoration of rooting competence. In vitro regenerated shoots of kokum obtained from nodal explants of mature trees were repeatedly grafted on to in vitro grown juvenile seedlings. After each grafting, some of the scions were tested for rooting competence and 75% regained the ability to root after the fifth grating.

19.6 Tissue Culture

Conventional vegetative propagation of mangosteen, Malabar tamarind and kokum is difficult, as cuttings cannot be rooted easily (Almeyda and Martin 1976); therefore, tissue culture is sought as an alternative for mass multiplication in these species.

19.6.1 Micropropagation of Mangosteen

Micropropagation of mangosteen was achieved through shoot tip, juvenile leaf segments, root segments, nodal explants and internode segments and seed sections (Goh et al. 1997, 1988a, b, 1990, 1994). Direct shoot organogenesis was described in most of the cases on MS medium (Murashige and Skoog 1962) or woody plant medium (WP medium) (Lloyd and McCown 1981) supplemented with cytokinins. Many factors are reported to affect shoot differentiation including concentration and type of auxin and cytokinin (Lakshmanan et al. 1977) and ethylene (Goh et al. 1997). Adventitious shoot induction occurs from leaf segments cultured on WP medium supplemented with 20 μM benzyladenine (BA) and 20 g/L sucrose. However, number and frequency of shoot formation and further growth of shoots depends on culture medium, phytohormones supplied to the medium, size and orientation of explants (Goh et al. 1990), source of explants (Goh et al. 1994) and ethylene content in the culture vessel (Goh et al. 1997; Lakshmanan et al. 1977). Callus mediated organogenesis is reported from leaf segments of mangosteen on MS medium supplemented with 2.22 μM benzyladenine (BA) and 2.25 μM thidiazuron (TDZ) (Te-Chato and Lim 2000). Callus formed on leaf explants developed shoots on WP medium with 0.44 μM BA. Shoot elongation was achieved on above said medium over-layered with half strength MS liquid medium containing 0.32 μM naphthaleneacetic acid (NAA) and 0.13 μM BA. Rooting of shoots was obtained on WP medium with 1.11 μM BA and 0.25% activated charcoal. The micropropagation protocols developed by various scientists are useful for large-scale multiplication of elite clones as well as conservation of germplasm as slow-growing shoot cultures (Fig. 19.11).

19.6.2 Micropropagation of Kokum

In vitro propagation of kokum was achieved by using immature/mature seed and root explants on WP or MS medium supplemented with benzylaminopurine (BAP) or benzyladenine (BA), kinetin and TDZ alone and in combination with NAA (Baskaran and Krishnan 2011; Chabukswar and Deodhar 2006b; Deodhar et al. 2008; Kulkarni and Deodhar 2002; Malik et al. 2005b). The shoot-forming capacity of seed was affected by the concentrations tested (5–50 μM) and optimal response was observe at different concentrations (12.5–50 μM) in different genotypes (Malik et al. 2005b). Elongation of shoots was accomplished on MS medium containing 0.2% activated charcoal. Optimum rooting was attained on half-strength MS medium supplemented with 10 μM indolebutyric acid (IBA). Induction of somatic embryogenesis was reported from immature seeds cultured on WP medium supplemented with BAP (4.44–22.10 μM) alone or in combination with NAA (2.69 μM) (Thengane et al. 2006a, b). Maturation of somatic embryos was achieved on a medium containing BAP (16.08 μM) in combination with IBA (2.85–5.71 μM) and/or kinetin (4.65 μM). Germination of somatic embryos was achieved on WP basal medium. Among the

Fig. 19.11 In vitro plant regeneration in mangosteen. **a** Induction of multiple shoots on seed segments on Woody Plant (WP) medium supplemented with 10 μM benzylaminopurine (BAP). **b** Elongated shoots. **c** Rooting of shoots on WP medium with 5 μM indole butyric acid (IBA). **d** Transplanted plantlets

above reports adventitious shoot induction from seed explants was highly reliable and it could be used propagation of kokum genotypes (Fig. 19.12).

19.7 Conclusions and Prospects

The main constraints to improving mangosteen, Malabar tamarind and kokum are strict climatic requirements, short viability of seeds, lack of rapid propagation methods, slow plant growth and delayed precocity of trees. Therefore, the main breeding objectives to improve these crops would be improving plant growth and development, shortening the juvenile period, increasing the yield and improving the fruit quality. These objectives are attainable using crop-improvement techniques such as natural selection, marker-assisted selection, mutation induction and selection of

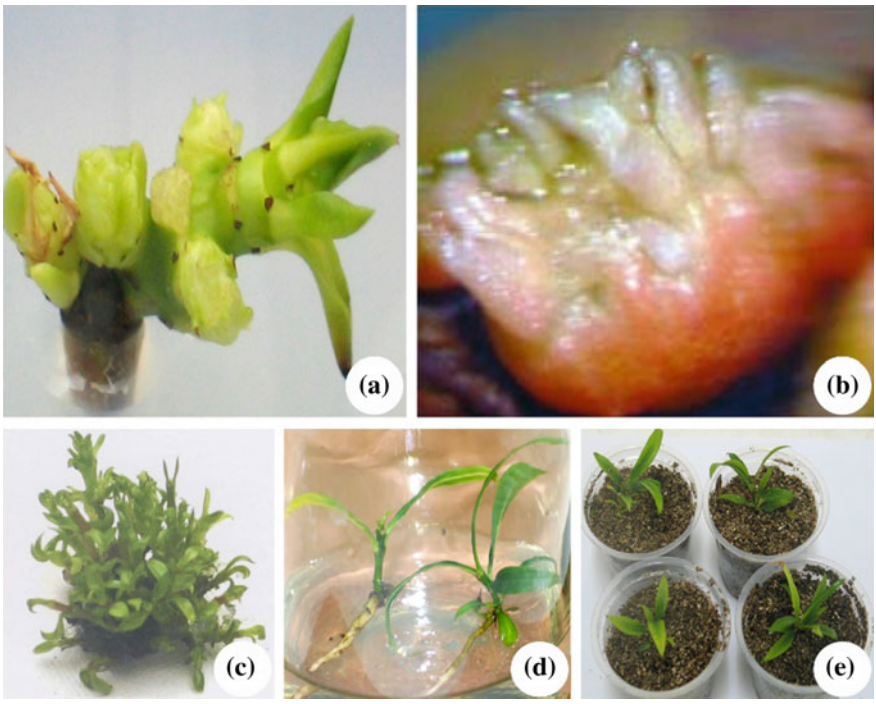

Fig. 19.12 Micropropagation of kokum. **a** Axillary bud induction on WP medium supplemented with 5 μM BAP. **b** Somatic embryos formed on seed explants on WP medium supplemented with BAP and naphthalene acetic acid (NAA). **c** Multiple shoots developed from seed explants. **d** Rooting of the shoots on WP medium with 5 μM IBA. **e** Acclimatized plants

useful mutants. Moreover, non-conventional crop improvement strategies such as in vitro mutagenesis, cell culture, somatic cell genetics, and genetic transformation should also be attempted. Identification of genes coding for important traits, including yield, fruit size and shape are quite useful to improve classical breeding by marker-assisted selection. Improvement of tissue culture techniques such as organogenesis and somatic embryogenesis could resolve limitations of seed as well as vegetative propagation.

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Appendix 1

Research institutes involved in germplasm collection, maintenance and research on mangosteen, Malarbar tamarind, and kokum and online resources

Institute	Location
Centre for Tropical Fruit Studies	Bogor Agricultural University, Kampu IBP, Barnangsiang, JI Pajajaran Bogor 16143, Indonesia
Indian Institute of Spices Research	Calicut 673012, Kerala, India
Prince of Songkla Universit,	Hat Yai 900112, Thailand
Dr. B. S. Sawant Konkan Krishi Vidyapeet	Dapoli, 415712, Maharashtra, India
College of Forestry	University Agricultural Sciences, Sirsi 481 401, Karnataka, India
National Bureau of Plant Genetic Resources (NBPGR) Regional Station	Kerala Agricultural University P.O., Vellanikkera 680 656, Thrissur, Kerala, India
Indian Council of Agricultural Research (ICAR)	Research Complex for Goa, Ela, Old Goa 403402, Goa, India
Central Horticultural Experimental Station	Chettalli 571248, Kodagu, Karnataka, India
Forest Research Institute of Malaysia	Kepong, 52109 Kuala Lumpur, Malaysia
Kasetsart University	Bangkok 10900, Thailand
Osman and Milan (2006) Mangosteen	*Garcinia mangostana*. Southampton Centre for Underutilised Crops, University of Southampton, Southampton, UK. ISBN: 0854328173

Appendix 2

Genetic Resources

1. Centre for Tropical Fruit Studies, Bogor Agricultural University, Kampu IBP, Barnangsiang, JI Pajajaran Bogor 16143 Indonesia has maintained *Garcinia mangostana* L. (mangosteen) germplasm and this center has released new varieties of mangosteen namely Mangosteen Wanayasa, Mangosteen Puspahiang and Mangosteen Malinau.
2. Dr. B. S. Sawant Konkan Krishi Vidyapeet, Dapoli, 415712, Maharashtra, India has maintained *Garcinia indica* Choisy (kokum) germplasm collected from Maharastra, Goa and Karnataka states and this institute has released two elite varieties of komum namely Konakan Amruta (early type, long shelf life and apple-shaped fruits) and Konkan Hatis (high yielding, bold size fruits with thick rind).
3. National Bureau of Plant Genetic Resources (NBPGR) Regional Station, Kerala Agricultural University P.O., Vellanikkera 680 656, Thrissur, Kerala, India has

maintained *Garcinia gummi-gutta* (Malabar tamarind) and G. indica (kokum) collections. This ICAR research center has released Malabar tamarind varieties i.e., IC244100-2 (INGR No.04061) and IC244111-1(INGR No. 04062) which are early fruit-bearing varieties (6–7 years) and kokum variety IC136687-3 (INGR No. 04063) which is a high-yielding variety.

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Chapter 20
Mango (*Mangifera indica* L.) Breeding

Ian S. E. Bally and Natalie L. Dillon

Abstract Mango *Mangifera indica* L. in the family Anacardiaceae is a large evergreen tree that grows throughout the tropical and subtropical regions of the world and bears a popular tropical fruit that is consumed locally and traded globally. Preferred mango varieties vary from country to country, with subcontinental Asian varieties typically being monoembryonic and South East Asian varieties being polyembryonic. The genus *Mangifera* consists of many species with *M. indica* being the most abundant, however several of the other species are graft and pollination compatible with *M. indica* and are useful as rootstocks or sources of novel genes for breeders. Mango presents several challenges for breeders; it is a recalcitrant species with seed viability declining rapidly, weeks, after fruit maturity, Polyembryonic varieties can only be used as pollen parents and one seed per fruit limits the number of progeny that can easily be generated per family. Incompatibility within and between species makes hand pollination success rates lower than many other species. Knowledge of the genes and gene markers associated with important traits is in its infancy, however there is considerable effort internationally to improve the knowledge of mango genetics. This chapter on mango breeding includes many recent advances in the biotechnological aspects of mango breeding that have occurred over the past decade. The chapter looks at the tools required to carry out a modern mango breeding program reviewing genetic resources, breeding program goals, stages and design and molecular breeding tools such as marker assisted selection, saturated linkage maps and transcriptome sequencing.

Keywords *Mangifera indica* · Fruit · Hybridization · Marker assisted breeding

I. S. E. Bally (✉) · N. L. Dillon
Department of Agriculture, Fisheries (DAF), Queensland Government,
28 Peters Street, Mareeba, QLD 4880, Australia
e-mail: ian.bally@daf.qld.gov.au

N. L. Dillon
e-mail: natalie.dillon@daf.qld.gov.au

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20.1 Introduction

Mango by production is the third largest tropical fruit crop in the world behind bananas and pineapple. Mangoes are produced in over 100 countries in the tropical and subtropical regions with global production estimated around 43 million mt per annum growing on 5.4 million ha according to Food and Agriculture Organization statistics. India dominates production accounting for some 41.6% of total global production with China the next largest producer with 10.0%. The international export trade in mangoes is 9.5 million tons per annum, which is about 3.5% of world production (Galán Saúco 2015). The reasons for the low export volumes lies in the highly perishable nature of mangoes and their importance to local rural economies of many tropical countries; in spite of large volumes of production almost all of the product is consumed domestically in the country of origin.

With the mangoes that are grown and traded internationally, the majority are cultivars originating in Florida, renowned for their strong blush color and longer shelf life. Cultivars such as Tommy Atkins, Kent and Keitt, and to a lesser extent Palmer, Haden and Irwin dominate global trade. These cultivars are well-colored with a well-balanced sugar/acid ratio, have transport resistance and long shelf life (Galán Saúco 2015). Markets with close proximity to the country of origin have enabled export markets to develop based on more perishable cultivars such as Carabao from the Philippines and Sindri from Pakistan. In recent years the USA has seen greater trade in cultivars such as Atlulfo, Madam Francis, Calypso; and European Union markets in cvs. Maya, Aya, Omer, Shelly, Chounsa, Shindri and Alphonso (Galán Saúco 2015). The developments in plant breeders' rights (PBR) has seen the exclusive commercial development of many new mango varieties and a renewed interest in breeding and selection programs.

Before initiating a mango breeding program, it is well worth considering the time (~20 years) and investment required before a new improved variety is produced. Other issues to consider are: the current status of the industry in the country; are current cultivars meeting market and grower requirements; or are there existing cultivars elsewhere that can meet market requirements. A breeding program needs to consider if it aims to replace cultivars in existing markets, to increase the variety available or to develop new domestic or international markets. These questions will greatly influence the way a mango genetic improvement or breeding program is conducted. It is also worth assessing the genetic resources available for breeding to determine if they have the diversity or genes and traits being targeting. Lastly, the availability of essential skills, capacity and financial backing to undertake a long-term program.

20.2 Botany

20.2.1 Morphological Description

The common mango (*Mangifera indica* L.) and closely related species, belong to the family Anacardiaceae that consists of dicotyledonous trees and shrubs. Mango trees are evergreen with branched, upright to spreading dense canopies that can grow to heights of 30 m. The tree is supported by one to several deep taproots and abundant surface feeder roots. Trees are long-lived with many specimens reaching more than 100 years of age. The canopy consists of dark green, simple, alternate leaves, oval-lanceolate to roundish-oblong in shape. New leaves are produced in periodic flushes with the color tone of expanding leaves varying between tan and red. Hundreds of hermaphrodite and male flowers are borne on branched conical panicles that grow from the branch terminals. Fruits are drupes variable in size, shape and color, with a fleshy mesocarp, the edible part of the fruit. Each fruit contains a single seed enclosed in a stony endocarp. Seed embryos can be either monoembryonic or polyembryonic depending on the genotype.

20.2.2 Cytology

Most *Mangifera* species are reported to be diploid with chromosome numbers of $2n = 40$ and $n = 20$ (Iyer and Degani 1997; Kostermans and Bompard 1993; Mukherjee 1950a, 1963), although there is some suggestion that mango evolved through amphidiploidy (Mathews and Litz 1992; Mukherjee 1950a; Yonemori et al. 2002, 2010), the high variability in leaf width and thickness and other morphological traits between varieties of *M. indica* supports a polyploidy and hybrid origin (Singh 1960). Recently two spontaneous tetraploid mango seedlings were identified in the Canary Islands, Spain. A putative tetraploid Gomera-1 was confirmed using both flow cytometry and chromosome count analyses (Galán Saúco et al. 2001). In Katherine, Australia, another tetraploid common mango was confirmed by chromosome count (Rayner and Stace, unpublished). Currently both these tetraploid mangoes are used for rootstock breeding purposes. The similarity of ploidy between the *Mangifera* species indicates that interspecific crossing should be feasible. Several authors have expressed the value and contribution that interspecific crosses between mango and many wild *Mangifera* species can improve cultivated varieties (Bompard 1993; Iyer and Degani 1997; Mukherjee 1963).

20.2.3 Anthesis and Pollination

The mango inflorescence is primarily terminal. On a panicle the total number of flowers may vary from 1000 to 6000 depending on the cultivar (Mukherjee 1953a). Flowers are usually small, 5–10 mm in diameter. Both perfect (hermaphrodite) and male flowers (Fig. 20.1) occur on the same panicle. The ratio of perfect to male flowers varies with cultivar (usually <50%) but is strongly influenced by environmental and cultural factors, e.g. cool temperatures usually reduce the number of perfect flowers, which is more pronounced in tropically evolved polyembryonic cultivars (Davenport and Nunez-Elisea 1997). Anthesis starts early in the morning and generally completes by noon. Stigma receptivity begins 18 h before anthesis and remains for about 72 h, but is most receptive during the first 6 h after anthesis (Iyer and Schnell 2009). Flowering on an individual panicle is sequential from the base to the distal end can occur over a 3-week period depending on temperature and other environmental conditions. Flowering within a tree can be extended for up to 8 weeks due to differential timing of panicle emergence, providing an extended period of pollination availability and fruit set.

20.2.4 Pollination and Compatibility

Pollination occurs when pollen attaches itself to the stigmatic surface of the style in hermaphrodite flowers (Ramírez and Davenport 2012) and pollen tube germination and growth can start as soon as 1½ h after attaching to the stigma (Sen et al. 1964; Singh 1954; Spencer and Kennard 1955), but varies with cultivars and temperature (Dutta et al. 2013b). Fertilization occurs 48–72 h after pollen deposition (Huang et al. 2010; Singh 1961). The viability of pollen is at its highest at dehiscence and degrades with time and cool temperatures (Davenport 2009). Sensitivity of pollen development to cool temperatures (<15 °C) causes reduced viability of 30–40% and flower deformation (Davenport 2009; Issarakraisila and Considine 1994). The optimum temperatures for pollination and fertilization are 22 °C day and 22 °C at night (Dag et al. 2000; De Wet et al. 1989; Huang et al. 2010).

Mango is generally pollinated by insects, but wind and gravity pollination cannot be excluded (Maheshwari 1934). The percentage of fruit set on a mango panicle varies. Usman et al. (2001) reporting less than 1% set, being dependent on pollination compatibility of cultivars, the number of hermaphrodite flowers, pollen viability and the environmental conditions during pollination. Pollination self-incompatibility has been demonstrated in the cvs. Dashehari, Langra, Chausa and Bombay Green (Desai and Bhandwalkar 1995; Mukherjee et al. 1968; Sharma and Singh 1970; Singh et al. 1962); however, the situation with cv. Langra is unclear for Narayana Swamy et al. (1989) found it to be self-fertile.

There are also many instances of differential fruit set and compatibility reported between cultivars. Bally et al. (2000) reported varying success rates of hybridization

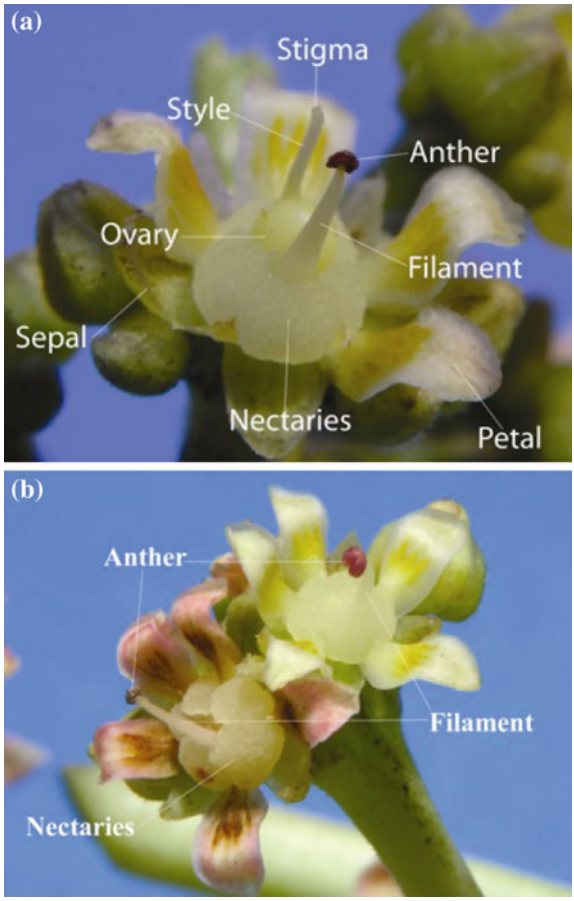

Fig. 20.1 Mango flowers. **a** Hermaphrodite. **b** Male

between different parental combinations due to a mixture of genetic, environmental and technical factors. They were unable to produce any hybrids from controlled crossing of cvs. Edward, Kensington Mono and Magovar as maternal parents with Kensington Pride. However, the bunch-bearing cultivars Creeping and Willard were more successful with 100% of crossed panicles bearing one or more hybrid fruit. Robbetse et al. (1994) suggested that there were clear indications of differences in receptivity existing among the cultivars, with the time of pollination and female parent receptivity playing an important role in pollination success. They found the pollination success rate of cvs. Tommy Atkins, Sensation and Kent to be much higher in the morning than in the afternoon, while in Isis and Keitt, the reverse was observed.

The source of pollen in outcrossing has also been shown to have an impact on the percentage of fruit set. Sensation cv. was shown to be the best pollinator among the Florida cultivars (Robbertse et al. 1993). Desai and Bhandwalkar (1995) showed that Kesar pollen increased the percentage of fruit set in cvs. Alphonso and Vanraj

but reduced it in Totapuri and Baramasi compared with selfing. Pérez et al. (2016) confirmed mangoes' preference for the retention of outcrossed embryos over self-pollinated ones. The many factors impacting on pollination make it difficult to predict the pollination success of any particular parental combination.

20.2.5 Heritability of Genetic Traits in Mango

In mango there have been relatively few studies on the genetic inheritance of traits due to the difficulties in generating suitable populations for the purpose. In open-pollinated populations the pollen parent is not easily identified. The high heterozygosity and small size of populations have also limited the suitable analyses; however, a few studies have provided some guidelines for breeders.

A study by Sharma and Majumdar (1988) of various hybrid populations made over 25 years indicated that recessive genes control traits such as dwarfing and regularity of bearing, while additive genes control traits such as fruit size and skin color. Lavi et al. (1998) studied correlations between genetic and phenotypic traits in an open-pollinated population of mainly Floridian cultivars and found the non-additive genetic variance was a major and significant component of total genetic variance and additive genetic variance was non-significant. They found the progeny performance was not easily predicted from parental performance and recommended a widening of the genetic background of the parents in breeding programs. However, in later work Brettell et al. (2004) demonstrated traits such as fruit weight, shape, flavor and color were highly heritable, giving confidence in systematic selection for these traits. The high heterozygosity and lack of information on heritability on many traits are a challenge for mango breeders. Hardner et al. (2013) successfully employed mixed linear model methods incorporating a pedigree to predict breeding values for average fruit weight from highly unbalanced data for genotypes planted over three field trials and assessed over several harvest seasons. The heritability of many other traits are discussed in the section on breeding objectives.

20.3 Genetic Resources

20.3.1 Origin and Dissemination

Today, *Mangifera* species are found growing throughout the tropical and subtropical world, with the greatest species diversity in the Southeast Asian regions of the Malay Peninsula, the Indonesian archipelago, Thailand, Indochina and the Philippines (Bompard 1989; Bompard and Schnell 1997; Mukerjee 1985). *Mangifera* is one of 76 genera in the family Anacadiaceae and was most recently classified into 69 species, split into two subgenera, *Mangifera* and *Limus,* by Kostermans and

Bompard (1993) using floral morphology. Since that time several authors have begun to classify sections of the *Mangifera* with molecular tools such as RFLPs and SSRs (Dillon et al. 2013a, b, 2014a, b; Eiadthong et al. 2000; Schnell et al. 2005, 2006; Sennhenn et al. 2011; Singh et al. 2012; Tsai et al. 2013; Viruel et al. 2010). The application of molecular phylogeny in the near future is expected to clarify much of the confusion between groupings of species found in earlier classification systems.

The most commonly cultivated species is *Mangifera indica* representing most of the commercially cultivated trees. Another 26 species also have edible fruit, including *M. altisima, M. caesia, M. foetida, M. kemang, M. laurina, M. odorata, M. panjang* and *M. pentandra* being traditionally consumed in various Southeast Asian communities (Bompard and Schnell 1997; Gruezo 1992; Mukherjee 1997).

The common mango (*Mangifera indica*) is thought to have originated in the Assam valley in Myanmar in the Quatenary Period (Mukherjee 1953a) and domesticated independently in several areas to the southwest and southeast of the center of origin. Domestication in the Indian region (southwest) gave rise to the monoembryonic varieties and domestication in the Indochina, Thailand and Myanmar regions (southeast) gave rise to the polyembryonic varieties (Bompard and Schnell 1997; Kanjilal and Kanjilal 1937). Cultivation of monoembryonic mangoes in India for over 4000 years has resulted in over 1000 named varieties (Mukherjee 1953a). Domestication and discrimination of the common mango throughout the tropical and subtropical world has been associated with the migration of people and trade within and between regions (Bompard and Schnell 1997; Duval et al. 2006; Mukherjee et al. 1983). Today mangoes are grown throughout the tropics on all continents and in many subtropical areas. Table 20.1 lists mango cultivars developed through breeding and selection and their significant characteristics.

Many of the current internationally-traded mango varieties grown in the world today originated from Florida, USA during the twentieth century. Mango varieties from many regions of the world were introduced to Florida from 1861, resulting in a wide diversity of mango germplasm in the area (Knight 1980). This diversity and mangoes' tendency for natural outcrossing, led to the production of hybrid seedlings with novel characteristics. In 1910 a high-yielding monoembryonic seedling from the variety Mulgoba, with an attractive red blush, was selected in Florida and named Haden. Following Haden. other seedling progeny selection were made from Mulgoba, Haden and other introduced cvs., such as Keitt, Kent, Tommy Atkins, Glenn, Lippens, Van Dyke, Parvin, Springfels and Zill (Campbell 1992; Knight and Schnell 1994). The success of the Florida varieties is due to their adaptability to many agroclimatic regions while retaining their fruit quality and regular-bearing characteristics. Detailed descriptions of Florida mango varieties have been published by Campbell (1973, 1992), Campbell and Campbell (1993), Campbell and Mallo (1976) and Knight and Schnell (1993).

Table 20.1 Mango cultivars developed by breeding and selection programs and details of their parentage, year developed, significant characteristics, breeding institution, and source of information

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
13/1 (rootstock)	Unknown	Unknown		Rootstock selected for salinity resistance	Israel	Gazit and Kadman (1980)
A2-A12			2005	PBR protected	ARC-Institute for Tropical and Subtropical Crops, South Africa	Human et al. (2009)
A2-B26			2005	PBR protected	ARC-Institute for Tropical and Subtropical Crops, South Africa	Human et al. (2009)
A2-CD28	Fascell		2005	PBR protected, high yielding, mid-season cultivar with an attractive pink blush	ARC-Institute for Tropical and Subtropical Crops, South Africa	Human and Rheeder (2004)
A67P	Sensation	Kensington Pride			Queensland, Australia	IP Australia (2006)
Akio	Kino (Chinqo, Taiwan)	Irwin		Fruit 600–700 g	Kinki University, Japan	Kanzaki S, personal communication
Akra Puneet				Similar to Alphonso with higher pulp yield	Indian Institute of Horticultural Research, Bangalore, India	Gowda and Huddar (2000), Iyer and Subramanyam (1993)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
Alfa (Embrapa 142)	Mallika	Van Dyke	1998	Semi-dwarf, high yielding and some resistance to fruit flies, anthracnose and powdery mildew	EMBRAPA, Brazil	Pinto et al. (2004, 2006), Rossetto et al. (2006)
Alfazli	Alphonso	Fazlicw	1980	Similar to Fazli but bears early	Bihar Agricultural College, Sabour Agricultural Institute, Sabour, Bihar, India	Hoda and Ram Kumar (1993)
Amrapali	Dashehari	Neelum		Dwarf, regular-bearing and late maturing	Indian Agricultural Research Institute (IARI), New Delhi, India	Singh et al. (1972)
Ambika	Amrapali	Janardhan Pasand		Low fiber, red blush	Central Institute of Subtropical Horticulture (CISH), Lucknow, India	Iyer and Schnell (2009)
Arka Anmol	Alphonso	Janardhan Pasand		Regular bearer and good yielder. Fruits are medium-sized with uniform yellow peel color, excellent keeping quality and free from spongy tissue	Indian Institute of Horticultural Research, Bangalore	Gowda and Huddar (2000), Iyer and Dinesh (1997), Iyer and Subramanyam (1993)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
Arka Aruna	Baganapalli	Alphonso		Dwarf, regular bearing, early bearing. Fruits are large with attractive skin color and red blush, free from spongy tissue	Indian Institute of Horticultural Research, Bangalore	Iyer and Subramanyam (1993)
Arka Neelkiran	Alphonso	Neelum		Late season, regular bearing variety with medium-sized fruits and attractive red blush, free from spongy tissue. Good pulp color, excellent skin color, and the tree is semi-vigorous	Indian Institute of Horticultural Research, Bangalore	Iyer and Schnell (2009)
Arka Puneet	Alphonso	Banganapalli		Regular and prolific bearer. Fruits are medium-sized with attractive skin color and red blush, and free from spongy tissue. Excellent keeping quality	Indian Institute of Horticultural Research, Bangalore	Iyer and Subramanyam (1993)
Arunima	Amrapali	Sensation			IARI, New-Delhi, India	Iyer and Schnell (2009)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
Au-Rumani	Rumani	Mulgoa		Large fruits with yellow cadmium skin color. Good eating quality	Fruit Research Station, Kodur, India	Iyer and Dinesh (1997)
B74P (Calypso ™)	Sensation	Kensington Pride		PBR protected	Department of Primary Industries Queensland, Australia	Whiley (2000)
Beta	Amrapali	Winter	1998	High yield, moderate resistance to powdery mildew	Embrapa Cerrados Brazil	Pinto et al. (2006)
Bundy special			2004	PBR protected	Private, Australia	IP Australia (2004)
C2-E17			2005	PBR protected	ARC-Institute for Tropical and Subtropical Crops, South Africa	Human et al. (2009)
CISH M1	Amrapali	Janardan Passand		Yellow fruit with red blush; firm and fibreless	CISH, Lucknow, India	Gunjate (2009)
CISH M2	Dashehari	Chousa		Dark yellow firm flesh fruit, late season, sooty mold free	CISH, Lucknow, India	Gunjate (2009)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
Carabao				Has low temperature resistance as a rootstock. Resistant to the disease mango sudden death, Ceratocystis fimbriata Ell & Halst.	Philippines	Ribeiro et al. (1995, 2002)
Ceriese					Citrus and Subtropical Fruit Research Institute, Nelspruit, South Africa	Snyman (1990)
Chene	Kent	Unknown	1996	Resistant to sun scorch and tolerant of bacterial black spot. Mid-season, weak flavor	ARC-Institute for Tropical and Subtropical Crops, South Africa	Human and Rheeder (2004), Le Lagadec and Kohne (2004)
CPAC 23/86	Amrapali	Winter	Not yet released	Very low susceptibility to fruit fly attack and very low resistance to malformation; high yield and very sweet	Embrapa Cerrados, Brazil	Pinto et al. (2005, 2006)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
CPAC 22/93	Amrapali	Winter	Not yet released	High yield, very sweet, excellent fruit color and low resistance to fruit fly attack	Embrapa Cerrados, Brazil	Pinto et al. (2005)
CPAC 165/93	T. Atkins	Mallika	Not yet released	Low fruit fly attack resistance and medium malformation, high yield and good pulp color	Embrapa Cerrados, Brazil	Pinto et al. (2005, 2006)
CPAC 329/94	T. Atkins	Mallika	Not yet released	Excellent fruit color, very sweet, medium resistance to fruit fly attack, and high yield	Embrapa Cerrados, Brazil	Pinto et al. (2005, 2006)
CPAC 58/95	Tommy Atkins	Winter	Not yet released	Very sweet, low fruit fly resistance and medium malformation resistance; absence of internal breakdown	Embrapa Cerrados, Brazil	Pinto et al. (2005, 2006)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
Delta R2E2	Kent	Kensington Pride	1982	Large fruit with firm flesh	Department of Primary Industries, Bowen, Australia	Bally (1998)
DolceP	Kensington Pride	Unknown	2008	PBR protected.	Private, Australia	IP Australia (2008a)
Heidi	Kent	Unknown	1990	Late season, high-yielding, large fruit (498–587 g)	Citrus and Subtropical Fruit Research Institute, Nelspruit South Africa	Cilliers et al. (1997), Snyman (1990)
Honey GemP	Kensington Pride	Ono	2005	PBR protected	Private, Australia	IP Australia (2005)
Honey GoldP	Kensington Pride	Unknown	1999	PBR protected	Private, Australia	IP Australia (1999)
IAC 101 Coquinho			1994	Rootstock resistant to the disease mango sudden death Ceratocystis fimbriata Ell & Halst.	APTA/Polo Noroeste Paulista Votuporanga, SP, Brazil	Rossetto et al. (1997)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
IAC 102 Touro			1994	Rootstock resistant to the disease mango sudden death Ceratocystis fimbriata Ell & Halst.	APTA/Polo Noroeste Paulista Votuporanga, SP, Brazil	Rossetto et al. (1997)
IAC 103 Mococa				Scion resistant to the disease mango sudden death Ceratocystis fimbriata Ell & Halst. and fruit fly	APTA/Polo Noroeste Paulista Votuporanga, SP, Brazil	Rossetto et al. (1997)
IAC 104 Dura				Rootstock resistant to the disease mango sudden death Ceratocystis fimbriata Ell & Halst.	APTA/Polo Noroeste Paulista Votuporanga, SP, Brazil	Rossetto et al. (1997)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
IAC 105 Campinas				Scion resistant to the disease mango sudden death Ceratocystis fimbriata Ell & Halst.	APTA/Polo Noroeste Paulista Votuporanga, SP, Brazil	Rossetto et al. (1997)
IAC 106 Jasmine				Rootstock resistant to the disease mango sudden death Ceratocystis fimbriata Ell & Halst.	APTA/Polo Noroeste Paulista Votuporanga, SP, Brazil	Rossetto et al. (1997)
IAC 107 Tiete				Fruit fly resistant	APTA/Polo Noroeste Paulista Votuporanga, SP, Brazil	Rossetto et al. (1997)
Imperial				Semi-dwarf variety	Brazil	
Jawahar	Gulabkhas	Mahmood Bahar	1989	Precocious bearer, fiberless, attractive shape and high pulp content. Early bearing	Bihar Agricultural College, Sabour	Hoda and Ram Kumar (1993)
Joa	Palmer		1996	Mid-season, PBR protected, tolerant to bacterial black spot	ARC-Institute for Tropical and Subtropical Crops, South Africa	Human and Rheeder (2004)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
Kensington Grossman	Kensington Pride	Kensington Pride	1988	Reputed to have some tolerance of bacterial black spot.	Australia	Mayers et al. (1984)
Kokan Ruchi	Neelum	Alphonso		Regular heavy fruiting, suitable for pickle	RFRS, Vengurla, India	Gunjate (2009)
Kensington red	Kensington Pride		1988	Improved red blush color	Private, Australia	IP Australia (1988)
Lita	Amrapali	Tommy Atkins	2002	High yielding, excellent fruit quality, late fruiting and regular bearing	EMBRAPA, Cerrados, Brazil	Pinto et al. (2006)
Maçã	Unknown	Unknown		Dwarf behavior, small fruit, highly susceptible to anthracnose	Brazil	
Mahmud Bahar	Bombay	Kalapady		Regular bearing	Sabour Agricultural Institute	Roy et al. (1956)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
Mallika	Neelum	Dashehari		Large mid-season variety, high TSS, regular bearer	IARI, New Delhi, India	Singh et al. (1972)
Manga d'Agua	Unknown	Unknown		Resistant to mango sudden death Ceratocystis fimbriata Ell & Halst.	Brazil	Ribeiro et al. (1995)
Manjeera	Rumani	Neelum		Good eating quality dwarf, regular and prolific bearer with firm and fibreless flesh	Fruit Research Station, Sangareddy, Andhra Pradesh, India	Iyer and Dinesh (1997)
Maxima	Fascel	Kensington Pride	2000s	PBR Protected, prolific, regular bearer, long shelf life, late maturing	Private, Australia	Aquilizan (2012)
MinjacP	Nam Doc Mai	Unknown	2007	PBR protected	Private, Australia	IP Australia (2007)
Namoi	Palmer				Israel	Tomer et al. (1993)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
Neeleshan	Neelum	Baneshan		Good eating quality, regular, heavy late fruiting	ARS, Paria Research Station, Gujarat, India	Sachan et al. (1989)
Neeleshwari	Neelum	Dashehari		Good eating quality, dwarf, regular, late fruiting	ARS, Paria Research Station, Gujarat, India	Sachan et al. (1989)
Neelgoa	Neelum	Yerra Mulgoa		Big fruit with red blush, regular cropping, moderate yield	ARS, Ananatrajupeth, Andhra Pradesh	Iyer and Schnell (2009)
Neelphonso	Neelum	Alphonso		Regular medium yield, small fruit, good eating quality	ARS, Paria Research Station, Gujarat, India	Sachan et al. (1989)
Neeludin	Neelum	Himayuddin			Kodur, AP, India	Iyer and Schnell (2009)
Neldawn			1990		Citrus and Subtropical Fruit Research Institute, Nelspruit, South Africa	Cilliers et al. (1997)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
Neldica	Palmer		1990	Mid-season, heavy regular bearer, prone to internal breakdown, lenticel spotting and off flavors	Institute for Tropical and Subtropical Crops, South Africa	Le Lagadec and Kohne (2004)
NMBP1201P	Irwin	Kensington Pride	2009	High yield with good blush color and Kensington flavor, PBR protected	QDPI, Australia	IP Australia (2008c)
NMBP1243P	Irwin	Kensington Pride	2009	Early cropping with good blush color and Kensington flavor, PBR protected	QDPI, Australia	IP Australia (2008d)
NMBP4069P	Van Dyke	Kensington Pride	2009	High yield with good blush color and Kensington flavor, PBR protected	QDPI, Australia	IP Australia (2008b)
Pico				Resistant to the disease mango sudden death, Ceratocystis fimbriata Ell & Halst.	Philippines	Ribeiro et al. (1995)

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
President P	Sensation	Sabre		Late season, deep crimson color, slightly elongated with a prominent beak	Mr Tabanelli, Tzaneen, South Africa	Le Lagadec and Kohne (2004)
Probha Shankar	Bombay	Kalapady		Resistant to fruit fly	Sabour Agricultural Institute, India	Roy et al. (1956)
Pusa Pratibha, Pusa Shreshta and Pusa lalima	Dashehari	Neelum		Resistant to mango malformation, and other pests, regular bearer, long shelf life, high yield	Indian Agricultural Research Institute	Rituraj and Bhosale (2012)
Pusa Surya	Eldon	?		Fruit is apricot yellow with red blush, late season maturing, heavy and regular bearing	IARI, New Delhi, India	Iyer and Schnell (2009)
Pusa Arunima	Amrapali	Sensation		Regular heavy bearing, semi-vigorous, red blush	IARI, New Delhi, India	Gunjate (2009)
Ramung	Xiangmang	Unknown			China	

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
Ratna	Neelum	Alphonso		Tree vigorous and precocious; fruits are medium sized, attractive in color and free from spongy tissue	Fruit Research Station, Vengurla, Maharashtra, India	Salvi and Gunjate (1989)
PKM-1	Chinna-swarnarekha	Neelum	?	Regular bearing, bunch-bearing, good quality	HRS, Periyakulam, TN, India	Gunjate (2009)
PKM-2	Neelum	Mulgoba		Regular bearing and good quality	HRS, Periyakulam, TN, India	Gunjate (2009)
Roxa (Embrapa 141)	Amrapali	Tommy Atkins	1998	High yield, fibreless, very sweet, low resistance to anthracnose and bacterial black spot	EMBRAPA Cerrados, Brazil	Pinto et al. (2000, 2006)
Sabri	Gulabkhas	Bombai	1989	Shape of Bombay, color of Gulabkhas, orange pulp and a regular bearer	Bihar Agricultural College, Sabour, India	

(continued)

Table 20.1 (continued)

Hybrid cultivar P (plant breeders' rights)	Maternal parent	Paternal parent	Year released	Comment	Breeding institution and country	Sources
TEF 02	Haden	Keitt		Late season, good bearer, PBR protected	South Africa	Le Lagadec et al. (2009)
Sonpari	Alphonso	Beneshan		Regular bearing, excellent quality, free from spongy tissue	ARS, Paria Research Station, Gujarat, India	Gunjate (2009)
Shelly	Tommy Atkins	Keitt	1997	Late season, small tree	ARO, Israel	Lavi et al. (1997b)
Sindhu	Ratna	Alphonso		Parthenocarpic, nonviable seed resulting in high flesh recovery	Fruit Research Station, Vengurla, Maharasatra, India	Salvi and Gunjate (1989)
Sundar Langra	Sundar pasand	Langra		Regular bearing	Sabour Agricultural Institute, Bihar, India	Hoda and Ram Kumar (1993)
Sundralangra	Langra	Sundar Prasad	1980	Similar to Langra but regular bearing	Bihar Agricultural College, Sabour	
Swarnajehangir	Jehangir	Chinnaswarnarekha		High yielding	Kodur, AP, India	Iyer and Schnell (2009)
Tango	Naomi	Keitt	1989		ARO, Israel	Lavi et al. (1997a)
TP1P	Kensington Pride	Unknown	1998	PBR protected, prolific, regular bearer, no stem end cavity	Private, Australia	IP Australia (1998)
Virginia	Van Dyke	Kensington Pride	2000s	PBR protected	Private Australia	Aquilizan (2012)

20.3.2 Genetic Resource Utilization

Although a relative few mangoes are considered good commercial cultivars suited to national and international trade, there are many other local minor varieties with interesting characteristics that form a vast genetic resource to the mango breeder. Local selected and wild varieties are often well-adapted to local environmental conditions, and provide a useful source of locally-adapted genes. There have been considerable efforts in the past to collect and document mango varieties in many countries.

Many wild species of *Mangifera* have been cultivated by local villagers in their native origins indicating they have traits of some benefit. Recently there has been a renewed focus on the potential of related *Mangifera* species as rootstock or for breeding. To date most breeding has been confined to *M. indica*. Eiadthong et al. (2000) and Yonemori et al. (2002) and Nishiyama et al. (2006) found several species (*M. laurina, M. sylvatica, M. obolongifolia, M. foetida, M. odorata* and *M. macrocarpa*) to be closely-related to *M. indica* by molecular studies and would be potentially comparable for breeding. The graft compatibility between *M. indica* and many of these species (Bompard 2009) also points to rootstock of breeding compatibility.

Bompard (2009) reports from various sources. Successful grafting of *Manginera indica* on to *M. foetida, M. odorata, M. zeylanica, M. laurina, M. casturi*, *M. cesia* and *M. kemanga*. Since then Campbell and Ledesma (2010) and Ledesma et al. (2017) have successfully crossed *M. rubropetala*, *M. casturi*, *M. lalijiwa*, *M. quadrifida* and one undetermined *Mangifera* species. The authors have also confirmed graft compatibilities between *M. laurina*, *M. quadrifida, M. casturi* and *M. rubropetala*. The use of these and other species may prove useful in poorly-drained or periodically-inundated soils, for other abiotic tolerances or for vigor reduction of scions.

20.4 Current Status and Breeding Efforts

20.4.1 Breeding Prospects

The prospects for breeding mangos suited to international production and trade are improving with time. Improvements in genetic sciences, genomics, progeny analyses and the existence of several mature mango breeding programs have advanced the understanding of mango genetics. Studies linking genetic expressions with essential aspects of mango physiology and phenology are also informing breeders. It is likely that in the future mango cultivars from breeding programs will have enhanced productivity, disease and pest resistance, improved shelf life and high fruit quality.

20.4.2 Breeding Principals

Mango, like many other tropical fruit crops, is relatively underdeveloped with most improvements coming from natural selection over many generations. However, with increasing costs of production and modern consumer and market demands on fruit, the traditional varieties are not always able to successfully compete. Systematic breeding programs are needed to improve the productivity and fruit quality of mango varieties to enable them to compete in markets today.

Improvement of mango through breeding has traditionally been slow and challenging, with breeding programs often taking more than 20 years to produce their first cultivars for commercial release. There are a number of characteristics that contribute to the slowness and small size of conventional breeding programs in mango, such as, long generation times, long juvenile stage, high heterozygosity, polyembryony, high natural fruit shedding and cultivar incompatibility. Management of these aspects in mango breeding programs is covered in more detail later in this chapter. Despite these difficulties, the wide genetic pool available in mango and the ability to vegetatively propagate improved selections at any stage of the breeding process (Iyer and Degani 1997) allows highly heterozygous progeny to be released as cultivars and genetic gain to be captured.

20.5 Breeding Program Stages and Design

Most mango-breeding programs can be divided into several stages such as varietal screening and utilization, progeny generation, primary evaluation, secondary evaluation, regional evaluation, new cultivar release and post-release support. Each stage can take several too many years, and efficiency at each phase is necessary to minimize the time taken to achieve genetic gain.

20.5.1 Varietal Screening and Utilization

Varietal screening and utilization is an essential part of any mango breeding program, especially in the early stages. The early phase enables the breeder to fully assess local and exotic varieties responses and adaptability to local growing conditions and to assess them for suitability. Varietal screening also identifies accessions with the necessary traits to achieve specific breeding goals and potential breeding parents.

20.5.2 Parental Selection

Parental selection is primarily dependent on the goals of the breeding program and the traits desired in the progeny. Parental selection has traditionally been guided by the phenotypic trait expression in potential parents, but with the general poor understanding of trait heritability in mango and their heterozygotic nature, prediction of progeny performance based on parental phenotypes is not always possible. However, analysis of current breeding populations for trait heritability is likely to produce improved knowledge of the inheritance of specific traits in specific families. Molecular genotyping is another technology that is beginning to help identify mango accessions that have a specific trait and are likely to pass on that trait to progeny.

Polyembryonic cultivars are not suitable as maternal parents as identification and recovery of the zygotic embryo among the nucellar embryos in the hybrid seed is not easily achieved without molecular identification and embryo-recovery techniques (Degain et al. 1993). Generally, polyembryonic varieties have been used as male parents.

20.5.3 Hybridization

There are several methods that can be used to generate breeding populations in mango. These are covered in more detail in the breeding techniques section below.

20.5.4 Preliminary Progeny Evaluation

Early selection of hybrids during the preliminary evaluation phase is essential to allow early setup of the secondary evaluation trials to reduce the overall time of the breeding program, even if additional years of evaluation are needed for confirmation of selections. Preliminary evaluation is usually done on a single tree basis to reduce the land, management and time resources required to grow large populations. Early selection can be achieved using traits that lend themselves to pre-selection, that is, traits that can be evaluated during the juvenile stage. Examples of pre-selectable traits are disease tolerance based on leaf assays, or fruit flavor based on the leaf volatiles (Majumder et al. 1972; Whiley et al. 1993). Another form of pre-selection is marker-assisted selection (MAS) that uses molecular markers linked to phenotypes or genes of interest. Although there are currently only a few developed molecular markers available for mango breeders, research and development of markers linked to traits of interest in mango is being conducted in several countries and the availability of functional molecular markers is expected to increase and provide many more pre-selection tools for breeders (Lavi et al. 2004).

20.5.5 Secondary/Regional Evaluation

Hybrids selected in the preliminary evaluation phase generally undergo a secondary evaluation phase to comparatively evaluate the hybrid performance against industry standard cultivars, parents and other hybrids. Secondary evaluations usually are done using multiple clonally propagated trees in several locations to obtain information on hybrid performance over a range of environments and seasons. Yield and productivity performance that are not easily measured on single trees in the preliminary evaluation (juvenile) phase are more reliable in secondary evaluation trials. The secondary evaluation trials are usually the first source of larger quantities of fruit for market testing and postharvest evaluation of fruit. Secondary evaluation trials are also a source of data for plant breeders' rights or patent application.

20.5.6 Cultivar Release and Post-release Support

Information from the evaluation phases will identify hybrids with suitable traits for release as new cultivars. There are many decisions to be made when releasing a new cultivar and the process will not be covered in this chapter. The commercial release of a new variety can be slow, especially if plant variety rights are involved. Early planning of release strategies will save much time at the stage of release. The release and successful uptake of a new cultivar is dependent on the comparative performance with existing cultivars, the availability of planting and propagation material and specific management information on the new cultivar. Many of the large-scale production issues of a new cultivar that are not apparent at the time of release can be managed post-release with a well-planned R&D support program for the new cultivar. The successful release of a new cultivar will necessitate the movement of plant material between growing regions. Production of clean bud wood or trees in a region with minimal quarantine restrictions will make dissemination of the new variety easier.

It is inevitable that any new cultivar will require some different management inputs than established cultivars. Determining the specific management requirements ahead of release and providing R&D support after release will facilitate uptake by growers and the ultimate success of the cultivar.

20.6 Breeding Strategies and Methodologies

20.6.1 Historical Overview

Historically most of the current cultivars grown world-wide, have originated from selection of open-pollinated seedlings, either as chance seedlings or specific selection

in breeding programs. A few cultivars have originated from closed-controlled pollination. The systematic breeding systems used in mango include techniques such as monoembryonic and polyembryonic seedling selection, controlled open pollination and controlled closed pollination.

Each of these techniques is influenced by the reproductive physiology of the mango that creates some significant challenges and limitations with conventional breeding techniques. Factors such as the perennial flowering habit, large number of flowers per panicle, polyembryony juvenility, polyploidy and inherent heterozygosity (Mathew and Dhandar 1997; Villegas 2002) can all potentially influence the management and success of mango-breeding programs. However, the ability to easily vegetatively reproduce clone trees allows any genetic gain to be captured in a new cultivar. The merits and drawbacks of each technique are discussed below.

20.6.2 Mutation Breeding

Natural, spontaneous mutations in mango have been reported with cultivars such as Davis-Haden, originating as a sport of Haden and Alphonso and Puthi originating as chimeras (Singh 1960). Gamma ray and chemical induction of mutations in mango for breeding purposes has been reported to induce dwarfness, firmer flesh, higher TSS (total soluble solids) and better sugar acid blends in a few of the treated plants (Sharma 1987).

20.6.3 Monoembryonic Seedling Selection

Many major cultivars are the result of selections that were made from naturally-occurring seedlings and exploited the natural outcrossing in open-pollinated seedlings. Cultivars originating from Florida and India during the twentieth century have often come from organized monoembryonic selection programs. In monoembryonic selection programs large numbers of seedlings from one or more desired monoembryonic maternal parents are grown, evaluated and screened for improved genotypes. The Australian cultivar R2E2 originated from a seedling-selection program based on cv. Kent (Bally 1998). Polyembryonic maternal parents are unsuitable as their seedlings are genetically similar to the maternal parent.

There are several limitations of the monoembryonic selection technique. The diversity amongst progeny is generally low due to self-pollination in many of the seedlings. Self-pollinated progeny have not been able to be easily detected until they begin to fruit, so large numbers of seedlings have to be field grown and screened before suitable improved selections can be identified. In addition to the low percentage of selectable seedlings, the breeder has no control over the paternal (pollen) parent of the hybrid progeny. These limitations are offset by the ease in generating large numbers of hybrids for testing; however, the high effort to reward ratio, and low

genetic control of this technique make it less favored by modern mango breeders. Molecular pre-selection techniques such as fingerprinting and MAS may be a useful way to selectively identify paternal parents and desirable types to avoid large field plantings of untargeted monoembryonic seedlings.

20.6.4 Polyembryonic Seedling Selection

Polyembryonic seedling selection aims to exploit the diversity in polyembryonic populations generated by natural mutation or out-crossed zygotic seedlings. It is well known that most polyembryonic seedlings are nucellar in origin and true to the maternal parent type. However, zygotic off-type seedlings have been reported of 2–47% in several polyembryonic cultivars (Beal 1981; Truscott et al. 1993). The main problem with generating progeny through polyembryonic seedling selection and the reason it has not been traditionally favored by plant breeders, is the ability to identify the zygotic embryos in the seed. Generally, polyembryonic selection programs have relied on identifying superior types within large commercial populations rather than selecting and growing zygotic seedlings for selection.

This method has been reported to produce some interesting cultivars in Florida such as Alica, Herman and Florigon, all of which originated from seedlings of Saigon (Knight and Schnell 1993), as well as some variations in the cv. Kensington Pride (Johnson 1995).

20.6.5 Controlled Open Pollination

This method exploits the natural outcrossing of monoembryonic cultivars with measures taken to encourage outcrossing with pollen from a desired source.

Several techniques can be used to maximize outcrossing to desired maternal (pollen) parent. Often the two parent trees are enclosed with pollinating insects in a large insect-proof cage. This technique was used by Sharma and Singh (1970) with the self-incompatible cv. Dashehari and others to produce the quality hybrids Mallika and Amprapali. This technique has also been employed in Israel (Degain et al. 1993). One major constraint on this technique is the requirement for synchronized flowering of the caged parent trees. An alternative method is to plant a single maternal parent tree in close proximity and surrounded by paternal pollinating trees, or to graft a branch of the maternal parent onto the paternal tree, thus allowing a higher probability of outcrossing to the desired paternal parent. This technique has been successfully used in Australia by Whiley et al. (1993), who produced cv. Calypso from a cross between Sensation ♀ and Kensington Pride ♂.

Controlled open pollination has been one of the more popular breeding techniques used in the last 25 years, however it has several shortcomings. As with all open-pollinated techniques, high numbers of self-pollinated progeny are often pro-

duced (Lavi et al. 1998) which have to be field grown and evaluated for several years before being recognized and culled, tying up valuable land and time resources. Positive identification of the male or pollen parent is not easy without DNA fingerprinting, making statistical analysis of segregation patterns in the breeding population difficult, and providing little improved understanding of mango genetics. With the development of several molecular markers for fingerprinting mango varieties, it is now possible to identify the paternal parents of controlled open-pollinated progeny before field planting.

20.6.6 Controlled Closed Pollination (Hand Pollination)

Controlled closed pollination uses hand-pollination techniques to cross two desired parents, delivering the desired pollen and excluding the undesired pollen to the female parent flowers.

Early attempts at hand pollination involved crossing a large number of flowers on a few panicles per tree, over several days, resulting in a very small number of hybrids, due to the large natural fruit thinning of the initial fruit set per panicle (Singh 1960). This technique was substantially improved by Mukherjee et al. (1961) who crossed fewer flowers per panicle on a larger number of panicles per tree. A further modification to the technique of Mukherjee et al. (1961) was suggested by Singh et al. (1980) who did not re-bag the crossed panicles after pollination. They claimed the re-bagging damaged the stigmas and styles of the crossed flowers, reducing the percentage of fruit set, and the risk of pollination from unwanted pollen was very low. Another modification to the Mukherjee et al. (1961) technique has been to replace the re-bagging step after crossing with application of gelatinous capsules to enclose the flowers (Bally et al. 2000). However, the gelatinous capsule technique has now also been dropped due to the difficulty in removing the capsules without damaging the crossed flowers.

The advantages of the hand-pollination technique are that it can be carried out on any desired female parent trees with a wide range of pollen sources not necessarily located in the same site, without setting up purposely-grown crossing blocks. Both parents of each hybrid generated through closed-pollination techniques are identified, making statistical analysis of segregation patterns possible. Unlike open-pollination techniques, resources are not wasted on growing and evaluating self-pollinated progeny.

The principal disadvantage of this technique is the relatively low number of hybrids generated per number of panicles crossed. However, success rates are dependent on the particular parental combination used in the cross. Kulkarni et al. (2002) reported success rates of 0–122% (percentage of mature hybrid seed obtained per crossed panicle) depending on the parents involved. The highest success rates (>100%) were seen in maternal parents that were bunch bearing such as cvs. Creeping and Willard. The reasons for the low success rates of the hand-crossing technique are not fully understood, but may include factors such as problems of incompatibil-

ity between parents and pollen viability (Dag et al. 2000; Sukhvibul et al. 2000a), stigmatic receptivity and protogyny, (maturation of stigmas before anthers) (Spencer and Kennard 1956) and low natural fruit set in mango.

20.6.7 A Method of Hand Pollination

Hand pollination (crossing) is a slow process that is limited by the time of day and shortness of the flowering season. Fresh pollen is generally not available until the anthers dehisce 2–3 h after sunrise and is unavailable after the filament and pollen begins to shrivel an about midday. A description of hand pollination in mango is outlined and illustrated below.

20.6.7.1 Selection of Panicles on Female Parent Trees to be Crossed

The ideal panicles for crossing are those that have minimal open flowers and 5–20 light tulip-shaped flower buds ready to open within 24 h (Fig. 20.2a, b). These panicles provide an adequate numbers of flowers for crossing the next day and easy removal of all other unwanted flowering buds. More developed panicles with larger numbers of open flowers can be used but they generally take longer to prepare and cross.

20.6.7.2 Removal of Opened Flowers

Selection and preparation of panicles is best done in the afternoon to minimize the chance of the selected flower buds opening before crossing the following morning. All open flowers are removed from the selected panicles by pinching them off with fine tweezers (Fig. 20.3a). This removes all pollen sources that may self-pollinate any flowers that open early the next morning. The panicles are then enclosed with a tight woven cloth bag to exclude external pollen and pollinating insects (Fig. 20.3b).

20.6.7.3 Pollen Collection

Pollen is collected early on the morning of hand crossing from open flowers of the male parent that have not yet dehisced (Fig. 20.4a). These flowers are removed and stored in a shallow container (petri dishes are ideal, Fig. 20.4b). Once an adequate number pollen flowers have been collected they are divided in two, with one-half being sealed and kept in a relatively cool spot. The other half are kept in the shallow dish that is placed is a well-ventilated warm position to encourage pollen dehiscence, which is indicated by a change in anther color from dark red to greyish blue (Fig. 20.4c). Once the anthers have begun to dehisce the shallow dish should be removed to a shaded position to slow subsequent dehiscence.

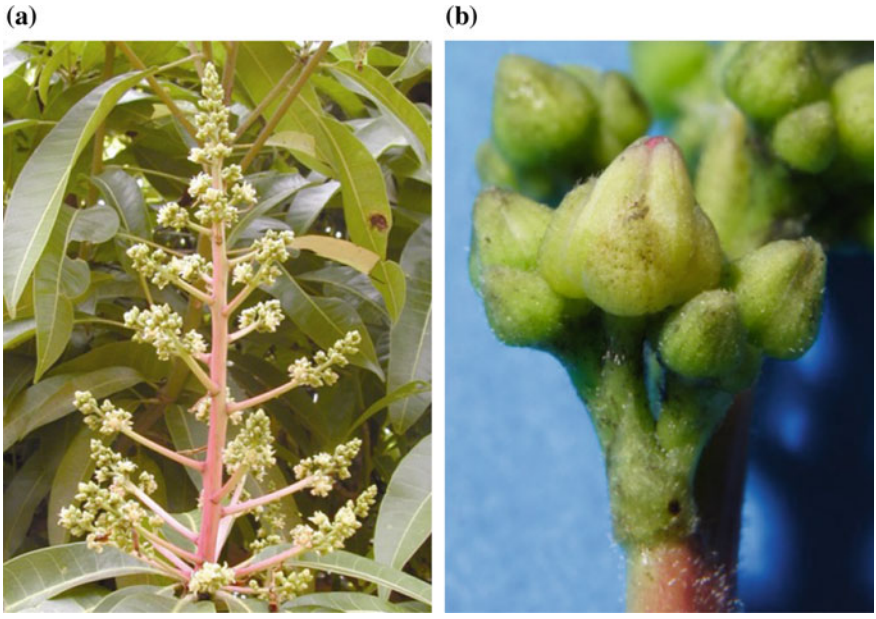

Fig. 20.2 **a** Mango panicle suitable for crossing with 5–20 open flowers and buds ready to open within 24 h, **b** Pale tulip-shaped flower bud ready to open in the next 24 h

20.6.7.4 Panicle Preparation for Pollination

Panicles are inspected and prepared for pollination in the early morning before the anthers on the opening flower buds dehisce. The bagged panicles are uncovered and inspected for suitable hermaphrodite open flowers in which the anthers have not yet dehisced (Fig. 20.4a). Five to ten flowers are selected and retained on the panicle for crossing. All other flower buds and unwanted panicle branchlets are removed by pinching them off with fine tweezers (Fig. 20.5a, b).

20.6.7.5 Emasculation of Hermaphrodite Flowers

Once the panicle has been prepared by removing all unwanted flower buds, the remaining hermaphrodite flowers are emasculated to remove the stamens before the anthers have a chance to dehisce. This is done by pinching off the anthers from their supporting filaments with a fine pair of tweezers (Fig. 20.6a). Care should be taken to NOT mistake the pistil for a stamen or damage it during the emasculation operation. Between flowers, tweezers should be washed and sterilized with ethanol to minimize unwanted pollen transfer.

Fig. 20.3 **a** Preparation of panicle the day before pollination by removal of all open flowers. **b** Subsequent placement of tightly-woven bags to exclude pollinators

20.6.7.6 Pollen Transfer

When the flowers on the female parent have been emasculated they are ready for pollination. This involves the transfer of pollen from the dehisced anthers of the male parent onto the stigma of the female parent (Fig. 20.6b). A pollinating flower with dehisced anthers from the male parent, collected earlier, is picked up with a freshly sterilized pair of tweezers, and the anthers are gently touched on the stigma of the female parent flower to dislodge pollen grains. One pollinating flower should

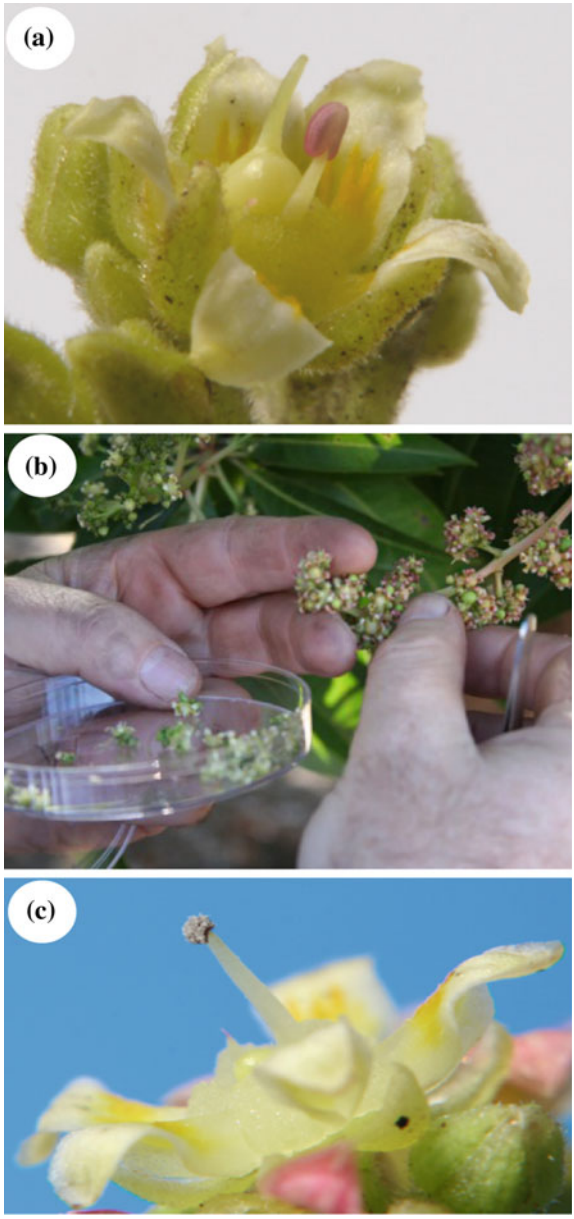

Fig. 20.4 **a** Freshly opened flower, prior to dehiscence of the anther. **b** collection of pollen flowers for hybridisation. Pollen donor flowers should be collected at this stage of development in the early morning. **c** Dehisced anther revealing grey pollen. Anther dehiscence is easily recognised by the change in colour of the anther from red to grayish blue

not be used on more than three female flowers to ensure adequate quantity of pollen is transferred.

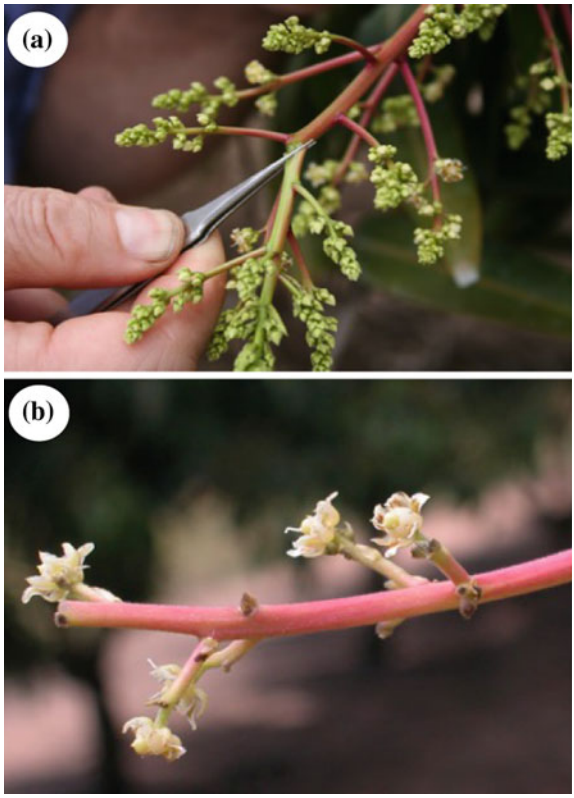

Fig. 20.5 **a** Removal of flower buds not selected for crossing with tweezers prior to pollination. **b** Panicle prepared for pollination. Only the flowers to be pollinated are retained, all other flowers are removed from the panicle

20.6.7.7 Post Pollination and Harvesting Hybrids

After pollination, a label indicating the parentage and date of the crossing is tied around the base of each crossed panicle. As the hybrid is nearing full maturity the fruit and panicle are covered with a fine mesh bag to exclude potential pests and to catch and retain identity of the fruit if it falls.

20.6.8 Polyembryony

Mangoes are classified as either monoembryonic or polyembryonic based on the numbers of embryos in the seed (Fig. 20.7). Monoembryonic cultivars have a single zygotic embryo whereas polyembryonic cultivars have multiple embryos one of which can be zygotic (usually a weak embryo) and the rest are nucellar in origin. The multiple nucellar embryos develop adventitiously from nucellar tissues surrounding the embryo, and, as a result, are genetically similar to the tree bearing the seed

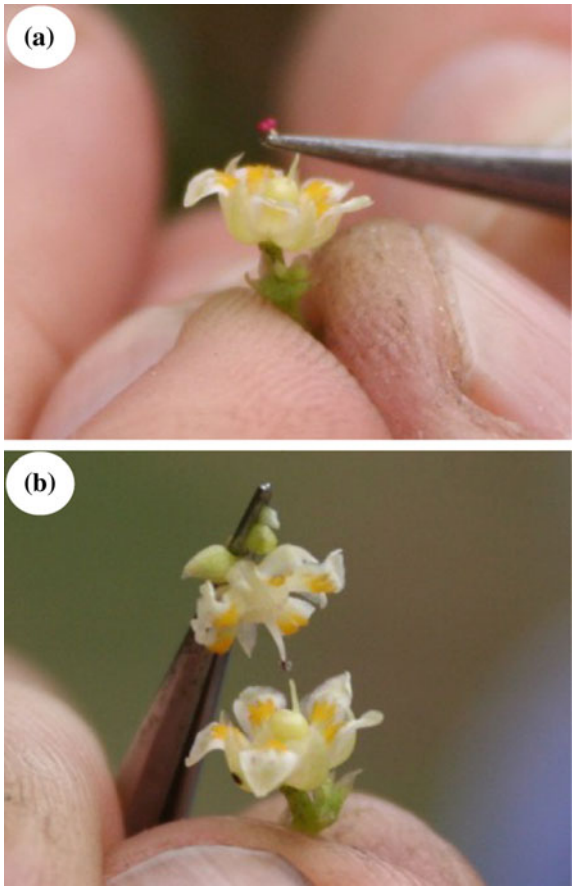

Fig. 20.6 **a** Emasculation of hermaphrodite flowers prior to pollination by removing the anthers from the filaments with a fine pair of tweezers. **b** Transfer of pollen from by gently touching the dehisced anther on the male flower onto the stigma of the female flower

(Juliano 1937). Polyembryonic seeds germinate as multiple separate seedlings, most of which are nucellar in origin and true to type for the maternal parent. Polyembryony is genetically inherited and thought to be influenced by a single dominant gene (Aron et al. 1998; Brettell et al. 2004); however, there are suggestions that high temperatures during flowering and fruit development cause some monoembryonic cultivars to produce polyembryonic seed (Brettell et al. 2004; Mathews and Litz 1992). The cv. R2E2 produces predominantly polyembryonic seed with up to 30% monoembryonic seed in some seasons (Bally 1998). Monoembryonic and polyembryonic mango varieties are thought to have originated separately with the monoembryonic types from the Indian subcontinent and the polyembryonic types from Southeast Asia (Ravishankar et al. 2004; Singh 1968).

Using polyembryonic cultivars for maternal parents in a hybridization program is problematic, as identification and recovery of the zygotic embryo is technically difficult and only possible by molecular screening techniques (Degain et al. 1993;

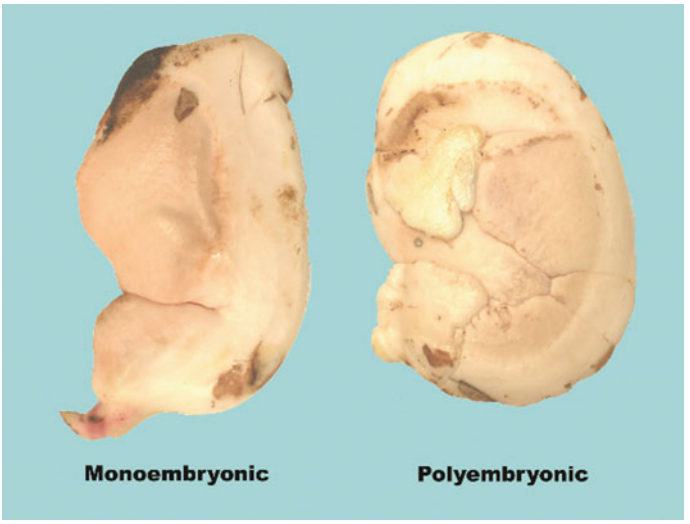

Fig. 20.7 Embryos of monoembryonic and polyembryonic mango seeds

Schnell and Knight 1992). For this reason, most hybridization breeding programs only use monoembryonic maternal parents.

20.6.9 Juvenility

Juvenility is the time taken for a seedling to reach a fruit-bearing stage. In mango juvenility is long and varies between cultivars. Mango seedlings normally take 3–10 years to bear fruit, making for a very long breeding program (Iyer and Degani 1997). Although the long juvenility can often be overcome by grafting onto mature trees (Singh 1957), girdling of young seedlings (Gaskins 1963) or by the application of growth retardants paclobutrazol or ethephon (Chacko et al. 1974; Iyer and Degani 1997), these methods can also bias the early evaluation of the hybrid's fruit quality attributes, such as size, color and flavor. Many productivity and fruit quality attributes cannot traditionally be measured in juvenile trees. However, molecular genomic tools such as marker-assisted selection will overcome some of these limitations as they are developed for mango.

20.6.10 Natural Fruit Shedding and Embryo Abortion

Mangoes bear multiple flowers on terminal inflorescences or panicles. Each panicle is capable of setting between a few to over 500 fruits, most of which are progressively

shed between fruit set and fruit maturity to leave one or several fruits per panicle at maturity. Early fruit shedding can be caused by poor pollination often associated with low temperatures (<10 °C) during flowering (Dag et al. 2000; Issarakraisila and Considine 1994; Sukhvibul et al. 2000b). Fruit shedding later in fruit development can be associated with embryo abortion that is not well understood. In a controlled-crossing situation, natural fruit shedding causes many of the hand-pollinated fruit to be shed before maturity, making the percentage of successful hand-crossed flowers very low. The effects of natural fruit shedding in controlled-crossing programs can be partly overcome by crossing fewer numbers of flowers on many panicles rather than crossing many flowers on fewer panicles. Protecting pollinated flowers from excessive heat, insects and anthracnose will also reduce the abortion of hand crossed flowers and fruit. Viable embryos may be recovered from later fruit shedding through embryo rescue in the laboratory (Perez-Hernandez and Grajal-Martin 2011; Sahijram et al. 2005).

20.6.11 Flower Synchronization

Flower synchronization between varieties is flexible in mango as flower anthesis occurs sequentially along a panicle with individual panicles flowering for up to 3 weeks. The timing of flower anthesis and stigma receptivity are covered in the section on pollination and compatibility in this chapter. In situations where flowering in varieties is not synchronized enough for pollination, pollen storage is a possibility.

20.6.12 Pollen Storage

Mango pollen cannot be stored at room temperatures and humidity. Dutta et al. (2013a,b) found that mango pollen of cvs. Sensation, Tommy Atkins and Janardan Pasand lost all viability when stored at room temperature for 4 weeks. However, they suggest that effective storage of pollen from 1 to 16 weeks within a flowering season can be achieved using storage temperatures of −4 to −20 °C. The storability of pollen was also found to be genotype-dependent (Abourayya et al. 2011).

20.7 Breeding Objectives

The objectives and aims of mango breeding programs will vary from region to region depending on market requirements and production constraints. In cooler subtropical regions regularity of production is often an issue whereas in warmer tropical regions poor flowering and low yields can be an issue (Iyer and Dinesh 1997). It is important to take a market approach when setting breeding goals because without market

acceptance, new cultivars cannot be considered successful. Future mango cultivars will need a set of traits that provide efficient, regular production and robust fruit quality at the consumer level. It is important for any new production traits to be integrated into new and existing production systems for successful adoption and their benefits to be realized. Some of the more desirable traits common to most mango breeding programs are: regular bearing, dwarf trees, attractive color, size, shape and flesh quality, resistance to pests, diseases and disorders, extended storage and shelf life, extended production period and tolerance of adverse soil conditions (Brown et al. 2009; Iyer and Degani 1997; Lespinasse and Bakry 1998). Fruit quality traits will vary between markets with some being more sensitive to fruit flavor and color than others. It is important to understand the sensitivity of target markets to fruit quality traits before designing a mango breeding program. The major desirable production and fruit quality traits are discussed below.

20.7.1 Productivity and Juvenility

Productivity in tree crops such as mango is made up of several contributing factors including early cropping (precocity) or juvenility. Precocity is often associated with high productivity and in many tree crops long juvenile periods are an issue for tree crop breeders (van Nocker and Gardiner 2014). In mango, the juvenility period of seedling progeny is 2–7 years. When Lavi et al. (1989) studied the heritability of juvenility, they found the female parent did not contribute to the distribution of the juvenility period in hybrid progeny. Precocious genotypes are identified by their early and regular flowering each season. Iyer and Degani (1997) suggested overcoming the delays caused by long juvenility in breeding programs by pre-selection of seedlings to avoid the need of maintaining large numbers of seedlings for long periods. Traits such as leaf volatiles can be used to predict fruit flavor and xylem to phloem ratios of less than 1 or high phenolics in the apical bud can be used to predict dwarfing (Iyer 1991; Kurian and Iyer 1992). Marker- assisted selection (MAS) will be a common pre-selection tool for mango breeders in the near future.

20.7.2 Breeding for Yield Improvement

Yields in mango are generally low compared with other fruit crops, and are characterized by high seasonal variability or biennially. High, seasonally uniform yielding cultivars are needed to improve farm productivity and provide an even market supply. The reasons for low and irregular productivity of mango are many, but include poor flowering and low photosynthetic efficiency during flowering and fruiting periods, and the failure to initiate new vegetative growth after flowering in late season cultivars (Chacko and Kohli 1984; Gonzalez and Blaikie 2003; Iyer and Degani 1997). Improvement in any of these traits is likely to contribute to a cultivar with improved

regularity of production. Selection for yield cannot be done early during the trees juvenility period and may require several years of assessment to account for the seasonal variation and biennial fluctuations. Currently assessment of a progeny yield potential is one of the reasons for the long-breeding cycle in mango. Because there are multiple genes that can impact on a tree's ability to yield, single markers are unlikely to provide enough information on which to base selection. Marker-assisted selection based on multiple markers may provide a way to assess yield potential.

20.7.3 Breeding for High Pack-Out

Pack-out refers to the percentage of harvested fruit that are packed into first, second or pulp grades. Returns to the grower are improved when most fruits are in extra or first grade. Many factors such as skin blemishes from sap, abrasion and temperature stress, misshapen fruit, small or large fruit size, and disease and disorders can downgrade fruit. Although many of the individual traits impacting on pack-out can be minor, attention to all of the fruit quality traits during selection of parents and progeny in a breeding program will assist in improvements in pack-out. Fruit uniformity is a major contributor to high pack-out.

20.7.4 Breeding for Harvest Time

A mix of cultivars with a spread of harvest times will extend the harvest season on the farm and provide a choice of cultivars to the consumer for a longer period. However, certain markets and production districts may have preferences for specific harvest times. There are mango cultivars with early through to late season production that can be used as parents in a breeding program. Very early season production is often desired in many industries, but there are only a few cultivars that can be considered very early.

20.7.5 Breeding for Disease Tolerance

Several diseases currently pose major constraints to production or limit the postharvest life and transportability of fruit. These can be divided up into preharvest and postharvest diseases. Diseases such as mango malformation (complex of *Fusarium* spp.), bacterial black spot (*Xanthamonas campestris*), powdery mildew (*Odium mangiferae*), mango scab (*Elsinoe mangiferae*), mango wilt (*Ceratocystis fimbriata* and *C. manginecans*), slow decline and sudden death and apical bud necrosis (*Pseudomonas syringar*) and bacterial canker (*P. mangiferaeindicae*) are all constraints to economic production. Anthracnose (*Colletotrichum gloeosporioides*) and

stem-end rots (*Lasiodiplodia* and *Dothiorella* sp.) are the primary postharvest diseases responsible for reduced saleable life and losses of fruit. Any improvements in postharvest disease tolerances will substantially contribute to cultivar reliability and quality. Anthracnose is generally the most significant mango disease as it affects flowers, twigs, leaf and flower growth as well as harvested fruit.

Bacterial black spot No cultivars have been identified with total resistance to bacterial black spot; however, Gagnevin and Pruvost (2001) found the cvs. Heidi and Sensation to be partially resistant. Bombay Green cv. has been reported as having some resistance to bacterial canker (Prakash and Srivastava 1987).

Mango wilt Several rootstock and scion varieties with resistance to mango wilt were identified and tested in Brazil between 1989 and 1997 (Rossetto et al. 1997). These may be a useful source of resistance as rootstocks or in crossing programs in countries where this disease is becoming established. Carabao, Manga D'agua, Espada Vemhelha and Voutpa are some of the better performing wilt resistance varieties (Carvalho et al. 2004; Rossetto and Ribeiro 1990).

Anthracnose Anthracnose is a preharvest disease of foliage, twigs, flowers, and fruit, and the most significant postharvest disease of fruit. Anthracnose affects mangoes throughout the world. There are many reports of varying susceptibility of varieties to anthracnose but no known significant resistance to the disease. There have been many reports of mango varieties that are more tolerant to anthracnose than others (Dinh et al. 2003; Paez Redondo 1996; Peterson 1986; Sharma and Badiyala 1999) with cvs. such as Alphonso, Brasamasi, Carabao, Carrie, Early Gold, Keaw, Kent, Krishan Bhog, Rad, Saigon, Tommy Atkins and Van Dyke being most tolerant. However, none has true resistance or levels of tolerance that significantly reduce the use of preharvest and postharvest fungicides. Yao et al. (2012) released Enong 2 as a mutant selected from natural seedlings of cv. American Hongmang with higher anthracnose resistance. They found the varieties Zihua, Chun Hwang, Zhixum No 4 and Spooner to be highly resistant to inoculation with the ZR64 strain of anthracnose. The variety Kensington Spooner has not proven to have any resistance in Australia. Interspecific crosses between *Mangifera laurina* and *M. indica* may improve the genetic tolerance to anthracnose. The wild mango species *M. laurina,* is closely related to *M. indica,* and is well adapted to wet climates and poor soil drainage. It sets fruit well and shows no signs of attack by anthracnose in areas where anthracnose prevents other mangoes from setting fruit (Bompard 1993). Bally et al. (2013) screened a range of mangoes for tolerance to anthracnose and found one accession of *M. laurina* to have superior tolerance to all others and they have hybridized the tolerant individual with advanced breeding lines.

Mango malformation Mango malformation is a serious disease in many countries in the Americas, Africa, and Asia, deforming shoot tips and panicles to prevent fruit set. Currently the disease is managed, but not controlled, by cultural methods. Sharma and Majumdar (1988) used cv. Bhaudauran as a source of resistance in a F1 crossing program, but found all the progeny to be susceptible to malformation. Other cultivars

have been reported as having tolerance to malformation, such as Bhyadayam Dula, Samar Bahist Rampur and Miam Sahib (Ram et al. 1987), but there have been no reports of their use in breeding programs.

Powdery mildew As with anthracnose and other diseases, there are differences in mango cultivars' responses to powdery mildew (Iyer and Degani 1997) but no known resistance.

20.7.6 Breeding for Pest Tolerance

Breeding mangoes for pest tolerance may involve altering the canopy architecture to a less favorable insect environment or incorporating tolerant genes into breeding progeny. Only a few examples of tolerance to pests have been documented. Rossetto et al. (2006) reported that cvs. Alfa, IAC 111 and Espada Stahl have strong tolerance to West Indian fruit fly (*Aanastrepha obliqua*). In this work Rossetto et al. (2009) found the main mechanism for fruit fly resistance was non-preference for oviposition. Angeles (1991) suggests *Mangifera altissima* may have resistance to hoppers and tip borers.

20.7.7 Breeding for Heat Treatment Tolerance

Access to many international markets require disinfestation quarantine protocols that heat the fruit for extended periods. Cultivars that can withstand heat treatments without loss of fruit quality will have a major competitive advantage in international trade.

20.7.8 Breeding for Abiotic Stress Tolerance

With increasing human population in many mango-producing nations, pressure on land and water resources is increasing, and as a result, mangoes are being grown on marginal soils and irrigated with poor quality saline water. Cultivars or rootstocks with tolerance of these conditions will help reduce the impact of declining conditions.

The best-known rootstock with tolerance to high pH and saline water is the Israeli cv. 13-1 (Gazit and Kadman 1980). Although most cultivars are sensitive to high concentrations of salt in soil or water (>EC 9 dS m^{-1}) cvs. such as Meho and Sheedi in Pakistan (Kashkelly et al., pers. comm.) and Babbaki, Olour and Kurukkan (Dubey et al. 2007) have shown some tolerance in low to medium saline conditions.

20.7.9 Breeding for Dwarfing

Reduced vegetative vigor and reduced tree size are desirable in commercial mango production because they maximize tree productivity, reduce tree management costs and allow greater tree densities per hectare. Dwarfing can be a cultivar trait or induced in a scion cultivar by the rootstock. Traits such as high phenolic levels, higher phloem/xylem ratio and low number of new shoots giving rise to flushes have been associated with dwarfing (Iyer and Kurian 1992; Singh et al. 1986). Trunk cross-sectional area have been correlated with vigor and cumulative fruit yield in mango (Reddy et al. 2003). These may be useful indicators for selection of dwarfing in a breeding program.

Several mango cultivars have been identified as potential dwarf parents in a breeding program, including the Indian cvs. Creeping, Kerala Dwarf, Janardan Pasand, Manjeera and Amrapali (Iyer and Degani 1997; Iyer and Subramanyam 1986). Sharma and Majumdar (1988) found dwarfing in Indian cultivars was controlled by a recessive gene. Pinto and Byrne (1993) found cvs. Amrapali and Imperial as male parents showed a high propensity to transfer the dwarf trait to their progeny, however the canopies of the progeny were often dense or spreading. Creeping cv. has been used as a female parent in the Australian National Mango Breeding Program, effectively reducing tree vigor in its progeny, however small fruit size and bunch bearing are also characteristics of the progeny. The cv. Vellaikulamban as rootstock has been shown to reduce tree vigor, and increase yields per unit canopy volume and per unit land in the cv. Alphonso (Reddy et al. 2003). With the increasing trend towards high-density orchards in many mango growing countries, the need for effective vigor-controlling rootstocks is greater than ever. Reducing mango canopy vigor can also be achieved through rootstocks and rootstock breeding as discusses below.

20.7.10 Breeding for Fruit Flavor

Flavor in mangoes, as in other fruits, is derived from sugars, acids and volatile aroma compounds. It is the mix and concentrations of the volatile aroma compounds that give the different mango cultivars their characteristic flavors. Over 370 free forms of aroma volatile compounds have been identified in mango and 70–140 as glycosidically-bound aromas. Monoterpenes and sesquiterpene hydrocarbons account for about 59% and esters about 20% of the identified compounds. Esters, alcohols, carbonyls and lactones contribute towards the unique aroma and flavor of certain cultivars. The unique flavor of cv. Kensington Pride has been attributed to the high predominance of monoterpenes such as α-terpinolene and *peach* flavors provided by esters and lactones (Lalel et al. 2003). Flavor preferences may be different between markets. Regardless of the flavor style, it is desirable for the flavor of a new cultivar to be robust and associated with strong aroma in ripening fruit. High

flavor quality is desirable in all fruit, not just the top 10% of tree-ripened fruit, if market reliability is to be achieved.

20.7.11 Breeding for Fruit Shelf Life

As most mango fruit is grown a long distance from the consuming markets, varieties that maintain quality after storage and transport will have an advantage by reducing retail shrinkage and maintaining consumer confidence. Many factors contribute to the shelf life and transportability of fruit such as susceptibility to postharvest rots, resistance to blemishing and retention of flavor.

20.7.12 Breeding for Fruit Size

The preferred mango by many retailers weighs 350–400 g, with smaller markets for other sizes of fruit. Size uniformity is also desirable as it contributes to higher pack-out percentages. Hardner et al. (2012) found average fruit weight to be under strong additive genetic control and predicted breeding values of 0.69–0.88 for average fruit weight among 35 cultivars.

20.7.13 Breeding for Multiple Fruit on a Panicle

Multiple bearing or bunch bearing can potentially increase the yield of a mango tree; however, when too many fruit are retained on a panicle, blemishes often occur where they touch or from insect infestation, reducing fruit quality. One to four fruit well-spaced on a panicle is ideal. Iyer and Schnell (2009) reported bunch bearing to be a dominant character over single fruiting.

20.7.14 Breeding for Fruit Color

Mango fruit color has three main aspects, background skin color when ripe, skin blush color and internal flesh color. Reliable de-greening of the background color during fruit ripening to a light yellow is desirable in many markets as it indicates ripeness to the consumer and lightens the overlying blush making it the color more vivid. Many current cultivars do not de-green well when grown under high nitrogen or if stored or ripened under non-optimal conditions.

The blush color is the overlying orange, pink, red or purple color that develops on fruit in the second half of its development and it is often dependent on expo-

sure to direct sunlight. Strong blush color that develops with minimal exposure to direct sunlight on the majority of fruit on a tree is desirable. Blush color is mainly the result of anthocyanin pigments that appear to be primarily the result of activation of cyaniding-3-*O*-galactaside synthesis (Berardini et al. (2005a, b) tentatively identified the compounds cyanidin 3-*O*-galactoside (Berardini et al. 2005a) and 7-*O*-methylcyanidin 3-*O*-β-D-galactopyranoside in the peels of red-colored mango cultivars such as Tommy Atkins. Many cultivars in Asia lack or are poorly blushed, however the cultivars originating out of Florida are highly colored and make good parents. Cultivars such as Tommy Atkins and Irwin have been used successfully to improve skin blush in progeny. Bally et al. (2006) reported broad-sense heritability of 0.41–0.59 for color in families with Tommy Atkins and Irwin as a female parent.

In ripe fruit, internal pulp color ranges from pale yellow green to deep orange. The intensity of flesh color varies with cultivar and appears to be the result of both carotenoids (Pott et al. 2003) and an orange anthocyanin (Proctor and Creasy 1969). Cultivars rich in α-carotene are characterized by a marked color change during ripening while cultivars poor in α-carotene have less pronounced red coloration (Vásquez-Caicedo et al. 2005). The carotenoid composition varies, depending on the cultivar, geographic or climatic effects, stage of maturity, fruit processing and storage conditions during shipment (Mercadante and Rodriguez-Amaya 1998). Iyer (1991) indicated that lighter yellow mesocarp colors were dominant over the orange yellow color. In addition to their aesthetic color attributes, these pigments have antioxidant activities with many health-promoting properties, including some cancers, atherosclerosis and age-related macular degeneration (Mayne 1996; Nishino et al. 1999).

20.7.15 Breeding for Fruit Internal Disorders

Internal fruit disorders in mango are difficult to manage and are generally undetectable until the consumer cuts open the fruit. A high incidence of internal fruit disorders in a cultivar reduces the confidence of consumers and retailers in that cultivar. The causes of internal fruit disorders are uncertain but factors such as nitrogen and calcium nutrition (Burdon et al. 1991, 1992), crop load and tree vigor (Simmons et al. 1998; Subramanyam et al. 1971) and fruit maturity at harvest (Galán Saúco and Calvo 1984) have all been implicated. The incidence and severity of internal disorders varies among mango cultivars with the highest incidences reported in those originating from Florida and India (Limaye et al. 1975; Oosthuyse 1993). Rane et al. (1976) reported that the cvs. Pairi, Kesar, Doophperda, Neelum and Dashehari, were free from the *spongy-tissue* form of internal breakdown. Any lowering of the incidences of internal disorders in new cultivars will contribute to consumer confidence in mangoes. Due to the seasonal variability in the expression of many forms of internal disorders, assessment of the genetic component of these disorders is difficult.

20.7.16 Breeding for Fruit Shape

Mango fruit shape varies from round to elongate, ovate, with shape being a characteristic cultivar trait. Fruit shape and color are the most recognizable features of a mango and can be important for consumer recognition of a cultivar. Longer-shaped fruits and those with prominent beak shapes can be more difficult to handle in a mechanized sorting and packing system. Iyer and Degani (1997) reported predominant beak shape being dominant over a less-pronounced beak shape in Indian breeding populations.

20.7.17 Breeding for Sap Burn

The lactiferous sap that spurts and oozes from the peduncle of mango fruit during harvest is toxic (Joel 1980) and when it comes into contact with the fruit burns the skin causing sap burn or skin browning (Loveys et al. 1992). These blemishes reduce the visual attractiveness and value of the fruit. The sap is also toxic to human skin causing skin dermatitis on contact (Calvert et al. 1996). Avoiding and removing sap contamination is a major expense in many mango-growing enterprises. Breeding mango cultivars with low sap toxicity would greatly improve both fruit quality and production efficiency. Sap toxicity differs between cultivars, regions and weather conditions (Hesse and Bowden 1995; Loveys et al. 1992). Loveys et al. (1992) found the toxic component in cv. Kensington Pride sap to be the volatile terpinolene which has also been identified as the main contributor to its characteristic flavor (Lalel et al. 2003). As some of the volatile monoterpenes in the latex are associated with sap burn damage, breeding for a reduction in sap toxicity may also alter the flavor profiles of progeny.

20.7.18 Breeding for Seedlessness

Seedlessness has been developed in many fruit species, for example, table grapes, citrus and watermelon. Although seedlessness occasionally occurs naturally in mango, it is usually associated with inferior and undersized fruit (nubbins). Hormonally-induced seedlessness in mango fruits of Dashehari were also undersized (Chacko and Singh 1969). Selection of seedless mango has been part of the Indian breeding program for several years. In the 1990s India commercially released the *seedless* cv. Sindhu, that bears medium-sized fruits with an extremely thin stone, devoid of embryo and endosperm (Gunjate and Burondkar 1993); however, this variety did not achieve commercial success (Anonymous 2006). Recently, in Hainan Island, China, commercial-scale production of seedless mangoes using plant growth regulator treatments has had great success in domestic and export markets (Lu et al. 2006) using

cvs. Tainong No. 1, White Ivory (Nang klang wan) and Guifei (Red-golden dragon), which usually produce fruits with fully developed seeds.

20.7.19 Breeding Rootstock

Requirements for mango rootstocks are somewhat different from those for scion cultivars. Effective rootstocks should be graft-compatible with scion cultivars and also confer some beneficial effects to the scion, such as dwarfing, or tolerance to calcareous soils and salinity (Litz and Gomez-Lim 2002). The poor understanding of the physiological causes of rootstock effects on scions in mango, especially in vigor reduction rootstock, limits the ability of the mango breeder to breed for or identify potential rootstock. For ease of commercial-scale propagation, polyembryonic rootstock cultivars are preferred. One of the most successful rootstock selections was cv. 13-1 selected in Israel for its tolerance to salinity (Gazit and Kadman 1980; Lavi et al. 1993). Smith et al. (2008) screened 64 mango rootstocks with Kensington Pride scion and found large variation (160%) in canopy growth and a 3.5 times difference in yield efficiency. They found no relationship between yield efficiency and tree size. Rootstocks Brody and MYP had high yield efficiency and small canopy size. Recent screening experiments for vigor-reducing rootstock in Australia have identified several accessions that reduce the size of scions, however yield testing of scions on these rootstocks is ongoing (Mizani et al. 2016).

20.8 Biotechnology

20.8.1 Biotechnology-Assisted Breeding

Biotechnology-assisted breeding refers to the application of molecular biology and somatic cell genetics to the improvement of plants (Litz and Lavi 1997). Use of the biotechnology-assisted breeding methods may overcome or alleviate many of the limitations in mango discussed above, such as, low fruit retention, polyembryony, heterozygosity, polyploidy, long juvenility, large tree size and long life cycle.

Molecular biology (genetics) approaches offer an effective alternative to conventional mango breeding/improvement in several ways. In conventional breeding marker-assisted selection (MAS) can be used for the identification of parents and selection of progeny, characterizing the relatedness or genetic diversity among cultivars or species of mango, and gene cloning can be used to transform existing mango cultivars with horticulturally-important genes of commercial interest (Litz 2004). Other biotechnological methodologies include the efficient somatic embryogenesis and plant recovery from elite (nucellar) material, induction of random mutations in embryogenic cultures and challenging for resistance to a specific selective agent, and

transformation with a gene that mediates a desired horticultural trait. On the basis of past and current research, it is probable that resistance to abiotic soil stress and certain diseases can be addressed by mutation breeding and the control of fruit ripening, seedlessness and certain diseases can be addressed by genetic transformation (Litz 2004). Progress has been made on controlling fruit ripening by inhibition of expression of genes encoding ethylene biosynthetic enzymes (Cruz-Hernandez et al. 1997).

20.8.2 Marker-Assisted Selection (MAS)

In classical genetic improvement programs, selection is carried out based on the observed phenotypic characteristics of individual candidates with little or no prior knowledge of the genes being selected. The development of molecular markers and, hence, marker-assisted selection (MAS), offers the potential to overcome this limitation to improve quantitative traits in plants and the efficiency of tree breeding. To be useful for MAS, desired agronomic traits must be associated with known molecular markers. Molecular markers are identifiable DNA sequences, found at specific locations within the genome and transmitted by the standard laws of inheritance from one generation to the next. They can be considered as constant landmarks within a genome (Semagn et al. 2006). There are clear advantages for the use of molecular markers in plant breeding, such as a decreased number of breeding generations, the availability of a uniform method for scoring, no need to use phenotypic scoring in the initial screening and, finally, the possibility of obtaining information on the percentage of the genome contributed by each parent in the offspring. Molecular-marker analysis enables the identification of genome segments, the quantitative trait loci (QTLs), which contribute to the genetic variance of a trait and thus to select superior genotypes at these loci without uncertainties due to interaction of genotype with the environment and experimental error. Selecting for favorable QTL effects based on MAS has great potential for improving specific traits.

Breeding mangoes is a long-term activity, complicated by a heterozygous genome, juvenility, low fruit set and retention rates, long evaluation periods, polyembryony and outcrossing behavior. These factors make genetic improvement through conventional parental selection and breeding slow and unpredictable. The adoption of molecular markers and genomics-based breeding strategies will likely improve predictability and breeding efficiency. In mango, there is currently a lack of basic genome sequence and DNA marker information which limits the practical application and adoption of molecular technologies. However, the lowering of cost and improvement in sequencing and marker identification are changing this and MAS is becoming a more attractive tool for mango breeders. Molecular markers linked to important phenotypic traits are especially useful when the traits are difficult, costly and time consuming to observe. Markers indicating DNA polymorphisms within a specific target gene are preferable as there is minimal risk in losing linkage due to recombination. Such markers are allele-specific and remain informative whatever the genetic

background, and are therefore more likely to be transferable across taxonomic boundaries.

In mango, molecular markers have been used to identify the pedigree of progeny and parents, for taxonomic purposes, and to assess the heritage of mango cultivars and species (Eiadthong et al. 2000; López-Valenzuela et al. 1997; Ravishankar et al. 2000; Schnell et al. 1995). The information generated is useful to assist in the design of mango-hybridization strategies for increased breeding efficiency; however, MAS has not yet been used for the selection of breeding traits in mango.

20.8.3 Diversity Analyses

There is a relatively poor understanding of the pedigree and genetic relatedness of many *Mangifera indica* cultivars and related *Mangifera* species. The distribution of mango cultivars in ancient and recent times, their adaption to different regions and adoption of local vernacular names makes tracing their origins and ancestry difficult. Traditional classification of the genus *Mangifera* has been based on phenological and morphological characteristics of flowers, leaves, fruits and seed. However, this mode of identification is exceedingly complicated by environmental effects on these characters and parallel selection for similar desired traits often being misleading (Smith and Smith 1992). Often plants need to be grown to full maturity prior to identification and evaluation of these traits, which may take up to 10 years as a result of a long juvenile period and may not unambiguously distinguish between closely-related genotypes or hybrids. As a result, several different classification systems have been proposed (Ding 1978; Kostermans and Bompard 1993; Mukherjee 1953b).

Recent application of molecular markers and nucleotide sequence analyses to determine phylogenetic relationships among *Mangifera* species such as by Díaz-Matallana et al. (2009), Duval et al. (2006), Eiadthong et al. (2000), Gálvez-López et al. (2009), Hidayat et al. (2011), Pandit et al. (2007), Schnell et al. (2006), Yamanaka et al. (2006), and Yonemori et al. (2002) are now beginning to reveal some of the predicted and new genetic relationships between mango cultivars. Nonetheless, the taxonomic classification of *Mangifera* remains uncertain.

20.8.4 Molecular Phylogeny

Every living organism contains nucleic acids (DNA and RNA) and proteins. In general, the molecular structures of these substances in organisms that are closely related have high similarity while in distantly related organisms they become more disparate. Over time, conserved sequences, such as mitochondrial (mt) or chloroplast (cp) DNA, are predicted to accrue mutations (assuming a constant rate of mutation) providing a *molecular clock* for dating divergence. Molecular phylogeny estimates and analyses these heritable molecular differences, mainly in DNA sequences, to generate a

framework of evolutionary relationships. With the innovation of Sanger sequencing in 1977 it became possible to isolate and identify these microstructural changes in organisms (Sanger and Coulson 1975; Sanger et al. 1977).

Microstructural changes, including insertions and deletions (indels) and inversions, in the genome of higher plants have been shown to be particularly useful for resolving phylogenetic relationships both between closely-related taxa and among more basal lineages (Ingvarsson et al. 2003). Analysis of the maternally-inherited chloroplast genome, due to the relatively small size, haploid nature, non-recombination and conservation, may overcome many of the disadvantages encountered in the analysis of nuclear DNA (nrDNA) markers. The rate of base pair substitutions in the chloroplast is extremely low (Muse 2000; Wolfe et al. 1987), approximately one-half that of plant nrDNA. However, non-coding regions such as gene spacers and introns are hot spots for mutation and provide a convenient source for detection of intra- and inter-specific variations in DNA sequences. Introns and intergenic spacers from the chloroplast genome are now increasingly used for phylogenetic and population genetic studies of populations from a single species. A number of studies have been undertaken in mango using chloroplast (cp) DNA sequence data.

Fitmawati (2010) sequenced the non-coding cpDNA trnL-trnF spacer in 3 *Mangifera laurina* and 3 related *Mangifera* species. The aligned length of all the samples was 433 nucleotides with 357 constant characters, 7 non-informative parsimony characters and 69 parsimony informative characters. The resulting phylogenetic tree was monophyletic (descended from the same common ancestor) with three groups determined. The three individuals of *M. laurina*, sourced from different regions within Indonesia, did not group together. This result did not agree to the clusters of morphological markers reported by Kostermans and Bompard (1993).

Khan and Azim (2011) sequenced the cpDNA intergenic spacer regions rpl20-rps12 and atp-rbcL of 19 *Mangifera indica* cvs. to assess intraspecific variation. Analysis of the atp-rbcL region showed no variation in the 510 nucleotides sequenced (Genbank GQ472294-GQ472309), while sequence data of the 768 nucleotide rpl20-rps12 region showed 1 intra-varietal and 2 inter-subspecific polymorphisms (Genbank FJ937738-FJ937756). At position 360 of the rpl20-rps12 spacer region, the inter-varietal SNP (C/G) was detected. The first inter-subspecific change detected was the insertion of cytosine (C) at position 714, detected only in cv. Langra.The second change was a C/A variation at position 755 in 16 of the 19 mango cultivars analyzed. The resulting dendogram was unclear whether cluster analysis separated the cultivars according to geographic origin or not.

More recently, the phylogenetic relationships among 5 *Mangifera* species (*M. indica*, *M. griffithii*, *M. camptosperma*, *M. odorata* and *M. andamanica)* were analyzed by employing chloroplast markers (*pet*B-*pet*D intergenic spacer, *rps*16 gene, and the *trn*L-*trn*F intergenic spacer) and a nuclear marker, external transcribed spacer (ETS). The nuclear and chloroplast markers based on phylogenetic analysis showed that the common mango, *M. indica,* was closely related to *M. griffithii* and *M. camptosperma*, belonging to subgenus *Mangifera*. However, *M. odorata* (belonging to subgenus *Limus*) grouped separately along with *M. andamanica*. The above results are consistent with the accepted classification of genus *Mangifera* by Kostermans

and Bompard (1993) with the exception of *M. andamanica*, which has been earlier classified under subgenus *Mangifera*.

Utilizing sequence comparison of the nrDNA internal transcribed spacer (ITS) region, Yonemori et al. (2002) investigated the phylogenetic relationships among 14 *Mangifera* species from Thailand. This nrDNA marker has proven useful due to its high copy number, small size, highly conserved flanking regions and rapid concerted evolution (Baldwin et al. 1995). In *Mangifera* the length of ITS1 ranged from 264 to 265 nucleotides while ITS2 was 228 to 230 nucleotides. The 5.8S gene region showed no variation in length with 163 nucleotides. A total of 659 nucleotides were aligned resulting in 55 and 41 polymorphic sites in ITS1 and ITS2, respectively. Only one site was polymorphic in the 5.8S gene region. Using the entire sequence of the ITS region, parsimony analysis showed that *M. indica* was closely related to *M. laurina*, *M. sylvatica* and *M. oblongifolia*. *Mangifera flava* formed a monophyletic group with *M. gedebe*, *M. foetida* and *M. odorata*. *Mangifera cochinchinensis* formed a monomorphic clade. This large group was separated from *M. macrocarpa*, *M. caloneura*, *M. gracilipes*, *M. pentandra* and *M. griffithii.* Hidayat et al. (2013) also studied the ITS region in 13 *M. indica* cultivars from Indonesia, Malaysia and Taiwan to reveal that the cultivars were classified into three major clusters based on their origin.

A phylogenetic tree from nucleotide variation in the nrDNA *rbcL* gene based on 16 *Mangifera* species from Indonesia and Thailand was developed by Supraman et al. (2013). Of the 905 aligned nucleotides, 807 were constant and 72 were considered parsimony informative. The phylogenetic tree was monophyletic with four main groups determined. The first group contained two species from Thailand, *M. macrocarpa* and *M. cochincinensis*; the second *M. indica*, *M. caesia*, *M. applanata* and *M. altissima*; the third *M. longipes*, M. laurina, *M. similis*, *M. gedebe*, and the Indonesian origin *M. macrocarpa*. The fourth group contained the Thai origin *M. laurina*, *M. foetida*, *M. casturi*, an unknown *Mangifera* species and *M. odorata*. Separation of the same species within the phylogenetic tree from different origins, such as *M. macrocarpa* from Thailand and Indonesia, may be due to geographical isolation and different mutations to the DNA at these origins. Alternately, these two *M. macrocarpa* individuals may actually be different species.

The phylogenetic studies to date clearly indicate that there are discrepancies between the results obtained of genetic relationships among *Mangifera* species when compared to the clusters of morphological markers reported by Kostermans and Bompard (1993). Further studies are required to clarify understanding of the taxonomic classification of *Mangifera*.

20.8.5 Molecular Genotyping

Molecular genotyping is a useful tool in establishing cultivar identity and relationships. Different kinds of molecular markers exist, such as restriction fragment length polymorphisms (RFLPs); amplified fragment length polymorphisms (AFLPs), ran-

dom amplified polymorphic DNA (RAPDs) markers, start codon targeted (SCoT) markers, cleaved amplified polymorphic sequence (CAPS) markers, microsatellite or simple sequence repeat (SSR) markers and single nucleotide polymorphisms (SNPs). The various types of molecular markers differ in a variety of ways such as their technical requirements (e.g. whether they can be automated, their detection method and level of reproducibility); the amount of time, money and labor needed; the number of genetic markers that can be detected throughout the genome; and the amount of genetic variation found at each marker in a given population. The degree of dominancy is one of the main differences among molecular markers. Dominant markers are able to estimate genotypes but not the allele frequency so are preferable for fingerprinting samples and identifying clones. Codominant markers are able to estimate allele frequencies and are able to estimate gene flow between populations. The choice of molecular marker depends, among other parameters mentioned, on its suitability to answer a particular ecological question.

20.8.5.1 Restriction Fragment Length Polymorphism (RFLP)

In the late 1980s, RFLPs were popular markers widely used in plant molecular genetics e.g. Helentjaris et al. (1986) and Weber and Helentjaris (1989). RFLPs are codominant, unaffected by the environment and are reproducible. Any source of DNA can be used for the analysis and markers can be mapped in a population that is not stressed by the effects of phenotypic mutations. RFLP marker analysis involves restriction enzyme digestion of DNA molecules resulting in a reproducible set of fragments of well-defined lengths. The pattern differences between DNA fragment sizes identifies individual organisms. With the advent of polymerase chain reaction (PCR) technology and its application for polymorphism detection, RFLPs proved to be expensive, labor- and time-consuming, and not amenable to automation, eventually making these low-throughput markers obsolete.

Two studies using RFLPs on cpDNA have been undertaken in mango (Eiadthong et al. 1999a; Ravishankar et al. 2004), while Chunwongse et al. (2000) used RFLPs to assess gDNA of 31 F1 hybrid progeny to develop a genetic linkage map.

Eiadthong et al. (1999a) used RFLP data of cpDNA to explore the phylogenetic relationship of 13 *Mangifera* species collected in Thailand. Analysis of the region bounded by conserved sequences in ribulose-1,5-bisphosphate (*rbc*L) and open reading frame (ORF) 106 genes revealed that these species could only be classified into two clusters. The two clusters were classified by 8 informative mutation sites detected within the 3.1 kb amplified region by digestion with four endonuclease enzymes, *Dra*I, *Msp*I, *Taq*I and *Hinf* I. The first cluster consisted of *M. indica* and *M. sylvatica*, and the second of *M. caloneura*, *M. cochinchinensis*, *M. flava*, *M. foetida*, *M. oblongifolia*, *M. macrocarpa*, *M. odorata*, *M. griffithii*, *M. collina*, *M. gedebe*, and *M. pentandra*. The 11 species belonging to the second cluster were monomorphic for digestion with all 20 restriction enzymes used, even though these species are scattered in different subgenera or sections, according to the classification by

Kostermans and Bompard (1993) based on the morphological characteristics of the shape of the flower disc.

Ravishankar et al. (2004) used RFLPs to assess the genetic relatedness of *Mangifera indica* cultivars traditionally grown on the southwestern coast of India. Chloroplast DNA of eight polyembryonic and eight monoembryonic cultivars were used in the study. The first cpDNA fragments, of around 3.1 kb, resulting from the ORF of the *rbc*L region and digested with *Pst*I showed no polymorphism within any of the mango cultivars. The second amplified regions, around 1.5 kb, were successfully digested with eight restriction enzymes. Dendrogram analysis clearly grouped the cultivars into two based on embryo type. The RFLP analysis was confirmed using RAPDs on the same cultivars in this study. The authors suggest that the polyembryonic types are unlikely to have originated in India and may have been introduced from other parts of Southeast Asia.

Chunwongse et al. (2000) utilized data from 197 genomic probes hybridized to restriction enzyme digested DNA of 31 F1 hybrid progeny of Alphonso x Palmer cvs. to determine segregation ratios. A 1:1 segregation ratio was determined for 175 probes while 43 and 31 probes showed a 1:3 and a 3.1 segregation ratio, respectively. These ratios were confirmed with AFLPs within the same study. The genetic linkage map developed from RFLPs and AFLPs is discussed later in this chapter.

20.8.5.2 Amplified Fragment Length Polymorphism (AFLP)

The AFLP reaction was first described by Zabeau and Vos (1993). This technique is considered to be a powerful, rapid and reliable assay to generate genetic data for intra- or inter-specific genetic diversity studies (Vos et al. 1995). The technique is based on the detection of genomic restriction fragments by PCR amplification allowing the detection of polymorphism at a large number of loci within a short period of time and requires very small amounts of DNA. AFLP technology is versatile and applicable to all organisms without previous sequence information. AFLP markers are mainly dominant but codominant inheritance may sometimes be evaluated. Due to the application of high stringency during PCR, robustness and high reproducibility is ensured (Hansen et al. 1999). AFLPs detect higher levels of polymorphism than RFLPs. One of the downsides of AFLPs is non-homology of co-migrating fragments in the gel.

AFLP markers have been used extensively for studying genetic diversity in different plant species because of their high reproducibility and multiplex ratio (Ellis et al. 1997; Erschadi et al. 2000; Maughan et al. 1996). These markers have also been widely used in genetic diversity and relationship analysis within mango cultivars (Archak et al. 2014; Fang et al. 2000, 2001; Gálvez-López et al. 2010; Gao et al. 2013; Mussane et al. 2011; Santos and Neto 2011; Santos et al. 2008), species identification (Eiadthong et al. 2000; Fang et al. 1999; Teo et al. 2002; Yamanaka et al. 2006) and the construction of genetic linkage maps (Chunwongse et al. 2015; Fang et al. 2003; Kashkush et al. 2001). A summary of the research undertaken in mango using AFLP markers is presented in Table 20.2.

Table 20.2 Summary of the research undertaken in mango using AFLP markers

No. *M. indica* cultivars (origin)	Known *Mangifera* species used in study (No.)	No. primer combinations	No. polymorphic bands (%)	Study	References
31 Alphonso x Palmer	Nil	106	650	Linkage map construction	Chunwongse et al. (2000)
7 local (Thailand)	13 *Mangifera* species	5	167 (77%)	Species identification	Eiadthong et al. (2000)
16 Keitt x TA	Nil	6	204	Genetic diversity	Fang et al. (2000)
23 introduced 29 Keitt x TA	Nil	6 14	204 105	Genetic diversity Linkage map construction	Kashkush et al. (2001)
4 Malaysia 6 Singapore	*M. foetida* (5) *M. odorata* (5)	7	332 (87%)	Species identification	Teo et al. (2002)
60 Keitt x TA	nil	ND	81	Linkage map construction	Fang et al. (2003)
6 local (Malaysia) 5 introduced	*M. caesia* (6) *M. foetida* (7) *M. odorata* (11)	8	499 (96.3%)	Species identification	Yamanaka et al. (2006)
105 local (Brazil) and introduced	*M. foetida* (1) *M. similis* (1)	13	157 (74.4%)	Genetic diversity	Santos and de Oliveira (2008)
47 local (Mexico) 12 introduced	*M. odorata*	4	331 (84.4%)	Genetic diversity	Gálvez-López et al. (2010)
30 local (Mozambique) and introduced	Nil	7	155 (74.9%)	Genetic diversity	Mussane et al. (2011)
108 Haden x TA	Nil	7	ND	Measurement of outcrossing rate	Santos and Neto (2011)
47 local (China) 151 introduced	*M. persiciformis* (1) *M. hiemalis* (1) *M. odorata* (1)	8	1539 (98.2%)	Genetic diversity	Gao et al. (2010)
23 local (India)	Nil	15	1073 (87.4%)	Genetic diversity	Archak et al. (2014)

Eiadthong et al. (2000) used 5 AFLP primer combinations, producing 217 amplified bands to assess the genetic diversity of 14 *Mangifera* species. Reproducibility of amplified bands was confirmed with repeated reactions using the same template DNA and primer combinations. Of the 217 scored bands, 77% (167) were polymorphic. The unweighted pair group method with arithmetic mean (UPGMA) grouped 7 species into a primary cluster including *M. indica*, *M. laurina*, *M. sylvatica*, followed by *M. oblongifolia*, *M. odorata*, *M. macrocarpa* and *M. foetida*. The species *M. collina*, *M. pentandra*, *M. flava, M. caloneura* and *M. cochinchinensis* formed a second cluster, while *M. gedebe*, *M. griffithii* sat with the two outgroup taxa, *Anacardium occidentale* and *Bouea macrophylla*. The only discrepancy between the UPGMA and a comparative neighbor-joining (NJ) analysis was that *M. cochinchinensis* sat with *M. gedebe*, *M. griffithii* in the NJ dendrogram. The groupings obtained in this study are somewhat in agreement with the classifications by Kostermans and Bompard (1993). According to Kostermans and Bompard (1993) *M. odorata*, *M. macrocarpa*, and *M. foetida* are within the subgenus *Limus*, however this study places these 3 *Mangifera* species into the section *Mangifera*, along with *M. indica*, *M. laurina*, *M. sylvatica* and *M. oblongifolia.* The taxonomic position of *M. collina* and *M. flava* may also need to be reconsidered as the molecular analysis suggests they are more closely related to species in the section *Euantherae* whereas Kostermans and Bompard (1993) have them in the section *Mangifera* based on morphological characters. These results complement the phylogenetic findings by Yonemori et al. (2002) with regard to taxonomic groupings of *Mangifera* species.

Yamanaka et al. (2006) used AFLPs to evaluate the genetic relationships among 4 *Mangifera* species, including *M. indica*. Using 8 AFLP primer combinations, a total of 518 bands were amplified from the 35 *Mangifera* accessions. Of these amplified bands 499 (96.3%) were polymorphic. The UPGMA clustering method grouped the accessions into the four, corresponding to the 4 *Mangifera* species used in the study, which are in agreement with their classification by classical methods. Yamanaka et al. (2006) found *M. odorata* accessions to have a high genetic similarity (0.95) to each other and display a low polymorphism ratio (7.2%). This similarity is also seen in the morphology of the *M. odorata* accessions assessed. *M. indica* accessions showed the most diversity (75.9%) followed by *M. caesia* (68.6%) and *M. foetida* (51.4%). AFLP analysis has shown to be a useful and robust tool for determining genetic diversity and genetic relationships within and among a wide range of *Mangifera* species.

20.8.5.3 Random Amplified Polymorphic DNA (RAPD)

Described by two independent research groups in 1990, the RAPD technique detects DNA polymorphism based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Welsh and McClelland 1990; Williams et al. 1990). Resolution of the resulting banding patterns results in a semi-unique profile for each sample. No prior knowledge of the DNA sequence of the targeted genome is required, as the primers will bind somewhere in the sequence, but it is uncertain exactly where. As a result, the nature of the amplified products will be

unknown. Simple and reproducible fingerprints of complex genomes can be generated using this marker system. This has made the method popular for comparing the DNA of organisms that have had little attention from the scientific community. As this technique relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. However, only small amounts of target DNA are required for analysis. Problems with reproducibility also result as RAPD suffers from sensitivity to changes in the quality and concentration of DNA, PCR components and PCR cycling conditions, resulting in changes of the amplified fragments. Thus, the RAPD technique requires carefully developed laboratory protocols to be reproducible.

RAPD markers are nearly all dominant and do not permit the scoring of heterozygous individuals. Amplification either occurs at a locus or it does not, leading to scores based on band presence or absence. This means that it is impossible to distinguish whether a DNA segment is amplified from a locus that is heterozygous (1 copy) or homozygous (2 copies). The presence of a band of identical molecular weight in different individuals is not evidence per se that the individuals share the same (homologous) DNA fragment. In addition, the absence of a band through mismatches between the primer and template sequence cannot be distinguished from that occurring through the lack of amplification for other reasons (e.g. poor quality DNA), contributing to ambiguity in the interpretation of results. Codominant RAPD markers, observed as different-sized DNA segments amplified from the same locus, are only rarely detected. RAPD markers have been a popular methodology used in mango genetic diversity studies and these are presented in Fifteen local Mexican and introduced *Mangifera indica* cvs. assessed by López-Valenzuela et al. (1997) using 13 primers produced 109 bands of which 81 (74%) were polymorphic. UPGMA analysis clustered the cultivars into groups according to their geographical origins. In this analysis one RAPD marker was present only in polyembryonic cultivars and not monoembryonic cultivars, suggesting a possible marker for this trait. No segregation studies were undertaken to validate this marker further.

Ravishankar et al. (2000) undertook RAPD analysis of 18 commercial mango cultivars from western, southern, northern and eastern parts of India. Nineteen primers produced 178 bands of which 130 (73%) were polymorphic. Cluster analysis showed two major groups of mango cultivars based on their geographical origin; one cluster of cultivars originating from the western, northern and eastern regions of India and a second cluster of those cultivars indigenous to the south of India. Similarly, Archak et al. (2014) identified southern Indian cultivars to be significantly different to northern and eastern Indian cultivars using AFLP and inter-simple sequence repeat (ISSR) markers. A summary of RAPD markers used in mango are listed in Table 20.3.

Fifteen local Mexican and introduced *Mangifera indica* cvs. assessed by Lopez-Valenzeula et al. (1997) using 13 primers produced 109 bands of which 81 (74%) were polymorphic. UPGMA analysis clustered the cultivars into groups according to their geographical origins. In this analysis one RAPD marker was present only in polyembryonic cultivars and not monoembryonic cultivars, suggesting a possible marker for this trait. No segregation studies were undertaken to validate this marker further.

Table 20.3 Summary of RAPD markers used in mango

No. *M. indica* cultivars (origin)	Known *Mangifera* species used in study	No. primers	No. polymorphic bands (%)	Study	References
1 local (USA)	*M. casturi* *M. decandra* *M. foetida* *M. quadrifida* *M. laurina* *M. odorata* *M. pajang* *M. torquenda*	10	109	Genetic diversity	Schnell and Knight (1993)
25 local (USA)	Nil	11	67 (44%)	Cultivar identification and genetic diversity	Schnell et al. (1995)
30 local (Australia)		7	107	Cultivar identification and genetic diversity	Bally et al. (1996)
15 local (Mexico) and introduced	Nil	13	109 (74%)	Genetic diversity	López-Valenzuela et al. (1997)
18 local (India)	Nil	19	130 (73%)	Genetic diversity	Ravishankar et al. (2000)
50 local (India)	Nil	10	139	Genetic diversity	Kumar et al. (2001)
29 local (India)	Nil	24	287 (91.4%)	Genetic diversity	Karihaloo et al. (2003)
14 local (Brazil) and introduced + 25 Rosa genotypes	Nil	13	116 (65.9%)	Cultivar identification and genetic diversity	de Souza and Lima (2004)
40 open pollinated Rosinha seedlings	Nil	9		Genetic origin of plantlets	Cordeiro et al. (2006a, b)
20 local (India)	*M. sylvatica*	15	134 (85%)	Genetic diversity	Srivastava et al. (2007)
46 local (India)	Nil	9	110 (87.3%)	Genetic diversity	Bajpai et al. (2008)

(continued)

Table 20.3 (continued)

No. *M. indica* cultivars (origin)	Known *Mangifera* species used in study	No. primers	No. polymorphic bands (%)	Study	References
25 local (Pakistan) and introduced	Nil	45	257 (80.3%)	Cultivar identification and genetic diversity	Rajwana et al. (2008)
112 local (cv. Hilacha, Colombia)	Nil	5	20	Cultivar identification and genetic diversity	Díaz-Matallana et al. (2009)
14 hybrids + parents local (India)	Nil	10	139	Cultivar identification and genetic diversity	Vasanthaiah (2009)
20 local (India)	Nil	10	89	Genetic diversity	Gajera et al. (2011)
35 local (Brazil) and 7 introduced	Nil	6	55	Genetic diversity	Ramessur and Ranghoo-Sanmukhiya (2011)
6 local (Mauritius) and 6 introduced	Nil	6	54 (77.1%)	Genetic diversity	Ramessur and Ranghoo-Sanmukhiya (2011)
63 local (India) and 2 introduced	Nil	15	129 (82.7%)	Genetic diversity	Samal et al. (2011)
10 local (Egypt)	Nil	11	72 (78%)	Genetic diversity	Mansour et al. (2014)
6 local (India)	Nil	5	120	Genetic diversity	Prasad and Sethi (2014)
19 local (India)	Nil	10	50 (80%)	Genetic diversity	Pruthvish and Chikkaswamy (2016)

Ravishankar et al. (2000) undertook RAPD analysis of 18 commercial mango cultivars from western, southern, northern and eastern parts of India. Nineteen primers produced 178 bands of which 130 (73%) were polymorphic. Cluster analysis showed two major groups of mango cultivars based on their geographical origin; one cluster of cultivars originating from the western, northern and eastern regions of India and a second cluster of those cultivars indigenous to the south of India. Similarly, Archak et al. (2014) identified southern Indian cultivars to be significantly different to northern and eastern Indian cultivars using AFLP and inter-simple sequence repeat (ISSR) markers. A summary of RAPD markers used in mango are listed in Table 20.2.

20.8.5.4 Start Codon Targeted (SCoT) Markers

The start codon targeted (SCoT) marker system is based on the short conserved region in plant genes surrounding the ATG translation start (or initiation) codon (Collard and Mackill 2009; Xiong et al. 2009). DNA polymorphism is based on amplification using a single primer (similar to RAPD or ISSR marker technologies) designed from the short conserved region flanking the ATG start codon that is conserved for all genes (Joshi et al. 1997; Sawant et al. 1999). Resolution of the resulting banding pattern is performed by standard gel electrophoresis with agarose gels and staining making this technique relatively cheap and simple to use.

Luo et al. (2010, 2011) concluded that the genetic relationships identified using SCoT markers in their analysis of mango were in agreement with the origin, pedigree and main phenotypic characteristics of the cultivars used in their studies. The first of these studies assessed the genetic variation and relationships among 47 *Mangifera indica* cultivars, of which 36 originated in China and developed from seed selections or hybridizations of a small number (4) of varieties held in a germplasm collection (Luo et al. 2010). One *M. persiciformis* and two *M. indica* x *M. persiciformis* hybrids were also included in this study (Luo et al. 2010). Using 33 selected SCoT primers 273 bands were generated of which 208 (76.2%) were polymorphic. UPGMA clustering grouped the 50 accessions into 6 clusters reflecting the history of breeding and selection of the cultivars studied as well as geographical origin and type.

A second study by Luo et al. (2011) compared ScoT and ISSR markers for identification and genetic comparison analysis of 23 mango cultivars. Using 18 selected SCoT primers 158 bands were generated, of which 104 (65.8%) were polymorphic. The 18 selected ISSR primers amplified 156 bands with 87 (55.8%) being polymorphic. UPGMA clustering grouped the 23 cultivars into 2 major groups based on the SCoT analysis and 3 major groups based on the ISSR analysis. Luo et al. (2011) concluded that SCoT markers were more efficient, in comparison with ISSR markers, due to the capacity to generate more polymorphic bands. However, both analyses gave similar results with grouping in accordance with their known origins and main phenotypic characteristics.

20.8.5.5 Cleaved Amplified Polymorphic Sequences (CAPS) Markers

The cleaved amplified polymorphic sequence (CAPS) method is a technique for the detection of single nucleotide polymorphisms (SNPs) at the recognition sites of restriction enzymes in polymerase chain reaction (PCR) products. This technique is an extension to the RFLP method, using PCR amplification prior to restriction enzyme analysis to more quickly analyze the results (Jarvis et al. 1994; Konieczny and Ausubel 1993). The principle behind CAPS is that genetic differences between individuals may create or abolish restriction endonuclease restriction sites. These differences can be detected in the resulting DNA fragment length after digestion.

In the CAPS method, PCR is directed across a section of chromosome with the altered restriction site and the products digested with a restriction enzyme. Resolution

of the resulting banding pattern is performed by standard gel electrophoresis with agarose gels and staining making this technique relatively cheap and simple to use. However, sequence information is required for the development of CAPS markers.

Shudo et al. (2013) developed CAPS markers for mango to identify true hybrids in F1 progeny. The sucrose synthase (MiSUS1) and sucrose phosphate synthase (MiSPS1) genes were cloned from 19 mango cultivars. In the MiSUS1 nucleotide sequences, 46 SNPs and 5 indels were detected. A total of 26 out of the 46 SNPs mapped on the restriction enzyme recognition sites were successfully converted to 25 CAPS markers. In the MiSPS1 nucleotide sequences, 99 SNPs and 6 indels were detected. Thirty out of 99 SNPs and 2 out of 6 indels mapped on the recognition sites were also converted to 32 CAPS markers. Two of the developed CAPS markers, SS-2798 and SPS-5745, were used to identify 37 true hybrids that had different genotypes to cv. Irwin (the maternal parent) from 82 F1 progeny. The method developed here to identify true hybrids is a simple, rapid and highly reliable tool for efficient mango breeding.

20.8.5.6 Simple Sequence Repeat (SSR) and Expressed Sequence Tag-Simple Sequence Repeat (EST-SSR) Markers

Simple sequence repeat (SSR) markers (also known as microsatellite markers) are based on repeating units of 2 (dinucleotide repeat) to 6 (hexanucleotide repeat) base pair DNA sequences. These sequences are codominantly inherited and thus, can differentiate homozygous genotypes from heterozygous for a particular locus. SSR markers are abundant, evenly distributed throughout the genome, highly polymorphic, and are believed to arise as a result of slipped-strand mispairing during replication (Levinson and Gutman 1987). Locus-specific primer pairs are utilized to amplify PCR products that are then separated on denaturing polyacrylamide gels and visualized by fluorometry, silver or ethidium bromide or autoradiography. Initially, the technique to use flanking PCR primers to amplify polymorphic tandem-repetitive DNA regions was successfully applied to minisatellites, consisting of repeat units of 15–50 nucleotides (Horn et al. 1989; Jeffreys et al. 1988). In 1989, four independent groups applied the same technique to identify shorter (dinucleotide) tandem repeats (Litt and Luty 1989; Smeets et al. 1989; Tautz 1989; Weber and May 1989). The downside to SSR primer development is that sequence information is required with the traditional techniques for developing SSR markers time consuming, labor intensive and of inefficient (Ellis and Burke 2007). However, once developed, SSRs are a popular marker choice for mango molecular genetics as they are highly polymorphic even between closely related lines, require a low amount of DNA, can be easily automated for high throughput screening, can be exchanged between laboratories and are highly transferable between populations (Gupta et al. 1999).

SSR variations within expressed sequence tags (ESTs) may also serve as potential molecular markers for traits influenced by these gene products. A key advantage of EST-SSRs is that they are often more transferable across closely-related genera compared to anonymous SSRs from untranslated regions (UTRs) or non-coding

sequences (e.g. Chapman et al. 2009; Pashley et al. 2006). This is due to the primer target sequences residing in the expressed DNA regions expected to be relatively well conserved, thereby increasing the chance of marker transferability across species boundaries (Chabane et al. 2005; Varshney et al. 2005). Despite their potential to represent selectively deleterious frame-shift mutations in coding regions, EST-SSRs appear to reveal equivalent levels of polymorphisms compared to SSRs located in UTRs, most likely due to an evolutionary trend towards tri-nucleotide repeats in these coding regions (Ellis and Burke 2007). EST-SSRs are physically linked to expressed genes and therefore represent potentially functional markers.

In mango studies using SSR and EST-SSR, markers have been undertaken for mapping, parentage assignment and verification, as well as inferring phylogenetic relationships between species and varieties (Dillon et al. 2013b, 2014; Duval et al. 2005, 2006; Gálvez-López et al. 2009; Honsho et al. 2005; Ravishankar et al. 2015; Schnell et al. 2005, 2006; Viruel et al. 2005).

Microsatellite enrichment libraries prepared from the cv. Tommy Atkins genomic DNA were used to examine the genetic relationship between 28 mango genotypes from diverse geographic regions (Viruel et al. 2005). The first reported 16 mango SSRs developed allowed the unambiguous identification of all mango genotypes studied. This discrimination of genotypes could be carried out with just three selected microsatellites. UPGMA cluster analysis grouped the genotypes according to their origin and their classification as monoembryonic or polyembryonic types reflecting the pedigree of the cultivars and the movement of mango germplasm.

Microsatellite enrichment libraries prepared from the mango cv. Haden genomic DNA for SSR development were used to examine the genetic relationship between 59 Florida mango genotypes and 4 related *Mangifera* species (Schnell et al. 2005). Of the 15 SSRs studied 2 were monomorphic in the Florida cultivars and 13 polymorphic. Further studies by Schnell et al. (2006) used 25 SSRs to assess the genetic diversity among 203 *M. indica* cultivars and 2 *Mangifera* species. When compared by origin, the Florida cultivars were more closely related to the Indian cultivars than to the Southeast Asian cultivars. SSR marker evidence suggests as few as 4 Indian cultivars and 1 landrace were involved in early selection of Florida cultivars.

Duval et al. (2005) examined the genetic relationship among 15 mango cultivars and related *Mangifera* species. Further diversity studies by Duval et al. (2006) of 307 mango accessions from India, Southeast Asia, Florida, Africa and the Caribbean (including 128 landraces of mango in the Guadeloupe Archipelago and in Martinique) using 19 SSRs demonstrated that 73% of the accessions displayed distinct genetic profiles. Diversity representation was constructed using neighbor-joining methodology and accessions clustered in accordance with their geographical origin and their known history (Duval et al. 2006).

Honsho et al. (2005) prepared microsatellite enrichment libraries from the mango cv. Irwin genomic DNA for SSR development. Six SSRs produced clear polymorphic patterns and were used to characterize 36 mango cultivars, mainly from Thailand. Of the 36 cultivars tested, 29 showed a unique pattern and 7 could not be identified due to genotype similarities.

Since 2005, a number of groups have developed new SSR markers for mango. Ravishankar et al. (2011) developed 36 new SSRs and characterized them using 30 diverse mango cultivars. Cross-species amplification was also demonstrated for 5 *Mangifera* species. (Ravishankar et al. 2015) went on to undertake next generation sequencing (NGS) of mango genomic DNA to identify a further 106,049 microsatellite repeats. Primers were designed for 84,118 of these SSRs and 90 were characterized among 64 mango cultivars and 4 *Mangifera* species. Chiang et al. (2012) developed 20 new SSRs for mango and assessed polymorphism in 22 mango cultivars. Chunwongse et al. (2015) developed 42 SSRs and assessed these on 6 cultivated and 2 *Mangifera* species showing cross-species amplification. Sherman et al. (2015a) identified 1903 SSRs in the Keitt reference transcriptome. The lengths of SSR motifs ranged from one to six with 70.1% being trinucleotide and 23.5% being dinuceotide repeats.

A growing number of diversity studies to assess the extant diversity have also been undertaken in mango growing countries around the world. (Hirano et al. 2011) compared the genetic structure of mango accessions from Myanmar with that of mango accessions from Florida, India, and Southeast Asia with 11 SSR markers. Myanmar's accessions were distinguishable from the other mango accessions studied and exhibited considerable genetic diversity. Further studies by Hirano et al. (2011) evaluated the genetic variation within two economically important traditional varieties of mango, Yin Kwe and Sein Ta Lone, from Myanmar. Using 12 SSRs, 94 individuals were genotyped. Higher levels of heterozygosity observed in Yin Kwe in comparison to Sein Ta Lone may be a result of propagation practices for each variety. Yin Kwe is often used as a rootstock for Sein Ta Lone and while clonal propagation is frequently observed for Sein Ta Lone it is not for Yin Kwe.

Wahdan et al. (2011) examined two new Egyptian mango cultivars, Hania and Aml, using 36 informative SSRs. Identified as local chance seedlings, these two genotypes showed promising commercial qualities and molecular characterization was undertaken to identify these cultivars.

Concerned for the genetic erosion of mango landrace diversity, Begum et al. (2012) examined 6 indigenous landraces and 5 commercial cultivars of mango from the Indian state of Andhra Pradesh using 109 SSRs. Fifty-seven of the SSRs were polymorphic with just 1 SSR able to distinguish between the 11 genotypes studied. UPGMA cluster analysis showed grouping of the 6 landraces associated with their geographical origin. Further work by Begum et al. (2014) assessed intra-varietal variability among 30 accessions of the mango cv. Cherukurasam collected from three eco-geological regions of Andhra Pradesh. Issues with variation in fruit size, shape and quality were reasons behind this study. Assessment of the 109 SSRs showed that 25 of these markers were highly polymorphic. UPGMA cluster analysis showed that the accessions did not group as per geographical origin and that the cultivar grown in this state was not clonal. Morphological assessment of the fruit among these 30 accessions had also revealed intra-varietal variations within cv. Cherukurasam.

In Iran, the diversity of mangoes present has been poorly studied. Shamili et al. (2012) used 16 SSR markers to analyze 41 mango genotypes, with most originating from India. The SSRs studied allowed the unambiguous identification of all the

mango genotypes. UPGMA and Bayesian cluster analysis indicated a clear separation between the Iranian cultivars originated from India and Pakistan.

Vasugi et al. (2012) used 14 SSRs to assess the genetic diversity of 34 unique indigenous mangoes from the Western Ghats, India, and 9 other mango genotypes. Specific to the humid, tropical rainforests, these highly acidic, fibrous, indigenous mangoes are known for tender, whole, fruit pickles locally called *appemidi*. UPGMA cluster analysis grouped the 43 genotypes into two: one group with highly acidic types and the second group with less acidic and high TSS types. Molecular characterization also revealed no correlation between geographical origin and clustering pattern for the mango genotypes studied.

In order to develop a reference database to support cultivar protection and breeding programs, dos Santos Riberio et al. (2012) examined genetic distances in a collection of 103 foreign and Brazilian mango accessions using 11 SSR markers. The 50 microsatellite alleles did not separate all 103 accessions, indicating that there are duplicates in this mango collection. Clustering analysis grouped the cultivars according to their pedigree. Tsai et al. (2013) investigated the genetic relationship among 22 Taiwanese mango cultivars using 37 SSRs. Most of the 40 cultivars grown in Taiwan have been selected by growers derived from open pollination with unknown pollen sources (Lee et al. 2009) or lines with unknown genetic background. UPGMA clustering method grouped the cultivars according to their pedigree and not based on embryony type.

Threatened by genetic erosion, local Kenyan landraces generally produce small and fibrous fruits with low market value, however they have high and stable yields, low management requirements, low susceptibility to pests and diseases and high drought tolerance (Kehlenbeck et al. 2012). Reliable and reproducible identification are a prerequisite to develop conservation strategies for these mango landraces and to retain their valuable genetic resources. Molecular characterization of 38 local mango landraces from Eastern and Central Kenya using 19 SSR markers revealed eight clusters (Sennhenn et al. 2014), partly supporting the morphological classification.

Surapaneni et al. (2013) investigated the genetic analysis of 90 mango cultivars, including juicy, table, dual (juicy/table) and pickle types, from different areas of Andhra Pradesh, India. Thirty-four new SSR primers were developed from microsatellite enrichment libraries prepared from the mango cv. Peddarasam. A further 109 SSRs from the previous studies were also used for the analysis. Of the 143 SSRs assessed, 106 (74.1%) were polymorphic. UPGMA cluster analysis showed two distinct groups, however it was unclear whether this clustering was related to geographic origin of the cultivars.

To understand the diversity held in a mango germplasm collection, Dillon et al. (2013b) analyzed over 300 mango accessions, including 7 *Mangifera* species, using 11 SSRs. Redundancy within the collection was identified with 226 unique molecular genotypes identified from the 254 accessions screened. The genetic diversity of accessions of Australia's main cv., Kensington Pride, was low with 41 of the 46 Kensington Pride accessions sharing all alleles. Bally et al. (1996) concluded from the lack of diversity in Kensington Pride that little was to be gained from selection within the Kensington Pride population, and the introduction of genes from other

cultivars may be the best way to improve the cultivar. The improved understanding of cultivar identity, parentage, genetic diversity and population structure should assist mango breeding strategies in selection of breeding stock, and confirming parentage of progeny.

Aiming to adopt the use of molecular markers and genomics-based breeding strategies to improve predictability and breeding efficiency, (Dillon et al. 2014a) further developed 25 EST-SSRs from 24,840 ESTs generated from five mango cDNA libraries. The markers were selected within genes involved in plant development, stress response, and fruit color and flavor development pathways, developed into PCR markers and characterized in a population of 32 mango selections including *Mangifera indica* varieties, and related *Mangifera* species. All *Mangifera* selections were discriminated and cluster analysis grouped the genotypes according to their origin. Correlation of the phenotypic data (categorical background skin, blush and pulp colors of fruit) measured with EST-SSR genotypes was not evident for these mango selections (Dillon et al. 2014b).

Eleven SSRs were evaluated for their usefulness to validate both hand- and open-pollinated mango hybrid progeny (Dillon et al. 2013a). Over 80% of all hand-pollinated hybrid individuals were successfully identified for the most likely candidate father using a strict (95%) confidence level. In 96 open pollinated mango hybrid progeny (assuming 75% of the candidate pollen donor population had been sampled and included in the analysis) parentage was assigned to 15 of the progeny from the single female tree sampled with 95% confidence and to almost half the progeny with 80% confidence or greater. Of the 96 progeny used in this study, 42 were assigned parentage with less than 80% confidence (Dillon et al. 2013a). The ability to use molecular markers to assess hybrids early in their life for confirmation of parentage will enable breeders the ability to cull undesirable self-pollinated and outcrossed hybrids early in the breeding process, thus reduce the population size for field testing, reduce costs and achieve improved efficiency of the selection process.

Characterization of genetic diversity and population structure among 387 Indian mango cultivars using 14 SSR markers revealed two distinct groups of populations, the cultivars from Southwest region and those from the Northeast region (Ravishankar et al. 2015). This grouping into two distinct clusters was further supported by different statistical analyses adopted in this study including distance-based analysis (dendrogram), model-based analysis (STRUCTURE) and analysis of molecular variance (AMOVA).

Faced with a growing threat of mango quick wilt disease (MQWD), caused by *Ceratocystis manginecans*, and the narrow genetic base of commercial mango cultivars in Pakistan, Azmat et al. (2016) studied 13 elite cultivars using 7 SSR markers. UPGMA cluster analysis grouped the cultivars according to their geographical origin. The knowledge gained from this study will be used for preliminary recommendation on MQWD tolerant cultivars for commercial cultivation.

20.8.5.7 Inter-simple Sequence Repeat (ISSR) Markers

The genome region located between microsatellite loci is referred to as the ISSR (inter-simple sequence repeat). The complementary sequences to two neighboring microsatellites are used as PCR primers (often with a degenerate 3′ anchor) and the variable region between them is amplified. ISSR markers are highly polymorphic and reproducible, relatively inexpensive and do not require previous sequence knowledge about the genome (González et al. 2002). Due to their ability to show differences among cultivars ISSRs may be used in studies on genetic diversity, phylogeny, gene mapping and breeding programs (Eiadthong et al. 1999b; Reddy et al. 2002; Ye et al. 2005).

Eiadthong et al. (1999b) assessed 22 mango cultivars using 7 ISSR markers to distinguish between the genotypes. A total of 56 scorable polymorphic markers were identified enabling all cultivars tested in this study to be distinguished from each other. Markers were not found to be unique to either monoembryonic or polyembryonic types, nor for the Thai cultivars selected for green harvest or for the cultivars selected for ripe fruit production.

González et al. (2002) studied the genetic variability of the cv. Kensington Pride using 8 ISSRs. Cultivars previously found to be indistinguishable using RAPDs (Bally et al. 1996) were found to display different DNA fingerprint using ISSRs. However, this method failed to detect small differences in amplification pattern produced by highly similar genotypes. Pandit et al. (2007) used 33 ISSR markers to study the genetic diversity among 70 mango cultivars, including 38 from southern India, 16 from north India and 10 introduced cultivars. These ISSR primers generated reproducible polymorphic bands in all genotypes. Of the 420 bands amplified, 408 (97.1%) were polymorphic and 12 monomorphic. UPGMA analysis separated the introduced cultivars from the Indian cultivars; however, north and south Indian cultivars could not be geographically separated.

Srivastava et al. (2007) compared 20 mango cultivars from India using 7 ISSRs. A total of 69 bands were detected, of which 44 (63.7%) were polymorphic. In comparison to the RAPD and DAMD analyses undertaken on the same mango cultivars, the ISSR method produced the least number of polymorphic bands. The neighbor-joining trees for the 20 cultivars using RAPD, ISSR and DAMD methods, also resulted in different groupings of the genotypes.

Singh et al. (2007) used 12 anchored-ISSRs to assess the genetic diversity of 12 Indian mango cultivars. A total of 161 bands were detected, of which, 113 (70.2%) were polymorphic. UPGMA analysis did not separate the cultivars according to geographic origin. Further studies by Singh et al. (2009) used 10 ISSR markers to assess the genetic diversity of 5 Indian mango cultivars. Between 2 and 20 trees per cultivar were assessed. Genetic fingerprints for 4 of the 5 cultivars were variable among trees indicating the possibility that this variation could have arisen due to accumulation of mutations over centuries of their existence or due to chance inter-cultivar hybridization.

Similarly, Samant et al. (2010) investigated the genetic diversity of 63 Indian mango genotypes using 28 ISSR primers. A total of 334 bands were detected of

which 331 (99.1%) were polymorphic. UPGMA clustering of ISSR data showed good correlation with pedigree, geographical separation and embryo type.

To gain precise information on the genetic relationships within germplasm diversity of mango, Tomar et al. (2011) assessed 20 mango cultivars, collected from the Gir region of Saurashtra, Gujarat, India, using 21 ISSR markers. UPGMA analysis clustered all mango landraces into 3 groups. This information will be useful for carrying out efficient breeding programs in the area. Comparison of the ISSR marker results with 10 RAPD markers was assessed for genetic variability and relationships among 20 *M. indica* genotypes representing 15 endangered and 5 cultivars (Gajera et al. 2011). RAPDs were more efficient then ISSRs for polymorphism detection as well as higher values for the average number of polymorphic loci per primer, average polymorphic information content (PIC) and primer index (PI) values.

To assess genetic diversity of cv. Ubá mango trees cultivated at the Zona da Mata of Minas Gerais State, Brazil, Rocha et al. (2012) evaluated 102 accessions with 9 ISSRs. Phenotypic diversity among the accessions (mainly grown from seed) was identified and subsequent genetic analysis.

Ariffin et al. (2015) employed ISSR markers to reveal genetic diversity and genetic relatedness among 28 *Mangifera* accessions collected from Malaysia. A total of 198 markers were generated using 9 anchored primers and 1 non-anchored primer, with 197 bands (99.4%) showing polymorphism. Genetic variation among the 28 accessions was high, with an average degree of polymorphism of 98%. Cluster analysis revealed separation of accessions based on species type and geographical origin.

Single Nucleotide Polymorphic (SNP) Markers

A single nucleotide polymorphism (SNP) marker is a single nucleotide base change in a DNA sequence, with a usual alternative of two possible nucleotides at a given position (bi-allelic). SNPs occur at high frequencies in plant genomes (Honsho et al. 2013). Yu et al. (2002) identified (on average) 1 SNP every 170 bp and 1 indel every 540 bp when comparing sequences from a japonica rice cultivar to those from an indica rice cultivar. The sheer abundance of these polymorphisms in plant genomes makes the SNP marker system an appealing tool for linkage mapping, marker-assisted breeding and map-based cloning (Batley et al. 2003; Gupta et al. 2001).

With SNP markers more abundant than microsatellite markers, easier to identify, easier to score and, being unambiguous markers, are appropriate for international databases as they show no platform bias, which means they can be assayed by any appropriate method and produce the same genotype. In contrast to all previous molecular-marker methods described, allele discrimination cannot be based on size differences on a gel, as a single base change in a DNA sequence cannot be detected by this method.

A large number of different SNP typing protocols are available for researchers, and there is no single protocol that meets all research needs. Aspects that need to be taken into account to determine the most suitable technology include sensitivity, reproducibility, accuracy, capability of multiplexing for high throughput analysis, cost effectiveness in terms of initial investment for equipment and cost per datapoint, flexibility of the technology for uses other than SNP discovery, and time-

consumption for analysis. The selection of technique is also dictated by the type of project envisaged as it is fairly different to perform genotypes with a limited number of SNPs on very large population samples, or a large number of SNPs on a limited number of individuals.

Sherman et al. (2015a) mapped read results from cvs. Keitt and Tommy Atkins mRNA resequencing (using Illumina HiSeq 2000) to the Keitt reference-transcriptome contigs developed (see transriptome sequencing section below). The resulting discovery of 332,016 SNPs are the first for mango. Tommy Atkins cv. was found to be more polymorphic than Keitt. More SNPs were discovered in the flanking regions of the open reading frames, than within them. A subset of 239 high quality SNPs (one SNP per contig) was used for genotyping 74 accessions of the Israeli mango collection, including cultivars originating from India, Southeast Asia, South America, Pacific Islands, Florida, Australia, and elite local hybrids. A dendrogram based on the proportion of shared alleles distance classified the accessions in the mango collection into two genetic subgroups, separating Indian and Southeast Asian accessions from the others. However, no clear division was observed between poly- and monoembryonic accessions.

Onto a reference transcriptome developed from cv. Tommy Atkins, Kuhn et al. (2016) mapped reads from 24 mango cvs. of diverse origins: Amin Abrahimpur (India), Aroemanis (Indonesia), Burma (Burma), CAC (Hawaii), Duncan (Florida), Edward (Florida), Everbearing (Florida), Gary (Florida), Hodson (Florida), Itamaraca (Brazil), Jakarata (Florida), Long (Jamaica), M. Casturi Purple (Borneo), Malindi (Kenya), Mulgoba (India), Neelum (India), Peach (unknown), Prieto (Cuba), Sandersha (India), Tete Nene (Puerto Rico), Thai Everbearing(Thailand), Toledo (Cuba), Tommy Atkins (Florida) and Turpentine (West Indies). Variants were identified from uniquely mapped reads with a 32x average coverage. At least one variant was identified for 28,959 of the 31,893 transcripts of the reference (91%). A total of 396,505 variants called in these 28,959 transcripts, of which 14,970 were indels and 381,535 SNPs. Stringent filtering of SNPs resulted in a total of 640 SNPs (0.17%) of the initial 381,535 evaluated. These SNPs have been used to genotype map populations from the USA, Brazil and Australia to develop a linkage map. A subset of 192 SNPs evenly spaced across the genome has since been identified for genotyping of all available germplasm of mango species Kuhn et al. (2016).

20.8.6 Transcriptome Sequencing

A transcriptome is the complete set of genes (or transcripts) in a cell and different cells of an organism showing different patterns of transcript expression. The variations in quantitative expression explain the wide range of physical, biochemical and developmental differences seen among various cells and tissues and may relate to a specific developmental stage or physiological condition. Understanding the transcriptome is essential for interpreting the functional elements of the genome. Thus, by collecting and comparing transcriptomes of different types of cells or tissues, a

deeper understanding may be gained of what constitutes a specific cell type, and how changes in transcriptional activity may reflect or contribute to a physical trait.

The key aims of transcriptome sequencing are: to catalogue all transcripts, including mRNAs, non-coding RNAs and small RNAs; to determine the transcriptional structure of genes, in terms of their start sites, 5′ and 3′ ends, mutations, splice variants and other post-transcriptional modifications; to quantify the changing expression levels of each transcript during development and under different conditions; and the discovery and analysis of rare or novel transcripts. By studying transcriptomes, researchers hope to determine when and where genes are turned on or off in various types of cells and tissues. Furthermore, by considering the transcriptome, it is possible to generate a comprehensive picture of what genes are active at various stages of development.

To date a number of mango transcriptomes from different plant tissues have been produced (Azim et al. 2014; Dautt-Castro et al. 2015; Kuhn et al. 2016; Luria et al. 2014; Sherman et al. 2015b; Wu et al. 2014).

Azim et al. (2014) characterized a mango leaf transcriptome of cv. Langra. De novo transcriptome assembly generated 30,509 unigenes with lengths in the range of 300 to ≥3000 nucleotides and 67-fold depth of coverage. *Citrus sinensis* was identified as closest neighbor of mango with 9141 (37%) matched sequences. This group has also sequenced and obtained a circular map the chloroplast (cp) genome using next generation DNA sequencing. The draft mango cp genome size is 151,173 bp with a pair of inverted repeats of 27,093 bp separated by small and large single copy regions, respectively. Within the cp genome, 91 of the 139 genes were found to be protein coding.

Wu et al. (2014) undertook transcriptome and proteomic analysis of pericarp and pulp of *Mangifera indica* cv. Zill at four development and ripening stages. RNA-seq generated 68,419,722 sequence reads that were assembled into 54,207 transcripts with a mean length of 858 bp, including 26,413 clusters and 27,794 singletons. A total of 42,515 (78.43%) transcripts were annotated using public protein databases.

De novo assembly of a mango fruit peel transcriptome of cv. Shelly revealed mechanisms of response to hot water brushing (HWB) treatment (Luria et al. 2014). The transcriptome was assembled from 8.6-Gbp sequence data (190-fold coverage), which generated 57,544 contigs with N50 of 1598 bases and an average length of 863.3 bases. A total of 35,719 transcripts (62.1%) showed significant similarity to known proteins in the NR database.

Dautt-Castro et al. (2015) also reported a transcriptome study using mango fruit mesocarp from *Mangifera indica* cv. Kent. The 67,682,269 clean reads generated enabled 33,142 coding sequences to be predicted. Differential expression patterns of several ripening related genes between unripe and ripe mangoes were identified.

A reference transcriptome, developed from six libraries of RNA from leaves, flowers and four tissues of the developing fruit (exocarp, mesocarp, seed coat, seed), of the Florida cv. Tommy Atkins was undertaken by Kuhn et al. (2016). A total of 493 M reads (246 M read pairs) were assembled and generated 31,816 transcripts.

Sherman et al. (2015a) characterized a mango reference transcriptome of the *Mangifera indica* cv. Keitt. Libraries from RNA of young leaves, young inflores-

cences, fruitlets, flesh and peel of mature fruit were sequenced on a 454-Titanium platform. The 1,329,313 raw sequence reads were filtered to remove low quality and empty reads. De novo assembly was performed on 1,113,875 clean reads resulting in a total of 47,956 contigs.

Srivastava et al. (2016) undertook a transcriptome analysis of the popular north Indian *Mangifera indica* cv. Dashehari at three ripening stages. A total of 71,733 contigs were assembled with an average length of 942 bp. A total of 46,412 assembled transcripts (74.7%) showed significant similarity to known proteins in the NR database of which most were related to *Citrus* sp. (59%). A total of 25,614 contigs showed no significant hits in any of the databases searched.

Sivankalyani et al. (2016) undertook transcriptome analysis of cv. Keitt in response to chilling stress. Fourteen libraries were prepared from three time points at two storage temperatures and sequenced. From 222,097,481 raw reads, a total of 57,576 transcripts were identified with a mean length of 863 bp. A summary of the transcriptome data for mango is presented in Table 20.4

20.8.7 Genetic Linkage Mapping

One of the significant applications of molecular markers described earlier is the construction of genetic linkage maps. A linkage map is a genetic map of a species or experimental population that shows the position of its known genes or genetic markers relative to each other in terms of recombination frequency rather than a specific physical distance along each chromosome. In other words, the linkage map describes the linear order of markers within linkage groups. The process of linkage mapping estimates how markers are grouped together based on the number of recombination breaks between them with one genetic map unit equal to the distance between marker pairs for which one product of meiosis out of one hundred is recombinant. Although mango has a high level of heterozygosity, it has a relatively small genome size (approximately 439 Mb) (Arumuganathan and Earle 1991). It is understood to be an allotetraploid with a diploid number of chromosomes of 20 and a total number of chromosomes of 40 ($2n = 4x = 40$) (Mukherjee 1950b).

Selection of parent plants is a crucial step in the process to generate a linkage map. Parent plants must be genetically divergent as to exhibit sufficient polymorphisms but not so different as to render the progeny sterile. Segregation analysis and linkage mapping is not possible when polymorphisms are absent in the parent plants. As mango naturally outcrosses it is assumed that large numbers of polymorphisms occur. This is seen with the marker analysis in *Mangifera indica* to date.

Another difficulty of creating a linkage map for mango is the generation of a substantial number of hybrid individuals in a mapping population in order to detect the effects of the QTLs responsible for traits of interest. Hand pollination is time consuming and costly, while low fruit set and high fruit drop impede hybrid progeny development. Another issue is the number of molecular markers currently available for the development of a saturated linkage map.

Table 20.4 Summary of the transcriptome data for mango

Mango variety	Tissue used	Total number of transcripts	Average length of transcripts (bp)	Number of unigenes obtained	Reference
Langra	Leaf	85,651	536	30,509	Azim et al. (2014)
Zill	Pericarp and peel	124,002	838	54,207	Wu et al. (2014)
Shelly	Peel	57,544	863	35,719	Luria et al. (2014)
Kent	Mesocarp	80,969	836	52,948	Dautt-Castro et al. (2015)
Tommy Atkins	Leaves, flowers and developing fruit (exocarp, mesocarp, seed coat, seed)	577,278	Unknown	31,816	Kuhn et al. (2016)
Keitt	Young leaves, young inflo-rescences, fruitlets, flesh and peel of mature fruit	60,997	Unknown	47,956	Sherman et al. (2016)
Dashehari	Inner zone of mesocarp	71,733	942	44,472	Srivastava et al. (2016)
Keitt	Peel	57,576	863	Unknown	Sivankalyani et al. (2016)

To date six genetic linkage maps have been generated for mango. The first was developed by Chunwongse et al. (2000) using 31 F1 individuals from a cross between cvs. Alphonso and Palmer. A total of 650 AFLP and 197 RFLP markers were used to construct a maternal (cv. Alphonso) linkage map and a paternal (cv. Palmer) linkage map. The two maps were generated according to a double pseudo-testcross strategy (Grattapaglia and Sederoff 1994). Hemmat et al. (1994) suggested that if any one of the mapping parents has recessive genes, then polymorphic marker segregation in the F1 hybrid progeny is a 1:1 (present and absent) ratio, and the 1:1 segregation type is based on the source of heterozygosis in order to construct the female and male linkage map. This means female and male maps can be constructed, respectively, but only the segregation 1:1 markers can be used to construct a female or male map. The 3:1 markers cannot determine the source of alleles and, therefore, are not used in analysis of individual genotypes. The cv. Alphonso map comprised of 205 linked markers, grouped into 63 LGs, covering 1437.7 centimorgans (cM) and the cv. Palmer map comprised of 246 linked, grouped into 59 LGs, covering 2561.9 cM.

A second linkage map was developed by Kashkush et al. (2001), based on AFLP markers segregating in 29 F1 hybrid progeny from a cross between Keitt x Tommy Atkins cvs. Fourteen primer combinations produced 105 clear bands for analysis. Of these bands, 85% showed Mendelian segregation and 15% showed distorted segregation. Thirty-four bands were mapped, with an average marker spacing of 4.75 cM, in 13 linkage groups (LGs) covering 161.5 cM. Fifty-four bands were un-mapped.

Fang et al. (2003) developed the third genetic map of mango using 81 AFLP markers based on 60 F1 individuals and covering a length of 354.1 cM with an average marker spacing of 4.37 cM. In this map the 3:1 segregating markers were analyzed in an interspecific hybridization and 14 linkage groups were constructed from a total of 39 loci based on the binomial distribution principal.

A mapping population of 115 full-sib (F1) progeny from hand-pollinated Irwin x Kensington Pride cvs. crosses was used to generate a linkage map using AFLP and SSR markers (Sheikh Jabbari, unpublished). A total of 10,431 AFLP loci were detected in the F1 progenies using 18 primer combinations. Of these, 400 markers (38.4%) were polymorphic between parents and 641 (61.6%) were present in both parents. The maternal map consisted of 18 LGs with 160 loci and covering an estimated 1711.5 cM, while the paternal map consisted of 21 LGs with 175 loci and covering an estimated 2091.7 cM. Taking all LGs into consideration the average length between markers was 6.2 and 7.9 cM for the female and male maps, respectively.

An update of the genetic maps by Chunwongse et al. (2000) has been more recently undertaken (Chunwongse et al. 2015), using the same mapping population of 31 F1 individuals obtained from a cross between cvs. Alphonso and Palmer (Chunwongse et al. 2000). A total of 214 codominant markers (69 SSRs and 145 RFLPs) were used for the analysis. Of these markers, 76 (9 SSRs and 67 RFLPs) were assigned to 29 LGs spanning a total distance of 529.9 cM.

These genetic maps have all been developed with small mapping populations or limited numbers of molecular markers resulting in a relatively low-density genetic map for future QTL analysis. With the advances in next generation sequencing (NGS) projects in mango, the development of a saturated linkage map has now been resolved. In the first of these genetic maps (Luo et al. 2016) organized 6594 specific locus amplified fragments (SLAFs) across 173 F1 plants from a cross between *Mangifera indica* cvs. Jun-Hwang and Irwin consisting of 20 LGs. The total length of the consensus map is 3148.28 cM and the average distance between adjacent markers 0.48 cM.

A second genetic linkage map developed by Kuhn et al. (pers. comm. 2016) has 726 SNP markers distributed across 20 LGs. Seven mapping populations, totaling 775 individuals, were used to create this consensus map. Assuming a haploid genome size of ~439 Mb and 20 chromosomes per haploid genome, the average size of a chromosome would be ~22 Mb. The total size of the map is 2890 cM. An estimate of the average size of a cM would be ~150 Kb. Further, a number of QTLs were identified in association with the genetic linkage map developed by Kuhn et al. (pers. comm. 2016). Embryony type, which is dimorphic (monoembryonic or polyembryonic),

showed significant association to a single locus on LG 8 in two of the three mapping populations that had phenotypic data associated with them. Significant associations of six other traits, bloom, pulp color, branch habit, ground skin color, blush intensity and beak shape, to specific loci on the genetic map were also seen. This is the first report of quantitative trait loci (QTLs) described in mango.

In the absence of a genome sequence, a high-density genetic map is a valuable tool to understand the genetic and genomic organization of mango, since it provides a framework of marker order and spacing. This in turn allows comparative analyses with the maps and sequence of other species. The genetic linkage map also serves as a starting point to map QTL for target traits. The estimation of QTL locations on a linkage map is still needed to bridge from markers to candidate gene models embedded in the assembled sequence data. The linkage map then provides information on linked markers that can be used during marker-assisted selection in breeding programs, making the selection process more efficient.

Over the last two decades the development of molecular techniques for genetic analysis has led to a great increase in our knowledge of mango genetics and diversity.

Many DNA markers have been developed for mango. The promise of marker-assisted selection in mango breeding still remains, but achieving practical benefits is taking longer than expected. The ability to monitor DNA sequence variation in and among species to create new sources of genetic variation by introducing new and favorable traits from landraces and related species is the ultimate goal. We are on the cusp of being able to identify traits linked to useful traits which, in turn, will have implications in breeding programs.

20.9 Conclusions and Prospects

Despite some of the constraints and long time frames of conventional breeding in mango, many opportunities exist to produce new cultivars with traits that will improve productivity, fruit quality and the economic competitiveness of mango industries. Recent and future development of molecular markers, especially those that are suitable for a high throughput marker-assisted selection, should substantially improve the efficiency of the conventional mango breeding by identifying the genetic linkage between marker allele and the gene of interest. Increased use of molecular markers and other pre-selection techniques has the potential to substantially reduce selection time frames in mango breeding programs. Identification and cloning of horticulturally-interesting genes from mango and its wide relatives, will enhanced the rate of conversion or plant survival from germinated transgenic somatic embryos, and would certainly revolutionize the genetic improvement of mango cultivar. Biotechnological developments and statistical analysis of current breeding populations are also substantially improving our understanding of the genetic traits and their inheritance in mango. These understandings make it easier for the breeder to select parents and design breeding programs with more specific breeding goals than has been possible in the past. However, there are still significant constraints in con-

trolled hybridization that need to be overcome if larger breeding populations are to be generated. Studies to improve our understanding of pollination compatibility, timing, and techniques can contribute to improved breeding efficiency.

Appendix: Research Institutes

Indian ICAR Central Institute of Subtropical Horticulture
http://www.cish.res.in/cib.php
Indonesian Tropical Fruit Research Institute
https://ccafs.cgiar.org/partners/indonesian-tropical-fruit-research-institute-0

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Chapter 21
Genetic Improvement of Papaya (*Carica papaya* L.)

Fredah Karambu Rimberia, Francis Kweya Ombwara, Naomi Nzilani Mumo and Elijah Miinda Ateka

Abstract Arising from a relatively isolated center of origin, papaya has spread throughout all tropical and subtropical countries through human intervention. This global dispersal has coincided with continuous improvement of the cultivated plants through breeding programs often designed to improve the agronomic characters and to address biotic and abiotic stresses that affect papaya production. Papaya production is threatened by a myriad of problems including devastating pests and diseases as well as the inability for both farmers and researchers alike to differentiate among the three sex types, male, female and hermaphrodite at the seedling stage, among others. Many attempts have been made by researchers over the years to resolve the problems through conventional and biotechnological techniques. Conventional plant breeding has given rise to varieties that are resistant to diseases as well as high yielders of quality fruits. However, conventional techniques require 12–14 years to develop new papaya varieties. Besides, devastating viral diseases like papaya ringspot virus (PRSV) have proved almost impossible to control through conventional means. The innovative technologies and growing understanding to manipulate the papaya phenotype at the molecular level provide new opportunities for the improvement of papaya. Through gene transfer technology, it is possible to develop transgenic papaya with pest and disease resistance as well as improved nutritional quality. This chapter provides insight into conventional breeding of papaya, the role of tissue and

F. K. Rimberia (✉) · F. K. Ombwara · N. N. Mumo · E. M. Ateka
Department of Horticulture, Jomo Kenyatta University of Agriculture and Technology, P.O Box 62000-00200 Nairobi, Kenya
e-mail: frenda@agr.jkuat.ac.ke; fredawanza@yahoo.com

F. K. Ombwara
e-mail: fombwara@agr.jkuat.ac.ke

N. N. Mumo
e-mail: naomi.mumo@jkuat.ac.ke

E. M. Ateka
e-mail: eateka@agr.jkuat.ac.ke

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protoplast culture as well as molecular techniques in papaya improvement such as genetic transformation, mutation breeding and marker assisted selection and breeding. In addition, the potential of parthenocarpy as well as polyploidy and somaclonal variation in papaya breeding are discussed.

Keywords Aluminum tolerance · Anther culture · *Carica papaya* L. · Carmine spider mite · Dwarf papaya · Genetic transformation · Mating systems

21.1 Introduction

21.1.1 Origin and Distribution

Papaya *(Carica papaya* L.) is a cosmopolitan tropical fruit tree, believed to have originated in the Southern Mexico/Central American region. It is well adapted to a wide range of environmental conditions, and readily cultivated throughout the tropical and subtropical regions of the world (Villegas 1997). Its global distribution is reflected by the numerous synonyms used to describe it, some of which include pawpaw, tree melon (English), mamão (Portuguese), melonenbaum (German), malakor (Thai) and apoyo in parts of East Africa.

Papaya is cultivated in the Americas, Asia, Africa and Oceania. The plant requires relatively little water for its establishment and needs only minimal levels of mineral nutrients for growth and development, making it suitable for cultivation under suboptimal nutrient conditions (Saran and Choudhary 2013). The plant is readily found under cultivation anywhere between 36°N and 36°S latitudes, but the greatest proportion of papaya production occurs between 26°N and 26°S (Nakasone and Paull 1998).

World production of papaya was estimated at over 12.8 million mt of fruit in 2016. India, Brazil, Indonesia, Nigeria and Mexico were ranked the five largest papaya producers, in that order, cumulatively accounting for 76% of the global production, with India alone contributing nearly 44% of the total (FAOSTAT 2017).

21.1.2 Taxonomy and Morphology

Papaya is a flowering plant, which belongs to the family Caricaceae. The family has an amphi-Atlantic distribution, with two species indigenous to tropical Africa and approximately 33 species endemic to Central and South America (Carvalho and Renner 2012). The family comprises six genera, namely *Cyclimorpha, Jacaratia, Jarilla, Vasconcellea, Horovitzia* and *Carica*. In the genus *Carica*, *Carica papaya* L. represents the cultivated papaya (Badillo 2000; Milind and Gurditta 2011). It is a member of the order Brassicales and shares a common ancestor with *Arabidopsis*

(Wikström et al. 2001). *Vasconcellea* spp. are the closest relatives to *Carica papaya* (Van Droogenbroeck et al. 2004) and its members include many species with edible fruits and have great importance for breeding and genetic studies, with a few cultivated varieties (Badillo 2000; Scheldeman and Van Damme 2003). Species of *Vasconcellea* which produce edible fruits include *V. cundinamarcensis* (chamburo) and *V. pubescens* (ababai) grown in the Americas and the fruits are normally cooked and flavored by adding sugar before eating. *Vasconcellea pentagona* (babaco), which is grown in western South America, New Zealand, South Africa, Spain and Italy, is commercially cultivated for its large parthenocarpic fruits. *Vasconcellea chrysopetala* also known as higacho or toronche, is found in Ecuador and New Zealand (Scheldeman and Van Damme 2003). Some *Vasconcellea* species such as *V. cauliflora* and *V. quercifolia* are resistant to papaya ringspot virus, which affects *Carica papaya* L.

21.1.3 Plant Description

Papaya is a herbaceous tree, with a soft, woody stem topped by a crown of leaves clustered at the top of the trunk. The plant is predominantly single-stemmed but will occasionally branch due to wounding of the terminal shoot or as the plant ages. The stem is hollow, light green to deep purple in color, with prominent leaf scars. The leaves are large, palmately-lobed or deeply incised, with entire margins borne on long, hollow petioles about 125 cm long, and open in sequence up the stem as the tree grows (Milind and Gurditta 2011). The papaya root system comprises two main components; a vertical tap root for absorbing water; and lateral fine feeder roots for the absorption of nutrients.

The papaya is polygamous, having, male, hermaphroditic or female plants, each of which bears flowers of unique morphology reflecting the plant's sexual diversity (Eustice et al. 2008). The male papaya's numerous flowers are borne on extended, branched peduncles, often greater than 30 cm in length, while the hermaphrodite tree bears several bisexual flowers, with an occasional male flower due to sex reversal. The female tree bears a few female flowers exclusively on short peduncles. Individual flowers are white, cream-colored, yellow, or purple-tinged, and are borne in inflorescences on the trunk in the leaf axils. Both male and female trees must be present to produce fruit, while hermaphrodite trees are self-pollinating.

The plant flowers 3–9 months after planting and its fruits mature 5–15 months later, depending on the cultivar and prevailing temperatures (Milind and Gurditta 2011; Paterson et al. 2008). Commercial varieties normally flower 5–6 months after transplanting and the fruits ripen 5–6 months after flowering. Papaya has a moderately small genome of 372 megabases (Mb) (Arumuganathan and Earle 1991) and diploid inheritance with nine pairs of chromosomes (Bennett and Leitch 2005; Wikström et al. 2001).

The plant bears fruits throughout the year and fruits are either borne singly or in clusters, in the axils of the stem. The fruits are attached to the trunk by a peduncle attached to the upper trunk, below the old leaves, and arranged in an acropetal manner

whereby younger fruits arise above the older fruit on the trunk. The fruits vary in the size, shape and quality depending on the sex of the fruit (Milind and Gurditta 2011; Yogiraj et al. 2014). The female plants produce medium to large round-shaped fruits with a relatively large seed cavity, while the hermaphrodites produce small to medium elongated fruits with smaller seed cavity. The color of the fruit ranges from green when unripe to yellow or red orange when ripe and the color of flesh ranges from yellow-orange or pinkish orange at maturity. The fruit is climacteric, with ripening being controlled in part by the synthesis of ethylene (Magdalita et al. 2002).

21.1.4 Utilization

Various parts of the papaya plant have numerous applications. The young leaves and shoots and flowers are often cooked and consumed as a vegetable (Oloyede 2005; Saran and Choudhary 2013). The ripe papaya fruit is eaten as a fresh fruit or desert. When consumed ripe, papaya fruits are rich in vitamins A, B and C, various minerals including potassium, magnesium and boron, and dietary fiber (Ming et al. 2008; Yogiraj et al. 2014). The actual chemical composition of papaya fruits is dependent on various factors, such as the variety, climate, cultural practices employed and location at which the plants are grown (Imungi and Wabule 1990). Studies indicate that one medium-sized papaya with an edible portion of approximately 350 g, exceeds the dietary reference intakes (DRI) of 3000 IU for vitamin A and 90 mg for vitamin C as recommended by the U.S. Food and Nutrition Board for adult minimum daily requirements (Farzana et al. 2008; Ming et al. 2008). The ripe fruit is also often processed into a range of food products which include canned tropical fruit cocktails, juices, wines, purees and jam. The fruit is also often processed blanched and dried for consumption as a sweet condiment. Papaya seeds are dried, ground and used as a spice due to their peppery flavor (Aravind et al. 2013).

The green fruit is rich in potassium, calcium and phosphorous and is peeled, deseeded and cooked as a vegetable or used in a variety of savory Asian dishes, including pickles and chutneys and for canning in sugar syrup (Manshardt 1992; Nakasone and Paull 1998). However, due to the toxic nature of its latex, unripe papaya fruit should never be eaten raw (Saran and Choudhary 2013). The latex of the immature green papaya fruit is used as a natural source of the proteolytic enzyme papain. The latex is tapped manually from green fruits and air or oven dried, before being refined for use in the food processing, pharmaceutical, cosmetic and manufacturing industries (Ming et al. 2008). Papain production, being a manual process, is predominantly carried out in developing nations which include Tanzania and India, due in part to the abundance of relatively cheap labor (Sankat and Maharaj 1997).

Papaya is reported to have numerous medicinal uses, some of which include: the use of the bark in treatment of jaundice, the dressing of wounds using the leaves of the plant, the use of the plant latex to cure diarrhea, the use of root infusions which act as

diuretics (dehydrants) and the consumption of seeds which act as a vermifuge (Boshra and Tajul 2013; Krishna et al. 2008). The latex from papaya is used to treat warts, cancers, tumors, corns and skin defects (Saran and Choudhary 2013). Oloyede (2005) reported that fruit and seed extracts possess pronounced bactericidal activity against *Staphylococcus aureus*, *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa* and *Shigella flexneri*.

21.1.5 Production Constraints

The papaya industry faces two main problems; diseases and unreliable methods of differentiating among the three sex types, male, female and hermaphrodite, at seedling stage. Papaya is mainly propagated sexually. Prior to flowering, the seedlings look alike irrespective of their sex. As such producers have to wait until flowering time to differentiate among the sex types. The general recommendation has been to plant 3 seedlings per hole and destroy the extra plants at the onset of flowering. Since only a few males are required for pollination (ratio of 1 male: 9 females), this recommendation is very uneconomical for producers in terms of both capital and time investment.

Molecular markers that can differentiate papaya sex types were developed by Lemos et al. (2002) and Urasaki et al. (2002a, b), but these procedures have not been commercialized. Their commercialization will ensure that producers purchase only the desired sex of seedlings. However, molecular markers are currently very expensive and it might be impractical to apply such a procedure in commercial production right away, especially in resource-poor developing countries.

Furthermore, the widespread nature of papaya ring spot potyvirus (PRSV) in all major papaya-producing areas of the world (Manhsardt 1992) has not spared the Kenyan papaya industry. As a result, papaya is being wiped out at a fast rate, and the devastation is already becoming obvious in both research institutions and farmer fields. Effective control has remained elusive since resistant cultivars are not available and chemical control of insect vectors using insecticides is impractical since PRSV is non-persistently transmitted. Viral diseases, particularly the papaya ringspot disease (PRSD) which causes yield losses of up to 30–40%, has caused enormous devastation of papaya fields in various countries worldwide resulting in decreased fruit production.

Other challenges facing papaya production include bacterial dieback (*Erwinia papaya*), fruit brown blotch (*Colletotrichum gloeosporioides*), fruit fly (*Bactrocera papaya*) and oriental fruit scale (*Aonidiella orientalis*) among others. Several attempts have been made to resolve these challenges through conventional and biotechnological crop improvement strategies as outlined below.

21.2 Conventional Breeding

21.2.1 Flower Types

The somatic chromosome number in the dicotyledonous genus *Carica*, is 2n = 18. Most *Carica* species are dioecious, except for *Carica papaya* L. (commercially-produced papaya). *Carica papaya* L. is characterized by various flower types and polygamous sexual types, pistillate (female), staminate (male) and hermaphrodite. Staminate trees produce long pendulous male inflorescences bearing 10 stamens in each flower, pistillate trees bear 1 or 2 flowers at each leaf axil, with a round to oblong ovary, while hermaphrodite trees normally bear one to several bisexual flowers characterized by an oblong ovary and usually 10 stamens. Based on the sex segregation ratios which resulted from cross- and self-pollination studies of the three basic sex forms. Storey (1938, 1953) and Hofmeyr (1938) symbolized sex genes of the three sex types respectively as M1 m (male), mm (female), and M2 m (hermaphrodite), and hypothesized that dominant homozygotes (M1M1, M1M2 and M2M2) were lethal. This theory was confirmed through in vitro culture of papaya anthers which generated only female plants (Rimberia et al. 2006). The absence of males among the shoot samples was presumably related to the lethality of dominant homozygotes, as proposed by Storey (1938, 1953) and Hofmeyr (1938). Pollen grains with M1 may have been impotent for embryo differentiation or differentiated embryos may have died at an early stage of development (Rimberia et al. 2006).

21.2.2 Sex Determination

Recent developments in molecular-marker techniques have made it possible for papaya sex types to be accurately differentiated at an early developmental stage.

Sex-linked DNA markers have been developed in different parts of the world through DNA analysis using polymerase chain reaction (PCR) that identified papaya sex type at an early developmental stage. These include randomly amplified polymorphic DNA (RAPD) markers (Chaves-Bedoya and Nunez 2007; Lemos et al. 2002; Parasnis et al. 2000; Reddy et al. 2012; Urasaki et al. 2002a); sequence characterized amplified region (SCAR) markers, converted from RAPD (Chaves-Bedoya and Nunez 2007; Deputy et al. 2002; Parasnis et al. 2000; Urasaki et al. 2002a, b) and simple sequence repeat (SSR) markers (Costa et al. 2011; Parasnis et al. 1999).

Sex type of papaya (*Carica papaya*) is determined by the pair of sex chromosomes XX, XY and XY^h for female, male and hermaphrodite, respectively, in which there is a non-recombining genomic region in the Y and Y^h chromosomes. This region is presumed to be involved in determination of males and hermaphrodites; it is designated as the male-specific region in the Y chromosome (MSY) and the hermaphrodite-specific region in the Y chromosome (HSY) (Ueno et al. 2015). They further reported

that the study of *short vegetative phase (SVP)-like* transcripts revealed that the MSY allele encoded an intact protein, while the HSY allele encoded a truncated protein.

Tsai et al. (2016) developed a rapid and efficient method for detecting the male-hermaphrodite-specific marker to examine papaya sex type based on the loop-mediated isothermal amplification (LAMP) assays without prior DNA purification. The molecular-marker technology is accurate, but expensive and out-of-reach for ordinary farmers. Recently, Abreu et al. (2015) developed a probe for fluorescence in situ hybridization (FISH) protocol for differentiating between hermaphrodite and female papaya, which gave fluorescent signals in hermaphrodite nuclei isolated from leaves, but no detectable intensity fluorescence signal in female nuclei. They further confirmed that this protocol is superior to the molecular-marker technology in that it has potential for commercialization and automation.

Development of physiological and biochemical markers has also shown promise. Soni et al. (2017) reported that the hermaphrodite plants had higher leaf chlorophyll content than their male counterparts. They further reported significantly higher total phenols and stomatal conductance in the leaves of the female plants, followed by the hermaphrodite plants, at the seedling stage. Further research needs to be done on physiological and biochemical sex identification markers.

21.2.3 Genetic Diversity

Genetic diversity is the basis for crop improvement and maintenance. Papaya germplasm in different parts of the world has reportedly been characterized using morphological, isozyme, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and PCR-RFLP markers (Asudi et al. 2013; Calderón et al. 2016). The simple sequence repeat (SSR) markers have been used successfully for determining the genetic relationship between *Carica papaya* L. cultivars (Ocampo et al. 2006). Most studies showed limited genetic diversity in the common papaya. Asudi et al. (2010, 2013), using both morphological and SSR markers reported limited genetic diversity in Kenyan papaya, with accessions from the Coast and Rift Valley presenting the highest diversity compared to accessions from other parts of the country. Elsewhere, Saran et al. (2015) reported a high degree of variation among Indian papaya germplasm using morphological and two different types of molecular markers (RAPD) and inter-simple sequence repeats (ISSR). They further concluded that both morphological and molecular markers were useful for inferring genetic diversity and relatedness in papaya.

21.2.4 Conservation of Germplasm

Conservation of papaya germplasm is essential for future breeding programs. Papaya bears recalcitrant seeds which have been reported to lose viability below moisture content of 8–10% (Ellis et al. 1991). Ashmore et al. (2011) investigated seed storage behavior from liquid nitrogen to 15 °C/15% RH and optimized treatments to break dormancy and to ensure germination after seed storage. Thus, conditions for ex situ conservation of papaya through cryopreservation, including conditions for maximum regrowth and recovery of plantlets following cryopreservation have already been established (Ashmore et al. 2011; Dhekney 2004).

21.2.5 Mating Systems and Commercial Seed Production

Dioecious papaya varieties are forced cross-pollinators because of physical separation of the androecium and gynoecium. It was previously assumed that dioecious papaya is wind pollinated due to the long pendulous male inflorescence which readily sheds pollen into the breeze. However, it was later shown that very little papaya pollen was airborne. Many insects have been credited with pollinating papaya including honeybees, skipper butterflies and hawkmoth (*Hyles* sp.) (Chan 2009). Papaya pollen is not a favorite for pollinators, so it is assumed that the native vegetation and cultivated crops attract these pollinators and increase fruit production in papaya (Chan 2009).

In gynodioecious populations, the stamens are packed inside the corolla tube and seldom protrude prominently out of the flower. Many gynodioecious varieties such as Sunrise Solo, Kapoho Solo and Eksotika are self-pollinated and are, therefore, pure lines (Chan 2009). The hermaphrodite flowers are mostly cleistogamous; anthers dehisce and release the pollen to effect self-pollination prior to anthesis of the flower (Rodriquez-Pastor et al. 1990).

Papaya pollen production and viability depends on variety and season. For instance, between the two inbred parental lines 19 and 20 of F1 hybrid Eksotika II, line 20 consistently produced more pollen. Additionally, pollen production decreased during winter and early spring because of the degeneration of pollen mother cells. Under ideal storage conditions, pollen remains viable for 5–6 years. Stigma receptivity remains high throughout the year and both female and hermaphrodite flowers pollinated with viable pollen successfully set fruit even in winter (Chan 2009).

Hermaphroditic cultivars are commercially cultivated in the tropics, while female cultivars are predominantly used in the subtropical regions such as South Africa, Australia and Okinawa, Japan. This difference in cultural adaptation between both types of papaya may be due to sex reversal. Hermaphrodite and male papaya plants seasonally reverse their flower sex by stamen carpellody and female sterility, resulting in poor quality and low yield of fruits. On the other hand, the female is stable with respect to sex expression throughout the year (Rimberia and Adaniya 2010).

Commercial seed production in gynodioecious varieties such as Sunrise Solo and Eksotika, is achieved through selfing hermaphrodite flowers or hybridization of hermaphrodite flowers with hermaphrodite pollen. Seed derived from these cross-combinations will have twice the number of hermaphrodites compared with females. For dioecious varieties, the preferred combination for seed production is mm × Mm, namely, to use pollen from male flowers for crossing female flowers. A ratio of 1:1 male to female will be obtained. Crosses between females and hermaphrodites may be used for hybrid seed production because it obviates emasculation when female flowers are used as the maternal parent (Chan 2009).

21.2.6 Parthenocarpy in Papaya Improvement

Female papaya plants are usually inter-cropped with males to allow pollination and increase fruit yield. Intercropping consumes extra space in small enterprises, or where papaya is produced within a windproof screen or glass houses for instance in Japan to protect against diseases like papaya leaf distortion, papaya ringspot virus (PRSV), papaya mosaic virus diseases and damage by frequent typhoons (Ogata et al. 2016). An alternative to pollination might be found in promoting parthenocarpic fruit production.

Nakasone and Paull (1998) and Ray (2002) reported the occurrence of natural parthenocarpy in female papaya. Rodriquez-Pastor et al. (1990) reported variation in parthenocarpic ability among female cultivars. More recently, Rimberia and Adaniya (2010) and Rimberia et al. (2006b) reported that both female and hermaphrodite plants had parthenocarpic ability and that this ability varied among cultivars. They further demonstrated that female plants had significantly higher parthenocarpic ability than their hermaphrodite counterparts (Fig. 21.1). This intra-specific variation of this trait suggests a possibility of improving papaya genotypes with high parthenocarpic ability. Rimberia et al. (2007) reported much variation in parthenocarpic ability among the anther-culture derived strains. The dwarf strains with high fruit yield showed high parthenocarpic ability, but the strains with low fruit yield had low parthenocarpic ability. The phenotypic variation in dwarf nature and parthenocarpic ability among the microspore-derived plants indicate that the anther culture technique has potential to contribute to the systematic breeding of papaya.

21.2.7 Varietal Development

All breeding methods useful for the improvement of self-pollinating species are applicable to the breeding of hermaphrodite papaya, while population improvement methods are useful in both females and hermaphrodites (Manshardt 1992). True-bred lines of gynodioecious varieties such as Kapoho, Sunrise Solo, Waimanalo, Kamiya and Eksotika, among others, were established through inbreeding by means of pedi-

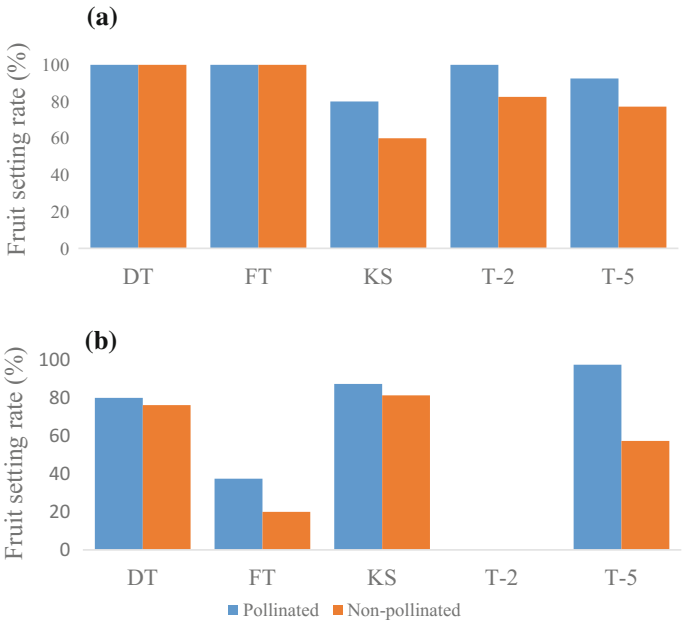

Fig. 21.1 Fruit setting rates of pollinated and non-pollinated female (**a**) and hermaphrodite (**b**) cultivars. DT: Dantesu, FT: Fruit tower, KS: Kansen, T-2: Taino-2, and T-5: Taino-5. *Source* Rimberia and Adaniya (2010), Rimberia et al. (2006b)

gree or backcross breeding methods (Chan 2009; Manshardt 1992). Self-pollination (inbreeding) for sustaining a variety is disadvantaged by inbreeding depression. Louw (2016) reported serious inbreeding depression resulting from continuous self-pollination, characterized by loss of productivity and poor or no seed germination.

Development of new varieties through inbreeding following selection has shown significant promise (Dinesh 2010). Four varieties, Pusa Delicious, Pusa Majesty (gynodioecious), Pusa Giant and Pusa Dwarf (dioecious) were developed through continuous sibmating and selection by conventional breeding in India (Singh et al. 2010), between 1966 and 1982. Commercial dioecious varieties like Hortus Gold, Honey Gold, Sunnybank, Hybrid No. 5, Cariflora, Co1 and Co2 were also developed through conventional plant breeding (Chan 2009).

Recently, Rimberia et al. (unpublished data) evaluated over 100 papaya accessions collected from within Kenya (both local and imported varieties), crossbred (controlled hand pollinated) those with potential for dwarfness, high yields and tolerance to viral diseases (Fig. 21.2), from 2008 to 2014. Four dioecious lines were successfully selected and are in the process of registration by the Kenya Plant Health Inspectorate Services (KEPHIS). Preliminary evaluation of the new Kenyan papaya varieties developed at Jomo Kenyatta University of Agriculture and Technology (JKUAT) indicate that they start flowering at a height of 58–79 cm, yield 13–20 kg of fruits per tree in the first season and have a mean Brix range of 11–12.

Fig. 21.2 Mature female JKUAT papaya varieties that are in the process of registration

21.3 In Vitro Applications

21.3.1 Tissue Culture

Papaya is commercially propagated by seed. Male, female and hermaphrodite papaya seedlings are indistinguishable before flowering. Farmers and researchers wait for about 6 months to differentiate among the sex types. Moreover, the papaya varieties cannot be maintained through sexual propagation due to the open pollination nature of the dioecious varieties and inbreeding depression among the hermaphrodite varieties (Louw 2016). Clonal propagation methods like tissues culture can overcome some of these difficulties in papaya cultivation and improvement. Additionally, tissue culture is useful for production of disease free planting materials.

The development of an efficient in vitro regeneration system for papaya represents remarkable progress for mass propagation and maintenance of uniform plants for both commercial and research purposes. The success of this technique depends on various factors such as (i) time of year, (ii) nature of the primary explant, (iii) the genotype and (iv) hormone component of the growth medium. Spring and early summer or tropical conditions offer the best natural conditions for rapid growth and development. Explants from actively growing papaya plants perform best under in vitro culture. Plant regeneration through tissue culture can be accomplished through shoot development from meristems, somatic embryogenesis and organogenesis.

21.3.2 Shoot Tip Meristems

The most reliable method of micropropagation in papaya is by shoot tip or axillary bud culture (Teixeira da Silva et al. 2007). Plants established from shoot tips have been shown to preserve the integrity of the parental genotype (Drew 1988).

Table 21.1 Axillary shoots induced from shoot tip explants of three papaya breeding lines cultured on MS media supplemented with different concentrations of BAP and NAA

BAP + NAA (mg/l)	Mean number of shoots formed per shoot tip		
	Line 1	Line 2	Line 3
0.0 + 0.0	0.8 ± 0.16^{h}	1.0 ± 0.44^{g}	0.6 ± 0.21^{h}
0.1 + 0.05	11.8 ± 0.91^{c}	8.8 ± 0.30^{c}	16.5 ± 1.54^{b}
0.5 + 0.05	13.8 ± 0.70^{b}	13.6 ± 0.95^{b}	16.8 ± 0.94^{b}
1.0 + 0.05	8.5 ± 0.22^{ef}	8.8 ± 0.47^{c}	6.8 ± 0.60^{efg}
2.0 + 0.05	6.5 ± 0.50^{fg}	7.8 ± 0.37^{cd}	6.5 ± 0.88^{efg}
0.1 + 0.1	11.0 ± 0.51^{cd}	12.3 ± 0.84^{b}	14.3 ± 0.91^{c}
0.5 + 0.1	24.3 ± 0.95^{a}	25.8 ± 2.08^{a}	19.3 ± 0.98^{a}
1.0 + 0.1	8.1 ± 0.47^{ef}	9.0 ± 0.25^{c}	10.6 ± 0.33^{d}
2.0 + 0.1	5.0 ± 0.36^{g}	7.6 ± 0.33^{cd}	5.8 ± 0.60^{efg}

Mean values ± SE within a column followed by the same letter are not significantly different by SNK ($P \leq 0.05$) within 12 weeks of subculture. *Source* Mumo et al. (2013)

In vitro regeneration of papaya is very much influenced by the genotype (Mishra et al. 2007). Reuveni et al. (1990) reported different rates of multiplication among 3 clones in the same media composition. Similarly, Mumo et al. (2013) demonstrated differences among 3 breeding lines in both rates of regeneration (Table 21.1) and elongation (Table 21.2) in the same media. Researchers from different parts of the world (Gatambia et al. 2016; Mumo et al. 2013; Panjaitan et al. 2007; Teixeira da Silva et al. 2007) established papaya shoots in vitro from shoot tip meristems of different genotypes of papaya, while Anandan et al. (2011) induced shoots from epicotyl segments.

Different phytohormones have been used successfully for shoot establishment, proliferation and elongation. Litz and Conover (1978) used solidified Murashige and Skoog (1962) (MS) basal medium with 50 μM kinetin and 10 μM naphthaleneacetic acid (NAA) for establishment, then subcultured into MS with 2 μM benzylaminopurine (BA) and 0.5 μM NAA for proliferation. All the media were solidified with 8 g/l Difco Bacto agar. Anandan et al. (2011) induced shoots on MS basal medium supplemented with 2.5 μM thidiazuron (TDZ), multiplied them in MS with 5 μM BAP and 0.05 μM NAA and elongated in 1.5 μM gibberellic acid (GA_3). All the media were fortified with 30 g-l sucrose and gelled with 0.7% agar. Caple and Cheah (2016), while using a Hawaiian variety Rainbow achieved initiation and multiplication of shoots in MS basal medium with vitamins, 3 mg/l benzyladenine purine (BAP) and elongation in 1.5X MS basal medium, 0.25 mg/l BAP, 0.3 mg/l GA3, maintaining the same concentration of sucrose (4% sucrose) and gelling agent (2.8 g/l Phytagel®). Mumo et al. (2013) used solidified MS with 0.5 mg/l 6-Benzyl Amino purine (BAP) and 0.1 mg/l NAA for both establishment and multiplication of 3 Kenyan papaya breeding lines through 3 weekly subcultures, followed by elongation in MS with

Table 21.2 Shoot length (cm) of shoot tip explants of three papaya breeding lines cultured on MS media supplemented with different concentrations of BAP and NAA

BAP + NAA (mg/l)	Mean shoot length (cm)		
	Line 1	Line 2	Line 3
0.0 + 0.0	1.31 ± 0.017^{g}	1.38 ± 0.031^{g}	1.58 ± 0.033^{f}
0.1 + 0.05	3.25 ± 0.085^{a}	3.30 ± 0.082^{a}	3.28 ± 0.070^{a}
0.5 + 0.05	2.63 ± 0.042^{b}	3.03 ± 0.105^{b}	3.01 ± 0.087^{b}
1.0 + 0.05	1.80 ± 0.037^{de}	2.05 ± 0.043^{d}	1.73 ± 0.042^{e}
2.0 + 0.05	1.76 ± 0.049^{e}	1.68 ± 0.031^{ef}	1.60 ± 0.037^{ef}
0.1 + 0.1	2.71 ± 0.048^{b}	2.78 ± 0.079^{c}	2.16 ± 0.033^{c}
0.5 + 0.1	2.26 ± 0.042^{c}	2.11 ± 0.031^{d}	2.13 ± 0.056^{c}
1.0 + 0.1	1.70 ± 0.026^{ef}	2.10 ± 0.058^{d}	2.13 ± 0.056^{c}
2.0 + 0.1	1.43 ± 0.033^{g}	1.83 ± 0.033^{e}	1.96 ± 0.071^{d}

Mean values ± SE within a column followed by the same letter are not significantly different by SNK ($P \leq 0.05$) within 12 weeks of subculture. *Source* Mumo et al. (2013)

0.1 mg/l BAP and 0.05 mg/l NAA. Both initiation/multiplication and elongation media had 30 g/l sucrose and solidified with 2.5 g/l gelrite.

Setargie et al. (2015) successfully used MS medium supplemented with 1 mg/l BAP and 0.5 mg/l NAA, solidified with 8 g/l agar for establishment, multiplication and elongation of hermaphroditic papaya cv. Maradol in Ethiopia. More recently, Gatambia et al. (2016) induced shoots from 3 local Kenyan papaya in MS basal media with 0.15 mg/l 2-Chloro-4-Pyridy-N Phenyl-urea (CPPU) with 30 g/l sucrose in a semi-solid/liquid double layer arrangement, solidified with 2.5 g/l gelrite mounted on an orbital shaker at a speed of 60 revolutions per minute (rpm). Multiplication and elongation of the shoots through bi-weekly sub culturing in the same media was achieved. CPPU, a synthetic cytokinin, has proved effective for shoot induction, multiplication and elongation. Cultures in liquid media on a shaker encourage close contact between the explant tissues and the culture medium, which facilitates the uptake of nutrients and phytohormones leading to better growth (Mehrotra et al. 2007). Additionally, continuous shaking improves aeration and eliminates apical dominance thus hastening the induction and proliferation of numerous axillary buds (Mehrotra et al. 2007). The semi-solid/liquid double layer technology has potential for automation of papaya micropropagation in bioreactors, as a strategy for reducing the cost of operation.

The shoots generated using solid media were successfully rooted in MS basal medium with 2.5 mg/l IBA (Anandan et al. 2011; Mumo et al. 2013; Teixeira da Silva et al. 2007), while those from solid/liquid bilayer were rooted in MS basal medium with 3 mg/l IBA (Gatambia et al. 2016). Different researchers (Mumo et al. 2013; Panjaitan et al. 2007; Yu et al. 2000) recommended the exposure of in vitro generated papaya shoots to MS basal medium with low concentration of IBA for 1 week, for root induction, followed by transfer to a medium supplemented with vermiculite (under

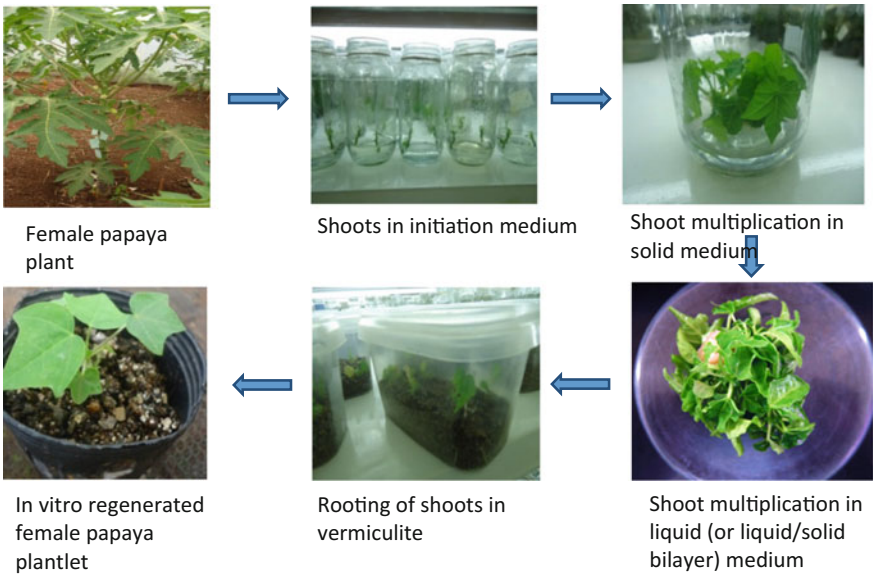

Fig. 21.3 Schematic representation of the processes involved in micropropagation of papaya plantlets

aerated conditions) for root development as being optimal for plantlet establishment. Fig. 21.3 illustrates the process of micropropagation of papaya plantlets of known sex using in vitro culture of shoot tip meristems. Since the shoots develop directly from the shoot meristem explant, which is genetically stable, somaclonal variation is eliminated. This makes micropropagation of papaya via meristem culture amenable for crop improvement, conservation of elite germplasm and commercial propagation.

21.3.3 Callus Induction and Somatic Embryogenesis

This method involves induction of embryogenic calli or embryos from which shoots are regenerated. Somatic embryos have been induced from different papaya explants such as the internode stem of seedlings, hypocotyl sections, seedling root explants, the midrib and lamina of cotyledons of seedlings, adventitious roots, immature zygotic embryos and immature seeds (Teixeira da Silva et al. 2007). Researchers have used different basal media with different concentrations and combinations of phytohormones to induce somatic embryogenesis such as MS basal medium with NAA and kinetin for callus induction, then MS with IAA and kinetin for embryo induction; MS with BA and NAA; Fitch's liquid medium with ½ MS and vitamins, myo-inositol, sucrose, 2,4-D and glutamine and de Fossard medium with BAP, NAA and GA3 (Abreu et al. 2014; Teixeira da Silva et al. 2007). Recently, Bukhori et al. (2013)

reported 77.5% embryogenic callus induction form immature zygotic embryos cultured on MS medium augmented with 10 mg/l 2,4-D and 250 mg/l carbenicillin. Auxins are critical for the initiation and subsequent growth of callus; NAA being the most effective, followed by 2,4-D and IAA.

Protoplast culture was attempted by Chen (1994) who successfully isolated protoplasts from highly regenerable suspension cultures from interspecific crosses of *Carica papaya* × *C. cauliflora* zygotic embryos. The somatic embryos proliferated and formed plantlets. The major challenge with somatic embryogenesis as a method of micropropagation is somaclonal variation which produces off-types and chimeras. However, it is an important procedure used in genetic transformation.

21.3.4 Anther Culture

This method is important for induction of haploid papaya plants which can be dihaploidized to generate homozygous parental breeding lines. The first attempt to produce haploids through anther culture in papaya was by Litz and Conover (1978), but did not specify the induction efficiency. A second attempt in 1979 (Litz and Conover 1979) managed a success rate of 0.4%. Tsay and Su (1985) also reported a low rate (0.67%) of embryo induction. Rimberia et al. (2005), while using anthers from Fl plants of a cross between a female cv. Wonder Blight and an unknown male strain reported an improved embryo induction rates in vitro to about 4.0% by pre-treating anthers on water or MS liquid medium with 2.0% sucrose at 35 °C for 1–5 days prior to culture on agar-solidified MS medium supplemented with 0.l mg/l BA, 0.1 mg/1 NAA and 3% sucrose. Gyanchand et al. (2015), used the same pretreatment and phytohormones to generate anther-derived embryos at a rate of 8.0% using papaya cv. Pusa Nanha. High temperature pretreatment improved the embryo induction rate from cultured anthers (Gyanchand et al. 2015; Rimberia et al. 2005), although the effects of nutrients and/or sucrose during the pre-treatment was ambiguous. The embryo induction rate was further improved to 13.8% by culturing anthers on agar-solidified MS medium with 0.01 mg/l CPPU and 0.1 mg/l NAA (Rimberia et al. 2006a). It appears that CPPU was more effective in embryo induction from anthers than high temperature pre-treatment. Additionally, genotypic differences have been demonstrated in embryo induction rates from cultured anthers (Gyanchand et al. 2015; Rimberia et al. 2005).

The sex diagnostic technique developed by Urasaki et al. (2002) and greenhouse evaluation were used to confirm that all the anther culture-derived papaya plants were female (Rimberia et al. 2007). This indicates that the plantlets were not derived from anther wall tissues but microspores. However, the optimum medium conditions for normal embryo growth was not defined because most of the embryos formed embryogenic calli (Fig. 21.4) (Gyanchand et al. 2015; Rimberia et al. 2005), that developed into fasciated shoots.

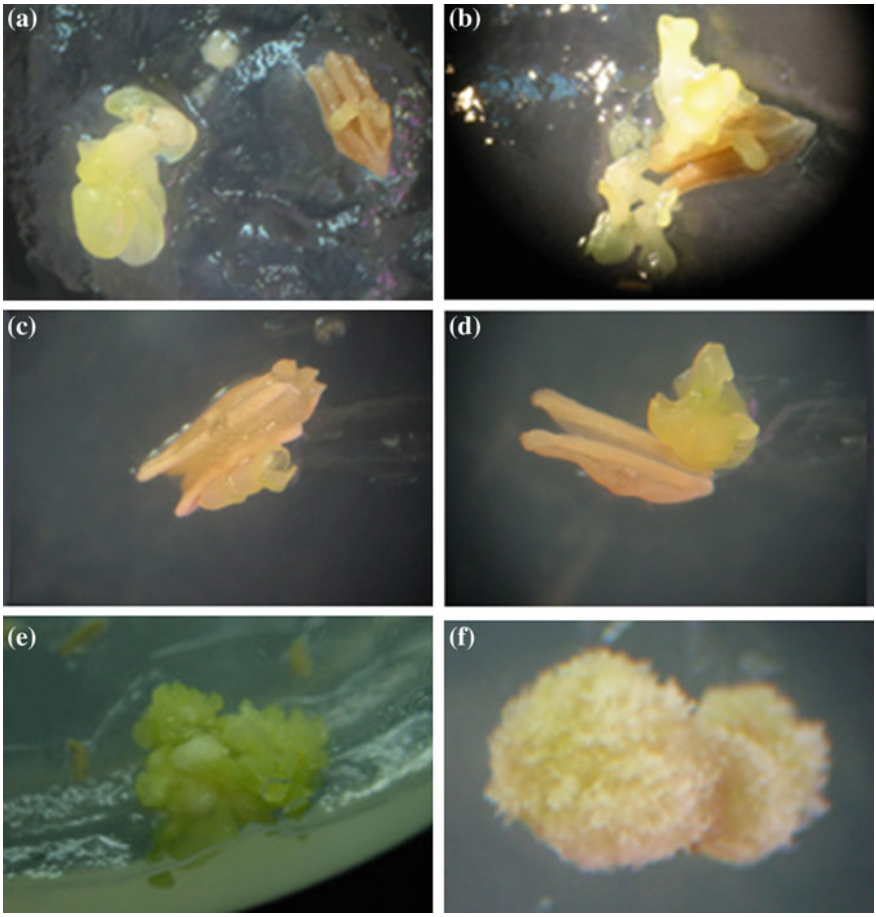

Fig. 21.4 Embryos and calli formed on anthers (**a**) Normally differentiated embryos, **b** multiple embryos, **c**–**e** Embryogenic calli, **f** Non-embryogenic callus *Source* Rimberia and Adaniya (2010)

21.3.5 Protoplast Culture

The first reports of successful protoplast culture in papaya were by Chen and Chen (1992) and Chen (1994), who isolated protoplasts from highly regenerable suspension cultures from interspecific crosses of *Carica papaya* × *C. cauliflora* zygotic embryos. Embryogenic cultures were digested with a mixture of cellulase R-10, macerozyme R-10 and driselase in 0.4 M mannitol, and plants were regenerated via somatic embryogenesis (Saksena 2013). Protoplast culture holds a lot of potential for somatic hybridization and genetic transformation.

21.3.6 Somaclonal Variation

Somaclonal variation usually accompanies in vitro plant regeneration via the callus phase and may be of great interest in crop improvement. Researchers over the years have managed to generate papaya plants of different ploidy levels using different explants. Clarindo et al. (2008) used immature papaya zygotic embryos to induce both somatic embryogenesis and somaclonal variants. The resulting plants were euploid (diploid, mixoploid, triploid and tetraploid) and aneuploidy that remained stable during the successive subcultures in multiplication medium. Sun et al. (2011) induced triploid papaya from immature endosperm with embryos. A total of 75% of the plants were triploids confirming their endosperm origin. Rimberia et al. (2006a) and Rimberia and Adaniya (2010) induced haploids, dihaploids, triploids, and tetraploids through in vitro culture of immature anthers. The triploids formed the bulk (87.5%) of the plantlets. Both triploids and tetraploids were evaluated in the greenhouse and they turned out to be females confirming their microspore origin. The triploids were variable in height and fruit bearing (Fig. 21.5). Some of the triplods were found to be dwarfs that produced seedless fruits of normal size (Rimberia and Adaniya 2010; Rimberia et al. 2007). The dwarf and parthenocarpic traits are important for the breeding of female papaya cultivars used for greenhouse cultivation. A system of propagation and maintenance of the triploids needs to be developed for them to be useful for commercialization.

21.3.7 Mutation Breeding

Mutation breeding is a useful tool for developing new varieties. In papaya, irradiation has been used to improve production (Chan et al. 2007; Hang and Chau 2010; Husselman et al. 2016; Kumar et al. 2017; Liao et al. 2017; Mahadevamma et al. 2012). In Malaysia, Chan et al. (2007) used gamma irradiation in variety Eksotika to produce several **M2** from which they screened for resistance to papaya ringspot virus (PRSV), dwarfness, lower fruit bearing stature and higher total soluble solids. No mutant was found to be resistant to PRSV (Chan et al. 2007). In Vietnam, Hang and Chau (2010) used gamma irradiation ranging from 10–60 Gy to improve precocity and reduction of plant height on a local papaya variety Dai Loan Tim. Four mutants were reported to be superior in plant height at first flower, plant vigor, fruit yield and quality compared to non-mutants. Irradiation has however been shown to result in poor germination and negatively affected the number of fruits and fruit quality compared to the control (Kumar et al. 2017). There is need for further research in this subject to develop resistance to devastating viral, bacterial and other diseases.

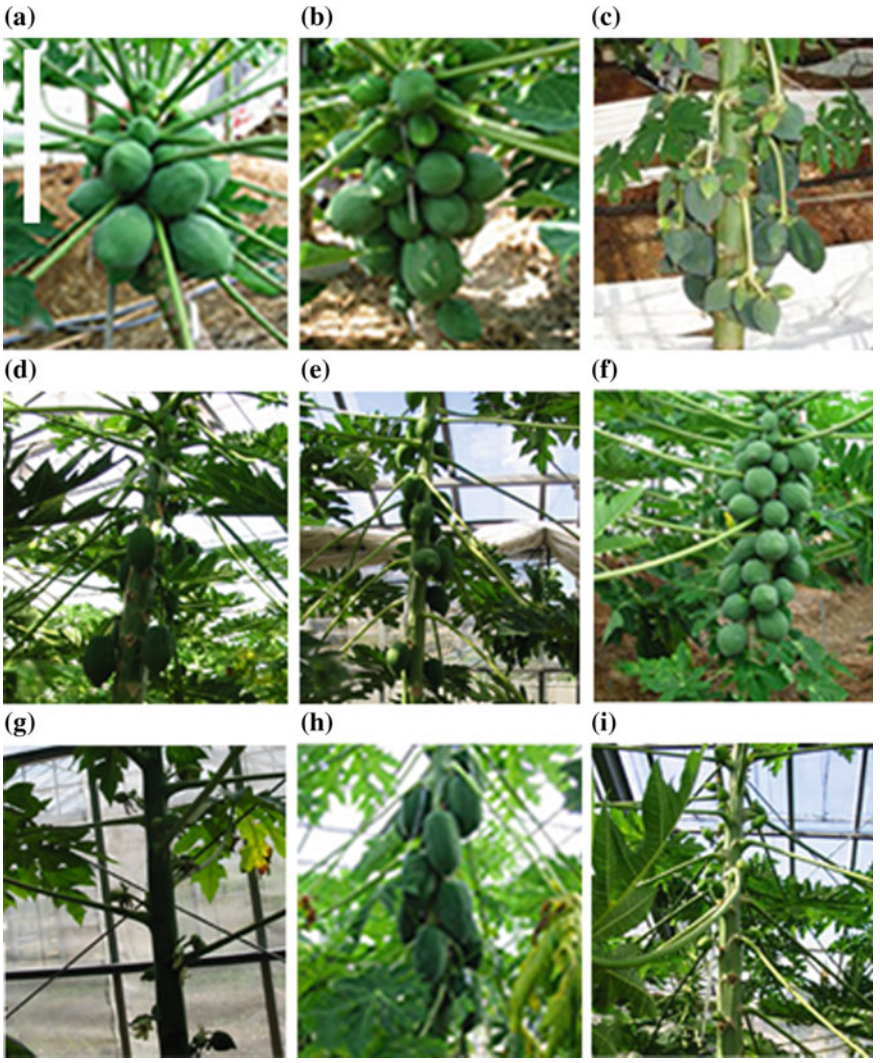

Fig. 21.5 Variation in fruiting habits among triploid female plants derived from anther culture. The strains shown are **a** EMB-1, **b** EMB-2, **c** EMB-7, **d** EMB-10, **e** EMB-15, **f** EMB-17, **g** EMB-19, **h** EMB-26, **i** EMB-30. Vertical bar indicates 50 cm *Source* Rimberia et al. (2007), Rimberia and Adaniya (2010)

21.4 Molecular Marker-Assisted Selection and Breeding

Molecular marker assisted selection and breeding have been applied in papaya, particularly in the selection for disease resistance. Although papaya ringspot virus (PRSV) is a major bottleneck of papaya production worldwide, no papaya cultivar is resistant

to the virus. Resistance has only been reported in wild relatives in the genus *Vasconcellea* such as *V. cauliflora*, *V. stipulata*, *V. pubescens* and *V. quercifolia* (Manshardt and Drew 1998). O'Brien and Drew (2009) showed that resistance to PRSV-p in *V. pubescens* was controlled by a single dominant gene (prsv-1) through successful backcrossing of *V. pubescens* to *V. parviflora* from F3 interspecific hybrids. The markers linked to prsv-1 have been used in breeding programs because of their dominant inheritance.

Carica papaya is also susceptible to other pathogens such as *Phytophthora palmivora*. Tolerance to *P. palmivora* has been identified and molecular markers linked to the tolerance developed using AFLPs (Noorda-Nguyen et al. 2010). This was identified from the F2 population achieved by crossing tolerant Hawaiian cv. Kamiya with highly susceptible cv. SunUp. Markers have also been used to select for other traits. For instance, Blas et al. (2010) found a major gene associated with yellow flesh of papaya which is dominant over the red flesh color in simple Mendelian fashion.

21.5 Genetic Engineering in Trait Improvement

21.5.1 Breeding for Resistance to Pests and Diseases

Papaya is mostly grown in the tropics and subtropics. The fruit like any other is affected by a couple of pathogens such as fungi, viruses and bacteria as well as insect pests such as aphids, leafhoppers, mites and nematodes (Nishijima 2002).

21.5.2 Breeding for Resistance to Carmine Spider Mite

Mites are some of the problematic pests in papaya due to various characteristics they possess such as being minute and their high fecundity. These mites colonize the papaya plant parts, and as a result of their feeding activity, they cause premature defoliation, reduced fruit yields and fruit-skin blemishes leading to reduced market value (Nishina et al. 2000).

Carmine spider mite (*Tetranychus cinnabarinus*) is of large economic importance due to its broad host range when compared with other *Tetranychidae* species because the mites are polyphagous and may be found on weeds and cultivated crops such as fruits, vegetables and ornamentals (Biswas et al. 2004). Genetically-transformed plants have shown to offer a long-term and sustainable control of pests such as the carmine spider mite and many other insect pests through targeting chitin metabolism (McCafferty et al. 2006). Chitin is an insoluble structural polysaccharide and forms part of the cell wall of fungi and cuticle of mites and nematodes (Kramer and Muthukrishnan 1997). The introduction of the insect chitinase gene into plants has shown

to improve resistance to insect attack. Tobacco budworm (*Heliothus virescens*) has been controlled using chitinase enzyme extracted from *Manduca sexta*; this caused a stunted growth of the larva (Ding et al. 1998).

A transgenic PRSV-resistant papaya cv. Rainbow which was bred from two parents SunUP (female) and Kapoho (male), both of which were susceptible to mites and leafhoppers, was enhanced to become tolerant to carmine spider mite. The cultivar was also transformed using chitinase from *Manduca sexta*. The chitinase activity was found to be high, up to 52%, in the transgenic leaf extracts when compared to the control. When tested under field conditions, there was a significant control of mites in the transformed lines when compared to the control (Kapoho). The chitinase activity was high in two lines in which high mite mortality was noted (McCafferty et al. 2006).

21.5.3 Breeding for Resistance to Phytophthora

Papaya is highly susceptible to root rot caused by *Phytophthora palmivora,* particularly in poorly-drained soils and during the rainy season. Papaya resistance to Phytophthora has been improved through the introduction of a defensin gene from *Dahlia merckii* through particle bombardment in embryogenic calli of papaya (McCafferty et al. 2006). Defensins are a family of peptides that occur in various plant species, *D. merckii* being one of them (Broekaert et al. 1995). These plant peptides have been shown to induce a response to fungal infection (Cociancich et al. 1993). Resistance to fungal attack has also been observed when the defensin gene was introduced to crops such as in the case of tobacco in a move to control *Alternaria longipes* (Terras et al. 1995).

To improve the resistance of papaya to *Phytophthora palmivora* (Zhu et al. 2007) used a gene extract containing the defensins gene from *Dahlia* which was driven from a CaMV 35Spromoter and nptII gene. This was under the nopaline synthase (NOS) promoter as a selectable marker. The papaya which had been transformed using this gene were found to contain total soluble protein of 0.07–0.14% and 0.05–0.08% in callus and leaves, respectively. The presence of this gene in genetically transformed papaya was able to inhibit mycelial growth of *P. palmivora* by up to 35–50% when leaf extract was used. Further tests in the greenhouse showed that papaya with the defensins gene had an improved resistance towards *P. palmivora* and this was associated with a decline in growth of the hyphae of the fungus on infected sites, thus indicating the capacity of the defensins to control the pathogen (Zhu et al. 2007).

21.5.4 Breeding for Resistance to PRSV

There are many economically important diseases of papaya: the most significant among them is papaya ring spot disease (Purcifull 1972). Papaya ringspot virus

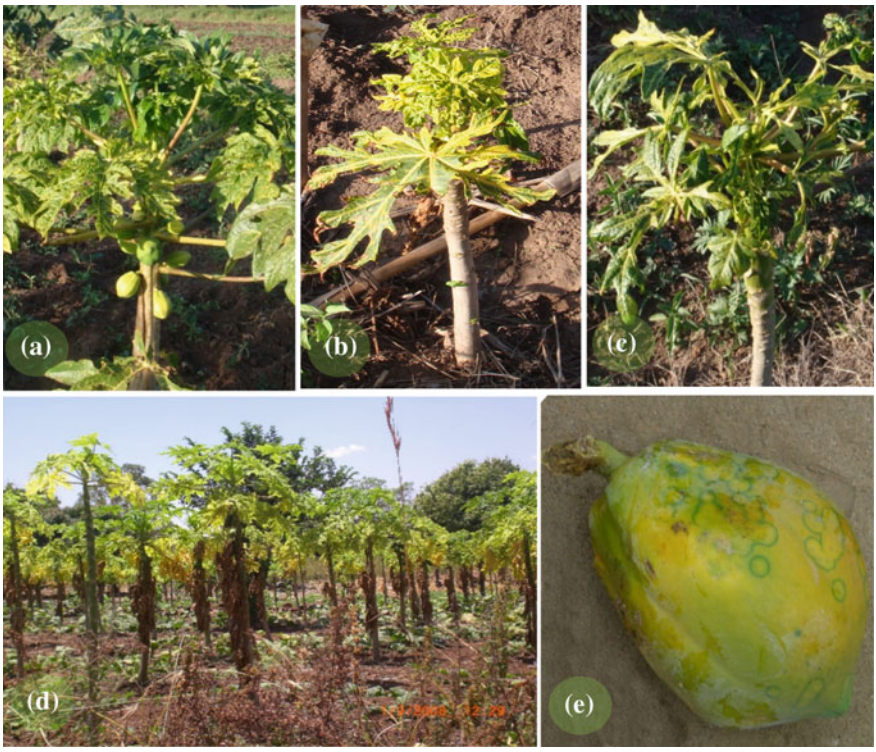

Fig. 21.6 Symptoms of PRSV infection in papaya. **a** Mosaic and leaf distortion, **b** Stunting and chlorosis as well as leaf distortion, **c** Stunting and leaf strapping, **d** A papaya orchard severely infected with PRSV, **e** Ringspot symptom on a papaya fruit

(PRSV) (genus *Potyvirus*, family Potyviridae) (Tripathi et al. 2008) infects papaya plants leading to symptoms including chlorotic leaves, deformation of leaves which resembles the damage of mites, ringed spots on the fruit (Fig. 21.6) and depressed fruit production which eventually leads to the death of the infected plants. In many parts of the world, PRSV is considered a serious threat to the papaya industry. The virus is transmitted by aphids in a non-persistent manner.

Management of PRSV through rouging of infected plants, quarantine regulations of restricting the plant movement, use of insecticides against insect vectors and cross protection, generally have not been effective in controlling the disease. Hitherto, no naturally-occurring resistance to PRSV has been identified in any papaya cultivar. Therefore, breeding for resistance to PRSV in papaya has only resulted in tolerant varieties (Conover 1976; Conover and Litz 1978). Large collections of papaya germplasm and cultivars representing the world's major production have been screened, but resistance has not been found. Because of the aforementioned, moderate levels of horizontal resistance were identified in papaya germplasm and used in papaya breeding programs with minimal success (Conover and Litz et al. 1978).

Breeding tolerant varieties with susceptible high yielding commercial varieties has improved yield under infectious conditions (Chan 2004).

It is common in plant breeding programs to obtain genes for plant disease resistance from wild relatives for introgression into acceptable high-yielding cultivars. Breeding of resistant varieties (by interspecific hybridization of *Carica papaya* with *Vasconcellea* sp.) therefore serves as a way of controlling PRSV. Numerous efforts have been made to incorporate the resistance genes from other genera in the Caricaceae namely, *Vasconcellea cauliflora*, *V. quercifolia*, *V. stipulate* and *V. pubescens*. Intergeneric hybrids between papaya and PRSV resistant species have been produced by a number of investigators with the aid of embryo rescue techniques (Horovitz and Jimenez 1967; Khuspe et al. 1980). However not much progress has been made.

In the mid-1980s, with the success in the development of genetically-modified crops such as cotton, maize and soybean, and the complete molecular characterization of PRSV (Yeh et al. 1992), scientists from Cornell University and the University of Hawaii initiated the development of PRSV-resistant papaya by genetic engineering. Transgenic papaya resistant to PRSV was developed and commercialized at Hawaii in 1998 (Gonsalves and Ferreira 2003). This was actualized through somatic embryogenesis and microprojectile transformation as one of the preferred methods of transformation; although the biolistic method of transformation also worked with high efficiency (Cai et al. 1999; Fitch et al. 1992).

Like numerous other dicotyledonous plant species, papaya can be transformed with *Agrobacterium tumefaciens* and regenerated into phenotypically normal appearing plants that express foreign genes. Yeh and Gonsalves (1994) developed a plant-expressible PRSV-cp gene construct from a Taiwanese PRSV strain. Transgenic plants expressing ß-glucuronidase (gus) were regenerated following co-cultivation of petiole explants with *A. tumefaciens*.

Pathogen-derived resistance is a phenomenon whereby transgenic plants containing genes or sequences of a pathogen are protected against adverse effects of the same or related pathogens. Coat protein mediated production is based on the phenomenon of cross-protection. Cross-protection is the term used for the phenomenon that a plant, when first inoculated with a mild strain of a given virus, becomes protected against the infection with a second, more severe strain of the same virus with which it has been infected (Fermin et al. 2010). Yeh et al. (2003) reported that the coat protein (cp) gene mediated transgenic resistance is the most promising approach for protecting papaya against the devastating effects of papaya ring spot viruses (PRSV). Viral cp gene imparting resistance against virus is known as coat protein mediated resistance (CPMR). Accumulation of the cp gene in transgenic crops has been proven to counter resistance to infection and/or disease development by the virus from which the cp gene was derived and by related viruses (Rosales et al. 2000).

21.5.5 Breeding for Aluminum and Herbicide Tolerance

Worldwide, up to about 40% of most agricultural lands are characterized by acidic soils. Aluminum is considered toxic to many plants. Organic acid excretion by plants is always associated with tolerance to aluminum. Therefore, overproduction of an organic acid in a plant either through genetic engineering or conventional breeding could address the problem of aluminum toxicity. De la Fuente et al. (1997) reported production of transgenic papayas by particle bombardment, with constructs driving the overexpression of the citrate synthase (cs) gene from *Pseudomonas aeruginosa*. There were lines expressing the cs gene that released 2–3 times more citrate than control plants. These transgenic lines were capable of forming roots and to grow in solutions containing up to 300 mM of aluminum, as compared to the control plants. The study indicated that excretion of the organic acid is a mechanism of aluminum tolerance in plants.

21.6 Conclusions and Prospects

Papaya is cultivated in all tropical and subtropical parts of the world where a majority of the people are poor and food insecure. It has therefore become a very important fruit crop with potential to alleviate human food and nutritional insecurity as well as provide some income. Compared to other fruit crops, papaya is one of the few fruit crops that bears fruits when less than 1 year old and continues to bear throughout the year under good management.

Papaya production is threatened by a myriad of problems including devastating pests and diseases, inability for farmers to differentiate among the three sex types, male, female and hermaphrodite at seedling stage, among others. Many attempts have been made by researchers over the years to resolve these problems through conventional and biotechnological techniques. Conventional plant breeding through introgression has given rise to many varieties with high yields and fruit quality.

Managing devastating viral diseases in papaya like PRSV requires efforts beyond conventional techniques as there is no known natural resistance available. Researchers in many parts of the world have managed to develop/introduce PRSV resistance to existing varieties through genetic transformation. However, genetically-modified fruits are still not accepted in most European countries and Japan. This notwithstanding, papaya plants tolerant to PRSV have been identified in Malaysia, USA (Florida) and recently Kenya. These tolerant plants hold important promise in resolving the devastating PRSV challenge although there is need for further development. On the other hand, tissue culture of papaya through shoot tip meristems has achieved a lot towards mass production of disease-free plantlets of known sex which will reduce the cost of production and enable growers to purchase the exact quantity of seedlings required. Somaclonal variation resulting from anther and embryo culture

derived calli hold huge potential for papaya improvement and needs to be explored further.

Dwarfness and parthenocarpy are two traits to be considered in the development of varieties for greenhouse production. Some varieties such as Kamiya Co 1 and Co 2 were released years ago. Where possible, the approach of developing varieties that combine dwarfness, parthenocarpy and PRSV tolerance needs to be adopted and encouraged to take advantage of greenhouse production as well as decreasing sizes of land available for production. Somaclonal variation probably holds considerable promise in research towards this direction.

A lot of effort has gone and continues to go into the improvement of papaya with each research group targeting different traits. It is widely agreed that the main challenge facing papaya productivity is PRSV which reduces orchard lifetime to about one year instead of three. Other priorities will include improvement of papaya for quality and yield. Whatever the location, the order of priorities may vary and therefore the priority of interventions may also change. Critical, however, is the choice of the most appropriate approach to solve the challenge.

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Appendix 1: Research Institutes and Online Resources Relevant to Papaya Genetic Improvement Research

Country	Institution	Specialization	Research activities	Contact information
Australia	Griffith University	Teaching and Research institute	Biotechnology of the papaya	Prof. Dr. Roderick A. Drew Griffith Sciences, Logan Campus, Griffith University, Meadowbrook, QLD 4131 Australia Telephone: (61)733821291 Fax: (61)737357618 E-mail: r.drew@griffith.edu.au
Brazil	Capixaba Institute for Research, Technical Assistance and Rural Extension (INCAPER)	Research, technical assistance and rural extension	Germplasm selection and improvement	Eng Agr, D. Sc., Luiz Augusto Lopes Serrano INCAPER/CRDR Nordeste, C. P. 62, 29900-970, Linhares-ES. E-mail: lalserrano@incaper.es.gov

Country	Institution	Specialization	Research activities	Contact information
India	Indian Agricultural Research Institute Regional Station, Pusa, Bihar, India	Research institute	Breeding papaya varieties of uniform, high yielding with better quality for wider adaptability	Dr. Tapas Ranjan Das Phone: 06274-240232 Fax: 06274-240236 E-mail: head_bihar@iari.res.in
Indonesia	Indonesian Tropical Fruit Research Institute (ITFRI)	Research institute	Breeding and biotechnology	Jl. Raya Solok, Aripan Km. 8, PO Box. 5, Solok 27301, West Sumatra Phone: 0755-20137 Fax: 0755-20592 Email: rif@padang.wasantara.net.id; balitbu@litbang.pertanian.go.id
Kenya	Jomo Kenyatta University of Agriculture and Technology	Teaching and Research institute	Varietal Development and Evaluation	Dr. Fredah K. Rimberia, P.O. Box 62000-00200 Nairobi Kenya Phone: +254726856304 Email: frenda@agr.jkuat.ac.ke or fredawanza@yahoo.com
Malaysia	Felda Agricultural Services Sdn Bhd	Commercial Agribusiness firm	Agricultural extension and Disease management in Papaya	Menara Felda, Platinum Park, Persiaran KLCC, Kuala Lumpur, Malaysia 50088 +60 3-2859 0366 Email: feldabiotech@felda.net.my
Nigeria	The National Horticultural Research institute	Research institutes	Papaya Varietal development and evaluation	Ms. Olubunmi Ibitoye National Horticultural Research Institute, P. M. B. 5432 Jericho Reservation Area, IDI-ISHIN, Oyo, Ibadan, Nigeria Telephone: (234)8023629104 E-mail bunmiajisafe@yahoo.com
Philippines	Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines, Los Baños	Research institute	National biotechnology research center and repository for all crops	Office of the director, 4031 College, Laguna, Philippines Telephone: (049) 536-5287; (049) 543-9571 Email: ipb.uplb@up.edu.ph
South Africa	Neofresh (Pty) Ltd.	Commercial fruit growers and exporters	Selection, hybridization and production of high quality papaya fruit for supermarkets and export outlets	Dr Aart Louw (Chief Researcher and Plant Breeder) PO Box 201 Sonpark 1206 Mpumalanga RSA T +27 13 590 0947 adminmanager@neofresh.net www.neofresh.net

Country	Institution	Specialization	Research activities	Contact information
Thailand	East West Seed Company	Commercial Seed company	Breeding	Lamai Yapanan Business Development Manager, 7 Moo 8, Chiang Mai Praw Road, 50290, Chiang mai, Thailand Email: lamai.yapanan@eastwestseed.com
United States of America-Hawaii	Hawaii Agricultural Research Centre (HARC)	Research Centre	Tissue culture and transformation of papaya	94-340 Kunia Rd, Waipahu, HI 96797, USA Phone:+1 808-677-5541
United States of America-Hawaii	University of Hawaii		Papaya breeding	Dr. Dennis Gonsalves 789 Hoolaulea Street, Hilo Hawaii 96720 USA E-mail dennisgonsal@gmail.com

Appendix 2: Genetic Resources

Country	Cultivar	Sex type	Flesh color
Australia	Improved Petersen	Dioecious	Yellow
	Guinea Gold	Hermaphrodite	Yellow
	Sunnybank/S7	Dioecious	Yellow
	Richter/Arline	Dioecious	Yellow
America – Mexico	Verde	–	–
	Gialla	–	–
	Cera	–	–
	Chincona	–	–
USA – Florida	Cariflora	Dioecious	Yellow
	Betty	Dioecious	Yellow
	Homestead	Dioecious	Yellow
USA – Hawaii	Kapoho Solo	Hermaphrodite	Yellow
	Sunrise	Hermaphrodite	Red
	Waimanalo	Hermaphrodite	Yellow
	Rainbow	Hermaphrodite	Yellow
Venezuela	Paraguanera	–	–
	Roja	–	Red
Caribbean – Barbados	Wakefield	–	–
	Graeme 5, and 7	–	–
Cuba	Maradol	Hermaphrodite	Red
Trinidad	Santa Cruz Giant	–	–
	Cedro	–	–

Country	Cultivar	Sex type	Flesh color
Dominican Republic	Cartagena	Hermaphrodite	Yellow
Asia – India	Coorg Honey Dew	Hermaphrodite	Yellow
	Coimbitor 2	Dioecious	Yellow
Indonesia	Semangka	Hermaphrodite	Red
	Dampit	Hermaphrodite	Red
Malaysia	Eksotika	Hermaphrodite	Red
	Sekaki	Hermaphrodite	Red
Philippines	Cavite/Sinta	Hermaphrodite	Red
Taiwan	Tainung No. 5	Hermaphrodite	Red
Thailand	Sai-nampueng	Hermaphrodite	Red
	Khaek Dam	Hermaphrodite	Red
South Africa	Hortus Gold	Dioecious	Yellow
	Kaapmuiden	–	Yellow
	Honey Gold	Dioecious	Yellow

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Chapter 22
Passion Fruit (*Passiflora* spp.) Breeding

Carlos Bernard Moreno Cerqueira-Silva, Fábio Gelape Faleiro, Onildo Nunes de Jesus, Elisa Susilene Lisboa dos Santos and Anete Pereira de Souza

Abstract The genus *Passiflora*, commonly known as passion fruit, is prominent in the family Passifloraceae due to its numerous species (approximately 520) and economic importance. The biodiversity of this genus is widely represented in the Americas, where Colombia and Brazil harbor approximately 170 and 150 species of *Passiflora*, respectively. The economic interest in passion fruit species emerged due to the beauty of their flowers, their active medicinal properties, their essential oils that can be extracted for the cosmetics industry and their production of fruit for consumption or for obtaining derivatives. Brazil is considered the largest producer of passion fruit, although its national productivity is low (an average of 14 mt/ha/year) compared with the potential for passion fruit cultivation (50 mt/ha/year). This low productivity is partly caused by a lack of cultivars adapted to different production regions and their susceptibility to major diseases. Although the number of passion fruit breeding programs has increased, the results obtained thus far have been modest compared with existing demands. Such programs therefore represent a burgeoning field of research and financial investment. Among the obstacles faced by breeders,

C. B. M. Cerqueira-Silva · E. S. L. dos Santos
Laboratório de Genética Molecular Aplicada, Departamento de Ciências Exatas e Naturais, Universidade Estadual do Sudoeste da Bahia (UESB), Itapetinga, Bahia, Brazil
e-mail: csilva@uesb.edu.br; cerqueirasilva@pq.cnpq.br

E. S. L. dos Santos
e-mail: elisa.lisboa@uesb.edu.br

F. G. Faleiro
Centro de Pesquisa Agropecuária do Cerrado, Empresa Brasileira de Pesquisa Agropecuária (Embrapa Cerrados), Brasília, Distrito Federal, Brazil
e-mail: fabio.faleiro@embrapa.br

O. N. de Jesus
Centro Nacional de Pesquisa em Mandioca e Fruticultura, Empresa Brasileira de Pesquisa Agropecuária (Embrapa Mandioca e Fruticultura), Cruz das Almas, Bahia, Brazil
e-mail: onildo.nunes@embrapa.br

A. P. de Souza (✉)
Departamento de Biologia Vegetal, Centro de Biologia Molecular e Engenharia Genética, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil
e-mail: anete@unicamp.br

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the low representation of *Passiflora* in germplasm banks (considering its species richness and wide geographical distribution) and the scarcity of biological and agronomic information for most accessions are the most salient. Despite the difficulties encountered in *Passiflora* research over the past two decades, there has been a notable increase in the use of molecular tools for the characterization of this genus and in the number of cultivars registered and effectively available for the large-scale production of passion fruit. Thus, in this chapter, we present an overview of innovations and modern technologies, including advances in breeding programs and molecular tools, related to the availability of genetic resources for *Passiflora*. These technologies can be used as strategies to improve every stage of breeding programs, from pre- to post-breeding. Finally, we discuss future perspectives for studies leading to the genetic breeding of passion fruit (*Passiflora* spp.).

Keywords Biotechnology · Molecular markers · Molecular tools · Passion flower · Passifloraceae · Plant breeding · Plant improvement

22.1 Introduction

22.1.1 Distribution and Biodiversity

The family Passifloraceae Juss. ex DC includes diverse species, numbering approximately 520–700, with highly variable leaves and flowers (Bernacci 2003; Feuillet 2004; Feuillet and MacDougal 2004), making this an exotic, complex group. These species are mainly categorized as lianas or climbing plants with tendrils, although some are trees or shrubs without tendrils (Cervi 1997). The family Passifloraceae exhibits great ecological importance not only for its numerous species but also for their ecological and evolutionary relationships with their pollinators, such as bees, wasps, hummingbirds and bats (Sazima et al. 1999; Semir and Brown Jr. 1975; Varassin et al. 2001) as well as their predators, such as butterflies (*Heliconidae*) (Benson et al. 1978; Plotze et al. 2005). These ecological relationships highlight the complex nature of interactions between plants and animals.

While many taxonomical uncertainties exist within the family Passifloraceae, the genus *Passiflora* is noted for its diversity, including approximately 520 species (MacDougal and Feuillet 2004), which is a consequence of the greater diversity (approximately 75%) that exists within the family (Bernacci et al. 2015; Cerqueira-Silva et al. 2014a; Feuillet 2004; Feuillet and MacDougal 2004). *Passiflora* species are commonly known as passion fruits and/or passion flowers (or "*flor da paixão*" in Portuguese). These denominations originate in the possible correlation between the morphology of the flower and the symbols of the passion of Christ (Faleiro et al 2005; Souza and Meletti, 1997). In South America, and especially in Brazil, the species of Passiflora are also known as "*maracujá*", indigenous name of Tupi origin that means food in the form of gourd (Faleiro et al. 2005). These species

are allogamous and exhibit self-incompatibility, although some species may be self-compatible (Bruckner et al. 2005; Varassin and Silva 1999).

The Passifloraceae family is widely distributed in the tropics and warm temperate regions (mainly in America and Africa). Specifically, the vast majority of *Passiflora* species are estimated to be distributed in tropical and subtropical regions, from the United States to Chile and Argentina, with the greatest occurrence and diversity being observed in South America (Cerqueira-Silva et al. 2016; Lorenzi et al. 2006; Martin and Nakasone 1970). There are also records of *Passiflora* spp. in India, China, Southeast Asia, Australia and the Pacific islands and neighboring regions, although they represent less than 5% of passion fruit species (Cerqueira-Silva et al. 2014a, 2016).

The importance of the genus *Passiflora* is not restricted to the ecological aspects inherent to the diversity and dispersion of its species, as many *Passiflora* species are recognized as natural resources due to their horticultural value. The beauty and diversity of the foliage and flowers of passion fruit, including over 400 ornamental hybrids, elicit significant economic interest (Abreu et al. 2009; Peixoto 2005). The economic interest in passion fruit species is also due to their medicinal and pharmacological traits (Costa and Tupinamba 2005; Dhawan et al. 2004), which represent an active field of research. Valuable oils and extracts obtained from passion fruit are utilized in the cosmetics industry for manufacturing creams, soaps and shampoos (Faleiro et al. 2011; Zeraik et al. 2010).

In addition to ornamental appeal, the economic value of the genus *Passiflora* can be attributed to production and commercialization of the fruits. At least 70 species of edible *Passiflora* fruits are recognized (Coppens d'Eeckenbrugge et al. 2001; Souza and Meletti 1997); however, the commercial production of passion fruit is based on the cultivation of only a few species, such as *Passiflora edulis* Sims, *P. alata* Curtis, *P. setacea* DC, *P. ligularis* A. Juss, *P. nitida* Kunth, *P. cincinnata* Mast, *P. tripartita* (Juss.) Poir, *P. maliformis* L. and *P. quadrangularis* L. (Fig. 22.1). Among these species, only *P. edulis* and *P. alata* are effectively grown on a commercial scale in Brazil, with the cultivation of *P. edulis* accounting for at least 90% of the area designated for Brazilian *Passiflora* cultivation, including the production of both yellow and purple fruits (Faleiro et al. 2011; Meletti et al. 2005). The other species are major components of the production chain of passion fruit in Colombia, which presents a greater diversification of *Passiflora* cultivation, despite its production being lower than that of Brazil.

Although incipient, in the context of national and international production, the cultivation and consumption of fruits and the derivatives of wild species such as *Passiflora cincinnata*, *P. nitida* and *P. setacea* have been described in previous studies, although this work is still in the early stages (Meletti et al. 2005; Souza and Meletti 1997). The biodiversity inherent to wild passion fruit trees and their resistance to biotic and abiotic stress factors are important components of passion fruit breeding programs (Faleiro et al. 2011; Junqueira et al. 2005).

Passion fruit biodiversity is potentially useful for the maintenance and expansion of passion fruit breeding programs, but additional studies are required to facilitate the exploitation of this characteristic as a natural resource. The lack of biological,

Fig. 22.1 Examples of the main species of passion fruit cultivated commercially: **a** *P. edulis* Sims, **b** *P. alata* Curtis, **c** *P. setacea* DC, **d** *P. ligularis* A. Juss., **e** *P. nitida* Kunth, **f** *P. cincinnata* Mast., **g** *P. tripartita* (Juss.) Poir., **h** *P. maliformis* L., **i** *P. edulis* Sims f. *edulis*, **j** *P. quadrangularis* L., **k** Ornamental hybrid *P. setacea* × P. coccinea cv. BRS Estrela do Cerrado, **l** Ornamental hybrid *P. edulis* × P. incarnata cv. BRS Céu do Cerrado-BRS CC.*Photos* Fábio Faleiro, Ana Maria Costa, Embrapa Cerrados

agronomic and molecular characterization for most wild species of *Passiflora* means that many species are omitted from germplasm banks and collections. An important advance for the morphological characterization of *Passiflora* was the elaboration of a list of descriptors with illustrations by Embrapa and partner institutions. This list aims to facilitate and standardize the morphological, agronomic characterization and identification of sources of resistance to diseases in germplasm banks (Jesus

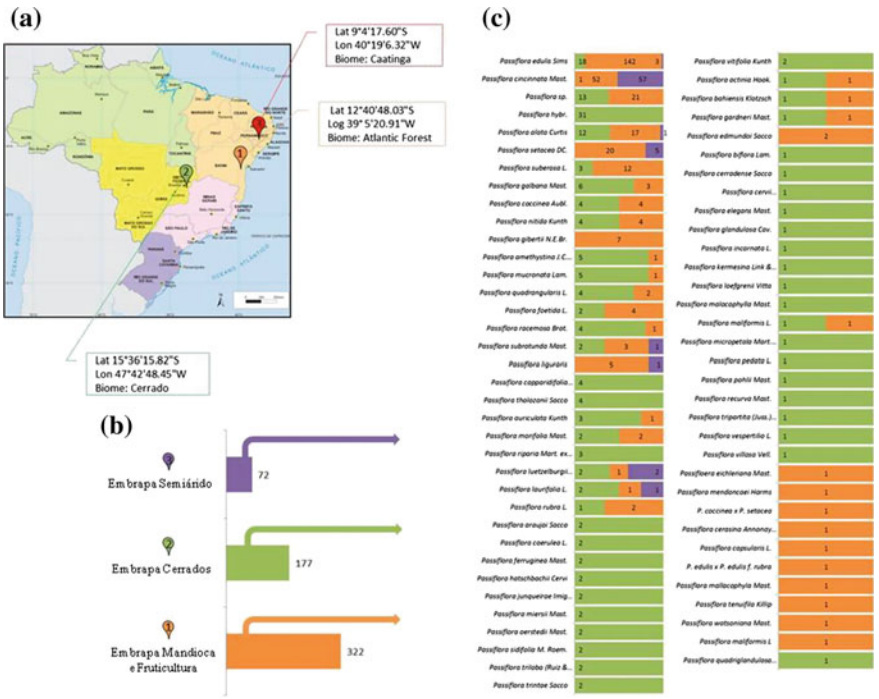

Fig. 22.2 Location of Embrapa Units with accessions preserved ex situ in gemplasm banks. **a** Number of passion fruit accessions preserved in germplasm banks in Embrapa Units. **b** Species and distribution of the number of accessions per Embrapa Unit. **c** The colors in **b** are related to those in **c**. *Source of map* IBGE maps

et al. 2017) Based on compilations performed by Ferreira (2005), it is estimated that only 15% of *Passiflora* species are represented at least once in one of the existing germplasm banks worldwide (Cerqueira-Silva et al. 2014a, 2016).

Brazilian passion fruit species are adapted to distinct biomes: semiarid, cerrado and Atlantic forest. Therefore, maintenance of the species in germplasm banks within these biomes is of value for broad-based genetic conservation. Embrapa has individual *Passiflora* germplasm banks (PGB) corresponding to the three biomes (Fig. 22.2a). Each bank has its own particularities as to type, genetic breeding use and frequency with which a certain species is represented. Embrapa has a total of 571 *Passiflora* accessions; 322 in Embrapa Mandioca e Fruticultura; 177 in Embrapa Cerrados; and 72 in Embrapa Seminárido (Fig. 22.2a, b). Most of the total accessions (163) are from the *P. edulis* Sims species (yellow and purple passion fruits) and another 110 from the *P. cincinnata*, also known as passion fruit from the savannas or brushlands (Fig. 22.2c). In regard to the total number of different taxa in the PGB, Embrapa Cerrados has 54, followed by Embrapa Mandioca e Fruticultura with 37 and Embrapa Semiarido with 9 (Fig. 22.2c).

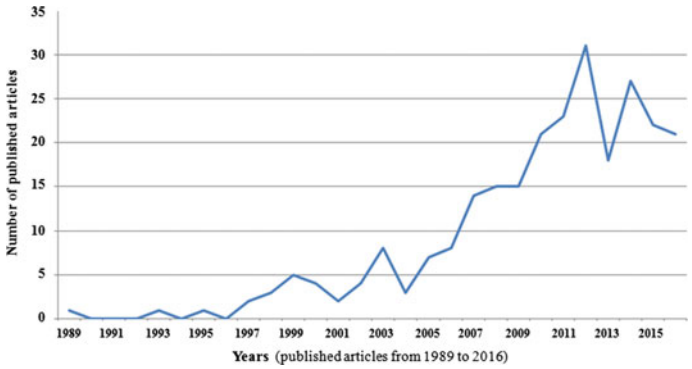

Fig. 22.3 Trend graph of the number of published articles reporting results related to the genetic characterization of passion fruit (*Passiflora* spp.) species. The values were calculated based on the SCOPUS database (www.scopus.com), adopting the search terms *Passiflora* and *genetic* and/or *molecular marker* as well as the corresponding Portuguese terms

Although there is a scarcity of available information related to the accessions maintained in collections, there has been an enormous growth in the number of studies dedicated to the genetic characterization of passion fruit species in past decades (Fig. 22.3). Moreover, there has been both quantitative and organizational growth of research groups and national and international partnerships established to enhance our knowledge and utilization of this natural resource. The First World Congress on Passion Fruit, held in March 2017 in Colombia (www.cepass.org), was a result of diligent research and genetic improvement programs for passion fruit.

The successful examples of research activities associated with the genetic improvement of passion fruit include the project Characterization and use of germplasm and genetic improvement of passion fruit (*Passiflora* spp.) with the aid of molecular markers (coordinated by Embrapa) as well as actions aimed at strengthening research networks (such as the Passion fruit: Germplasm and Improvement and Molecular Genetics of Passion Fruit networks), which receive support from researchers at different public institutions [Universidade Estadual do Sudoeste da Bahia (UESB), Universidade Estadual de Santa Cruz (UESC), Universidade Estadual de Campinas (UNICAMP), Empresa Brasileira de Pesquisa Agropecuaria (Embrapa), Universidade Estadual da Bahia (UNEB), Universidade de Brasilia (UNB) and others], and financial support from development agencies such as the Fundação de Amparo a Pesquisa do Estado da Bahia (FAPESB) and the Conselho Nacional de Pesquisa Científica (CNPQ).

In general terms, the actions proposed in these projects strengthen and contribute to the scientific and technological advancement of the passion fruit crop as well as accelerate the production of new cultivars for genetic resistance to diseases. The activities being developed are expected to obtain and use microsatellite markers to ensure the estimation of the genetic diversity contained in the main germplasm banks of passion fruit, the proposition of a nuclear collection and the identification

and protection of cultivars. New generation technologies are also being used for the construction and analysis of transcriptomes from contrasting passion fruit in reaction to passion fruit woodiness virus, allowing the identification of single nucleotide polymorphisms (SNPs) and differentially-expressed genes. In addition, the reaction of the passion fruit plants kept in the main germplasm banks is being evaluated for the virus (CABMV).

22.1.2 Objectives and Challenges of Breeding Programs

Considering the richness of the species available in the *Passiflora* genus and their potential genetic variability, together with the factors limiting their expansion and the maintenance of their cultivation, it is possible to highlight the major challenges faced by the breeding programs in the development of varieties and hybrids of passion fruit. These challenges include (i) the production of fresh fruits for market or industrial purposes (with the physical-chemical quality of fruits and multiple resistance to diseases) and ensuring suitability for (ii) ornamental purposes, (iii) use in medicinal applications and (iv) the functional foods market.

In view of these challenges, there is a need for morpho-agronomic and molecular characterization of both economically/commercially important species and wild passion fruit species to support the advancement of breeding programs. Wild species may also contribute to activities that are complementary to breeding, such as the use of rootstocks (Lima et al. 2017). Therefore, activities recognized as pre-improvement actions (prospecting and characterization of germplasm), in addition to domestication and the development of new commercial passion fruit, are current critical demands of research associated with the use of this natural resource.

The number of accessions available for breeding programs is lower than the total number of accessions registered in active germplasm banks (AGBs), mainly due to the technical and financial difficulties in the maintenance and characterization of AGB accessions. The lack of information related to the accessions maintained in collections and AGBs is the main impediment for their use in programs focused on genetic variability in breeding (Duvick 1984; Nass 2011).

The biology, agronomic characteristics, and genetics of wild passion fruit species as well as domestication and breeding activities can contribute to increasing the number of passion fruit species marketed in Brazil and other countries, thus increasing fruit production. The potential for diversification in the production of passion fruit is illustrated by the discrepancy in the number of species cultivated on a commercial scale in Colombia in relation to the small number of species cultivated and effectively marketed in Brazil. Moreover, the prominence of countries such as Brazil in fruit production does not always stem from efficiency of orchard productivity but from the area of the land devoted to passion fruit cultivation. The productivity of the Brazilian crop can reach 40–50 mt/ha/year (Meletti et al. 2005; Melo et al. 2001; Neves et al. 2013) but has become much lower (approximately 14 mt/ha/year) in recent years.

Over 400 ornamental passion fruit hybrids are registered worldwide. However, regarding the effective commercialization of passion fruit species as ornamental plants in the Brazilian market, improved techniques for launching cultivars adapted to shaded environments, potting and large-scale flower production are lacking.

Theoretically, the limiting factors for the expansion and maintenance of passion fruit cultivation can be classified as biotic stress factors (pests and pathogens) and abiotic stress factors (low water availability, which is a characteristic of certain Brazilian regions). The losses resulting from pathogen attacks cannot be ameliorated by adopting better cultivation techniques for the use of pesticides; thus, special attention must be paid to these stress factors by researchers.

Among the common diseases that affect passion fruit, the following can be highlighted due to their widespread occurrence in the national territory or the difficulty of combat and control activities: anthracnose (*Glomerella cingulate*); anamorfo (*Colletotrichum gloeosporioides*); verrugose (*Cladosporium cladosporioides* and *C. herbarum*); fusariosis (*Fusarium oxysporum* f. sp. *passiflorae*); bacteriosis (*Xanthomonas axonopodis pv. passiflorae*); and passion fruit woodiness disease (*Cowpea aphid-borne mosaic virus*) (Fischer and Resende 2008; Junqueira et al. 2005; Meletti et al. 2005). Although some studies on these diseases have focused on their pathosystems, the great majority are limited to the identification and epidemiological aspects of pathogens and do not involve research programs allowing the continuous evaluation of plant diseases. Furthermore, Fischer and Resende (2008) noted that passion fruit production is generally associated with small producers, who sometimes have other growing priorities.

Finally, the scarcity of commercial cultivars exploiting the potential of wild passion fruit is the primary cause of the underutilization of this biodiversity. Faleiro et al. (2015) mainly attribute the lack of cultivars available to producers to the lack of consistency in the production chain of passion fruit, leading to high market prices (Faleiro et al. 2015). Thus, increasing the number and quality of available cultivars will allow producers to at least cope with crop restrictions imposed by pathogens and water scarcity, increasing consistency in the production chain and commercial stability for crop diversification.

22.2 State of the Art in Genetic Improvement

Passion fruit has been commercially cultivated in Brazil for over 40 years, and regional varieties, such as Maguary, Golden Star, Amafrutas and the composite IAC-27, have been available since the 1990s. However, the first cultivars of passion fruit are more recent, having been introduced approximately 15 years ago (Faleiro et al. 2011; Meletti 2011), and are associated with *Passiflora edulis* Sims. The first cultivar related to a wild species was introduced as recently as 2013, from *P. setacea*, and a second cultivar related to a wild species (*P. cincinnata*) was released in 2016.

Currently, 34 entries are cataloged in the national cultivar registration system, Ministério da Agricultura, Pecuária e Abastecimento—MAPA. These entries are associ-

ated with the term passion fruit (consulted in May 2017, http://extranet.agricultura.gov.br/php/snpc/cultivarweb/cultivares_registradas.php). Approximately half of these records are related to cultivars that are available to producers or have been evaluated by the scientific community. In Table 22.1, 19 cultivars are presented, for which dissemination and commercialization information is available. Data from scientific evaluations of the fruit production, ornamental use and disease reactions of these cultivars have also been recorded.

The advances that have been made in the genetic improvement of passion fruit are due to the establishment of research groups and networks dedicated to characterization of the genus *Passiflora*, in public institutions such as Embrapa, UESB, UESC, UNICAMP, Universidade de São Paulo (USP), and Universidade Estadual do Norte Fluminense (UENF) in Brazil. In these institutions, steps have been taken to support pre-breeding and breeding programs that effectively contribute to the release of cultivars and hybrids in the market.

Among the many possibilities for using biotechnology in genetic improvement programs for passion fruit, molecular markers are mainly employed to (i) characterize genetic diversity among accessions of wild species (Cerqueira-Silva et al. 2010a, b, 2012; Junqueira et al. 2007; Pérez-Almeida et al. 2010) and commercial varieties (Bellon et al. 2007, 2009; Cerqueira-Silva et al. 2010c; Fonseca-Trujillon et al. 2009; Ortiz et al. 2012) present in collections and germplasm banks; (ii) monitor the variability and recovery of the parent genome over recurrent selection cycles (Fonseca et al. 2009; Reis et al. 2011, 2012); (iii) map genes and genomic regions associated with characteristics of interest (Carneiro et al. 2002; Lopes et al. 2006; Oliveira et al. 2008; Penha et al. 2013); (iv) confirm hybrids (Conceição et al. 2011; Junqueira et al. 2008); and (v) monitor introduced/marketed varieties, as discussed by Ferreira and Rangel (2011) and in the next subsection of this chapter.

Practical examples of the use of molecular markers in breeding programs include reduction of the time and resources required to retrieve the genomes of parents of interest in backcrossing cycles (Faleiro et al. 2011). Thus, molecular markers have contributed to the selection of intra- and interspecific passion fruit hybrids with desirable characteristics (including disease resistance) and a decreased genetic distance from the recurrent parent for various passion fruit species (e.g. *Passiflora edulis*, *P. setacea*, *P. caerulea*, and *P. coccinea*) (Faleiro et al. 2007, 2011; Fonseca et al. 2009).

Estimates of diversity based on microsatellite markers, performed in conjunction with assessments of resistance to the major diseases that affect passion fruit crops, are also being used to assist to target breeding programs (Cerqueira-Silva et al. 2014b, c). For example, Cerqueira-Silva et al. (2014c) used 23 microsatellite loci and determined the scale based on specific symptoms for each of the diseases assessed (woodiness virus, scab and anthracnose), which aided in the establishment of a nuclear collection including the accessions of *Passiflora edulis* showing the highest levels of resistance to diseases and exhibiting greater representativeness of the microsatellite alleles identified in the germplasm. In addition to the identification of accessions with a higher level of resistance to each of the diseases, we also identified

Table 22.1 Main Brazilian cultivars of passion fruit (varieties and hybrids of *Passiflora* spp.) available for cultivation

Cultivar identification	Year of release	Website information	Responsible company
Monte Alegre (IAC-273)	1999	http://www.iac.sp.gov.br/publicacoes/agronomico/maracuja_amarelo.php	Instituto Agronômico de Campinas http://www.iac.sp.gov.br/
Maravilha (IAC-275)			
Joia (IAC-277)			
IAC Paulista	2005	http://www.iac.sp.gov.br/publicacoes/agronomico/pdf/v58_Maracuja_Roxo.pdf	
FB 200—Yellow Master	2008	http://www.viveiroflorabrasil.com.br/site/produtos-2/	Viveiro Flora Brasil http://www.viveiroflorabrasil.com.br
FB 300—Araguari			
CPATU Casca Fina	2002	http://dx.doi.org/10.1590/S0100-29452003000100052	Embrapa http://www.embrapa.br
BRS Sol do Cerrado (BRS SC1)	2008	http://www.cpac.embrapa.br/lancamentoazedo/	
BRS Gigante Amarelo (BRS GA1)			
BRS Ouro Vermelho (BRS OV1)			
BRS Rubi do Cerrado (BRS RC)	2012	http://www.cpac.embrapa.br/lancamentobrsrubidocerrado/	
BRS Perola do Cerrado (BRS PC)	2013	http://www.cpac.embrapa.br/lancamentoperola/	
BRS Sertão Forte (BRS SF)	2016	http://www.cpac.embrapa.br/lancamentosertaoforte/	
BRS Estrela do Cerrado	2007	http://www.cpac.embrapa.br/lancamentoornamental/	
BRS Rubiflora			
BRS Roseflora			

(continued)

Table 22.1 (continued)

Cultivar identification	Year of release	Website information	Responsible company
BRS Céu do Cerrado (BRS CC)	2017	http://www.cpac.embrapa.br/lancamentoornamental2016/	
BRS Rosea Púrpura (BRS RP)			
BRS Mel do Cerrado (BRS MC)	2017	http://www.cpac.embrapa.br/lancamentomeldocerrado/	
Sul Brasil	2010		AFRUVEC/Fundo *Passiflora*
SCS437 Catarina	2016	http://www.epagri.sc.gov.br/?p=13866	EPAGRI (http://www.epagri.sc.gov.br/)

accessions with a reasonable resistance profile for the three diseases considered in the study.

22.3 Genetic Diversity Based on Molecular Markers and Characteristics of Agronomic Traits

Estimates of genetic diversity are based on molecular data and agronomic characterization and are essential for the effective targeting and use of genetic resources. Such assessments are standard in pre-breeding stages, and when effectively conducted, they facilitate the use of available accessions in germplasm banks for breeding programs (Lopes et al. 2011). Among the advances in passion fruit cultivation achieved in recent decades, we highlight the increasing use of molecular markers to obtain basic knowledge of the genetic diversity of the genus (Fig. 22.4). Additionally, we note the effort of different research groups to associate molecular and agronomic data for more robust and useful characterization, to prioritize accessions in breeding programs.

In this context, we have (as practical examples and directly associated with the genetic improvement) the identification of potential nuclear collections for some species of passion fruit (*Passiflora edulis*, *P. cincinnata P. setacea*), which provide information that can aid in their genetic improvement (Cerqueira-Silva et al. 2014b, c). For these species, the authors report the possibility of representing 100% of the allelic richness identified with microsatellite markers through the maintenance growth of approximately 27% (for *P. edulis*), 75% (for *P. cincinnata*) and 47% (for *P.*

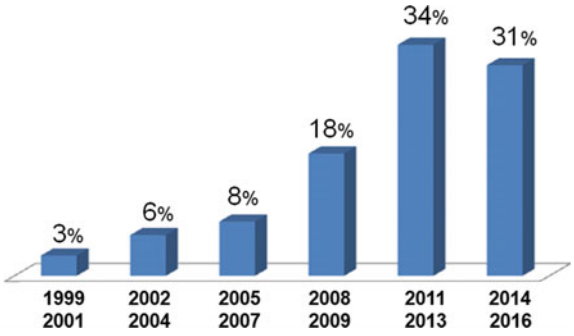

Fig. 22.4 Histogram of the percentages of reports associated with molecular genetic studies of *Passiflora* species published 1999–2016. The percentages were calculated based on the SCOPUS database (www.scopus.com), adopting the search terms *Passiflora* and *molecular marker* as well as the corresponding Portuguese terms

setacea) of the accessions maintained in the germplasm bank of the Embrapa cassava and fruit unit.

Although only a few studies have been conducted with the aim of proposing nuclear collections (or working collections), various authors have presented genetic estimates based on molecular markers and *Passiflora* spp. accessions maintained in germplasm banks (Bellon et al. 2009; Cerqueira et al. 2010a, b, c, 2012, 2014b, c; Ortiz et al. 2012; Santos et al. 2011). In summary, despite the specific objectives of each study, the estimates of genetic diversity contribute to the understanding of the variability available in the germplasm banks and the direction of convergent and divergent crosses that drive the initial stages of breeding.

22.4 Genetic Mapping

The first genetic maps were published by Carneiro et al. (2002) and were based on the characterization of a segregant population of *Passiflora edulis* through random amplification of polymorphic DNA (RAPD) markers. The population used to construct the first map helped identify the first quantitative resistance locus (QRL) linked to the genus *Passiflora*, based on amplified fragment length polymorphism (AFLP) markers (Lopes et al. 2006), which explained approximately 16% of the phenotypic variation related to the symptoms of bacterial spot disease caused by *Xanthomonas axonopodis pv. passiflorae*.

Oliveira et al. (2008) employed map integration strategies to construct a single map, which was a landmark in genetic mapping studies and the identification of QTLs in passion fruit. An important advantage of the integrated construction of a single representative map for a segregant population is an increase in map saturation and length. Recently, this research was extended to construct maps for passion fruit

plants of another species of this genus, i.e. *Passiflora alata* (Vieira et al. 2005). The use of variable AFLP and simple sequence repeat (SSR) analyses as well as the first characterization of SNPs in passion fruit plants were included in this map.

The first research dedicated to the construction of physical maps was based on putative genes identified from a passion fruit genomic library inserted into bacterial artificial chromosomes (BACs) (Penha 2012; Santos 2013). These studies are a new development in genetic and genomic research in passion fruit, allowing the characterization of genomes and the identification of evolutionary relationships among species and aiding in the development of new molecular markers, including SSR markers, expressed sequence tags (ESTs) and SNPs (Cerqueira-Silva et al. 2014a).

Advances in the genetic mapping of passion fruit associated with the reduction of next-generation sequencing (NGS) costs should enable genetic characterization based on genotyping-by-sequencing (GBS), at least for commercial species. Thus, alternative methodologies that decrease costs and enable GBS, such as the use of restriction enzymes to reduce genome complexity (Elshire et al. 2011), should be considered in future genetic studies of passion fruit. These methods can contribute to both population studies and genome-wide selection in collections and germplasm banks (Cerqueira-Silva et al. 2014a).

22.5 Genetic Engineering

Genetic engineering in passion fruit has been employed as a strategy for the development of resistance to diseases such as passion fruit woodiness (PWD) (caused by the cowpea aphid-borne mosaic virus in Brazil) and bacterial spot disease (caused by *Xanthomonas axonopodis pv. passiflorae*), against which chemical control measures are not effective, and resistant cultivars are not available (Alfenas et al. 2005; Freitas et al. 2007; Monteiro-Hara et al. 2011; Trevisan et al. 2006). These studies were initiated in the late 1990s and early 2000s, and their first results published (Alfenas et al. 2005; Trevisan et al. 2006); in these studies, *Passiflora edulis* plants were transformed with fragments of genes obtained from viral isolates known to cause PWD.

The first transgenic *Passiflora alata* plants were later generated by Pinto (2010), who demonstrated the possibility of obtaining transgenic *P. alata* plants using methods similar to those employed in studies conducted in *P. edulis* (Alfenas et al. 2005; Trevisan et al. 2006). More recently Correa et al. (2015) demonstrated that it is possible to select *P. alata* plants with gain of resistance to the CABMV from the incorporation of CABMV-gene fragments into the genome of *P. alata* lines.

Although the available studies dedicated to the production of transgenic passion fruits are not conclusive, they indicate that, at least for PWD (in species *Passiflora edulis* and *P. alata*), it is possible to obtain resistant plants for cultivation and/or for use in breeding programs dedicated to the production of resistant cultivars with higher productivity. However, it was not observed in the release of cultivars related to plants of transgenic passion fruits.

22.6 Biotechnology Tools in Pre- and Post-breeding Programs

The various stages of pre-breeding, especially the characterization of material from natural populations and germplasm banks, are essential for extending the genetic basis of breeding programs (Ferreira and Rangel 2011; Pereira et al. 2005). Moreover, these steps aid in the identification of genetic variability, which further facilitates the study of resistance to biotic and abiotic stress factors (Ferreira and Rangel 2011). Thus, biotechnology and molecular tools (especially molecular markers) effectively contribute to estimation of the available variability in germplasm banks, to identify duplicate accessions, monitor the percentage of the genome recovered in backcrossing cycles and confirm inter- or intra-specific hybrids (Cerqueira-Silva et al. 2014a, 2016; Ferreira and Rangel 2011).

The real success of genetic improvement is associated with the use of the developed products and their acceptance and consumption. Thus, post-breeding activities essentially highlight (i) the preparation of marketing plans; (ii) the definition of strategies and logistics for upscaling the production and commercialization of seeds; (iii) the registration and protection of cultivars by legal and competent bodies; (iv) the performance of market promotion and insertion actions; and (v) the strengthening of partnerships with the private sector, aiming at the validation and application of technologies. In this context, especially regarding items (ii), (iii) and (v), molecular markers have the potential to accelerate and provide security in the genetic identification and consequent protection of these products.

Thus, biotechnology and molecular tools can contribute to the effectiveness of genetic improvement programs and significantly reduce the amount of time necessary to complete the stages inherent to breeding programs, thereby bestowing confidence in the monitoring of the marketed technological product (Cerqueira-Silva et al. 2016; Faleiro et al. 2006; Ferreira and Rangel 2011; Pereira et al. 2005).

22.7 Conclusions and Prospects

Although population studies of *Passiflora* species are highly relevant to conservation and breeding activities, this research is in its nascent stage. Population studies, associated with increasing numbers of microsatellite markers and recent advances in the development and use of SNP markers, are important strategies for achieving sequencing and genotyping on a large scale. The development and application of large-scale genotyping should allow the use of strategies for genome-wide selection (or simply genomic selection), enhancing the association of genetic diversity data with characteristics of agronomic interest. Moreover, the use of GBS strategies reduces the costs and time required for germplasm characterization and the selection of passion fruit genotypes.

In tandem with the population estimates that must be performed for the genus *Passiflora* using molecular tools, strategies for the exploration and conservation of germplasm collections are also necessary to enable morpho-agronomic characterization and to improve the understanding of diversity. Thus, interactions involving the accessions present in germplasm banks and collections and the elite materials used in breeding programs will be enhanced.

Finally, the molecular characterization and evaluation of agronomic characteristics, particularly those related to disease resistance, will promote better utilization of *Passiflora* biodiversity in breeding programs. In this context, resistance characteristics must be introduced not only to species such as *P. edulis* and *P. alata* but also, more importantly, to cultivars related to new wild species that can potentiate the diversification of passion fruit cultivation should be made available.

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Appendix 1

Major institutes engaged in research on Passion fruit (*Passiflora* spp.)

Institution	Specialization Research Activities	Contact information and website
Centro Internacional de Agricultura Tropical, 6713, CIAT/Cali, Colombia	Breeding Conservation Genetics and Molecular Biology	jaocampop@unal.edu.co http://ciat.cgiar.org/
Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina, Florianópolis, 88034-901, Santa Catarina, Brazil	Breeding Conservation Genetics and Molecular Biology Production and commercialization	http://www.epagri.sc.gov.br/

Institution	Specialization Research Activities	Contact information and website
Empresa Brasileira de Pesquisa Agropecuária, Cruz das Almas, 44380000, Bahia, Brazil	Breeding Conservation Genetics and Molecular Biology Production and commercialization	onildo.nunes@embrapa.br https://www.embrapa.br/mandioca-e-fruticultura
Empresa Brasileira de Pesquisa Agropecuária, Brasília, 70770901, Distrito Federal, Brazil	Breeding Conservation Genetics and Molecular Biology Production and commercialization	fabio.faleiro@embrapa.br https://www.embrapa.br/cerrados
Instituto Agronômico de Campinas, Campinas, 13001970, São Paulo, Brazil	Breeding Conservation Genetics and Molecular Biology Production and commercialization	lmmm@iac.sp.gov.br http://www.iac.sp.gov.br/
Universidade Estadual de Santa Cruz, Ilhéus, 45662900, Bahia, Brazil	Breeding Genetics and Molecular Biology	ronanxc@uesc.br www.uesc.br
Universidade Estadual do Sudoeste da Bahia, Itapetinga, 45700000, Bahia, Brazil	Breeding Genetics and Molecular Biology	csilva@uesb.edu.br www.uesb.br
Universidade Estadual do Sudoeste da Bahia, Vitória da Conquista, 45100000, Bahia, Brazil	Breeding Genetics and Molecular Biology	abelsj@edu.uesb.br www.uesb.br
Universidade Estadual de Campinas, Campinas, 13083860, São Paulo, Brazil	Genetics and Molecular Biology	anete@unicamp.br www.unicamp.br
Universidade de São Paulo, Piracicaba, 13418900, São Paulo, Brazil.	Genetics and Molecular Biology	mlcvieir@usp.br www.esalq.usp.br
Universidade Federal de Viçosa, Viçosa, 36571000, Minas Gerais, Brazil	Breeding Genetics and Molecular Biology	bruckner@ufv.br www.ufv.br
Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, 28013602, Rio de Janeiro, Brazil	Breeding Genetics and Molecular Biology	pirapora@uenf.br www.uenf.br
Universidade Federal do Rio Grande do Sul, Porto Alegre, 91501970, Rio Grande do Sul, Brazil	Conservation Genetics and Molecular Biology	loreta.freitas@ufrgs.br www.ufrgs.br

Institution	Specialization Research Activities	Contact information and website
Universidad Jorge Tadeo Lozano, 22-61, Bogotá, Colombia	Breeding Genetics and Molecular Biology	javier.hernandez@utadeo.edu.co http://www.utadeo.edu.co/es
Viveiro Flora Brasil, Araguari, Minas Gerais, Brazil	Production and commercialization	florabrasil@viveiroflorabrasil.com.br www.viveiroflorabrasil.com.br

Appendix 2

Genetic resources of passion fruit

Cultivar	Important traits	Cultivation location
Monte Alegre (IAC-273)	Quality of fruit and productivity Average weight of the fruit between 180 and 220 g Average productivity of 45–50 mt/ha/year	Southeastern Brazil
Maravilha (IAC-275)		
Joia (IAC-277)		
IAC Paulista	Quality of fruit and productivity Average weight of the fruit between 100 and 160 g Average productivity of 25 mt/ha/year	Southeastern Brazil
FB 200—Yellow Master	Quality of fruit and productivity Average weight of the fruit 240 g Average productivity of 50 mt/ha/year	Brazil Does not suit regions of frost
FB 300—Araguari	Quality of fruit and productivity Average weight of the fruit 120 g Average productivity of 50 mt/ha/year	Brazil Does not suit regions of frost

Cultivar	Important traits	Cultivation location
CPATU Casca Fina	Quality of fruit and productivity Average weight of the fruit between 118 and 227 g	North Brazil
BRS Sol do Cerrado (BRS SC1)	Quality of fruit and productivity Tolerant to foliar diseases Average weight of the fruit between 150 and 340 g Average productivity of 40 mt/ha/year	Brazil Does not suit regions of frost
BRS Gigante Amarelo (BRS GA1)	Quality of fruit and productivity Tolerance to anthracnose and to soil pathogens Average weight of the fruit between 120 and 350 g Average productivity of 42 mt/ha/year	Brazil Does not suit regions of frost
BRS Ouro Vermelho (BRS OV1)	Quality of fruit and productivity Tolerant to foliar diseases Average weight of the fruit between 120 and 350 g Average productivity of 40 mt/ha/year	Brazil Does not suit regions of frost
BRS Rubi do Cerrado (BRS RC)	Quality of fruit and productivity Tolerant to diseases Average weight of the fruit between 120 and 300 g Average productivity of 50 mt/ha/year	Brazil, especially in the State of Mato Grosso and in the Federal District.
BRS Perola do Cerrado (BRS PC)	Quality of fruit and productivity Resistance to diseases Average weight of the fruit between 50 and 120 g Average productivity of 10–25 mt/ha/year	Brazil Does not suit regions of frost
BRS Sertão Forte (BRS SF)	Quality of fruit and productivity Resistance to *Fusarium* sp. Average weight of the fruit between 109 and 212 g Average productivity of 30 mt/ha/year	Brazil Does not suit regions of frost

Cultivar	Important traits	Cultivation location
BRS Mel do Cerrado (BRS MC)	Quality of fruit and productivity Ornamental, for landscaping of large areas Average productivity of 15 mt/ha/year	Brazil Does not suit regions of frost
BRS Estrela do Cerrado	Ornamental Production of large quantities of flowers and few fruits	Brazil
BRS Rubiflora	Ornamental Production of large quantity of flowers Potential also as rootstock	Brazil
BRS Roseflora	Ornamental Production of large quantities of flowers and few fruits	Brazil
BRS Céu do Cerrado (BRS CC)	Ornamental Production of large quantities of flowers	Brazil
BRS Rosea Púrpura (BRS RP)	Ornamental Production of large quantities of flowers	Brazil

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Chapter 23
Genetics and Breeding of Fruit Crops in the Sapindaceae Family: Lychee (*Litchi chinensis* Sonn.) and Longan (*Dimocarpus longan* Lour.)

Jorge Lora, Van The Pham and José I. Hormaza

Abstract The Sapindaceae or soapberry family, which contains about 2000 species in more than 125 genera, includes several important subtropical and tropical crops, among them, litchi or lychee (*Litchi chinensis* Sonn.) and longan (*Dimocarpus longan* Lour.) are the most popular. Litchi and longan exhibit three different flower types and the fruits have a whitish pulp, sweet flavor and a very aromatic aril. Both crops have been traditionally produced and marketed in China, which is the largest litchi and longan producer and consumer country. Litchi and longan have similar environmental requirements but due to their limited environmental adaptation, cultivation is restricted to countries with subtropical climates. The Asia-Pacific region accounts for more than 95% of the world production. Over the last several decades, litchi and longan cultivation has undergone a considerable expansion worldwide. However, only a few cultivars are used for production, usually with low yield. There are hundreds of cultivars of litchi and longan collected and preserved in China; important germplasm banks have also been established in other countries. In this review we discuss several aspects of genetics and breeding of litchi and longan.

Keywords Genetics · Litchi · Longan · Plant breeding
Sapindaceae · Subtropical crops

J. Lora · J. I. Hormaza (✉)
Department of Subtropical Fruit Crops, Instituto de Hortofruticultura Subtropical y Mediterránea La Mayora, (IHSM La Mayora-CSIC-UMA), 29750 Algarrobo-Costa, Málaga, Spain
e-mail: ihormaza@eelm.csic.es

J. Lora
e-mail: jlora@eelm.csic.es

V. T. Pham
Department of Plant Resources, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Ha Noi, Vietnam
e-mail: phamvthe@gmail.com

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23.1 Introduction

The Sapindaceae or soapberry family, which comprises about 2000 species of trees, shrubs and vines, includes several subtropical and tropical crops with edible fruits such as litchi or lychee (*Litchi chinensis* Sonn.), longan (*Dimocarpus longan* Lour.), rambutan (*Nephelium lappaceum* L.), guarana (*Paullinia cupana* Kunth), korlan (*Nephelium hypoleucum* Kurz.), pitomba (*Talisia esculenta* Radlk.), Spanish lime (*Melicoccus bijugatus* Jacq.), pulasan (*Nephelium mutabile* Blume) or ackee (*Blighia sapida* K. D. Koenig). Among them, litchi and longan are the most popular worldwide. Both have whitish pulp, sweet flavor and a very aromatic aril, an outgrowth of the ovule that envelops the seed (Endress 1973). Litchi is generally more acidic and juicer than longan. Longan fruit is similar to litchi but is usually smaller, rounder in shape and has smooth skin. Litchi and longan can be eaten fresh, frozen, canned, dried and, due to a short shelf life and often excessive supply at peak harvesting season, also processed into juice, wine, pickles, preserves, ice cream and yoghurt (Zhao et al. 2014).

Ancient documents, seeds from tombs and old living specimens confirm litchi and longan cultivation in China for more than 2000 years (Huang et al. 2005; Wu et al. 2007). Thus, litchi and longan were supplied to the emperor during the Han dynasty 200 BC and the first record of an unsuccessful attempt to introduce litchi from subtropical to temperate areas was documented in 116 BC when the emperor Wu of Han conquered Guandong (China) (Huang et al. 2005).

The first description of litchi and 32 cultivars from Fujian province was reported by Cai Xiang during the Son dynasty in 1059 (Papademetriou and Dent 2002) but it was not until the eighteenth century when the Jesuit missionary and botanist Joannis de Loureiro provided the first description of longan and litchi for western countries in the first comprehensive flora of Southeastern Asia, *Flora Cochinchinensis* (Loureiro 1790). However, the fruit was rarely seen in western markets until the late 1980s (Huang et al. 2005). The name *litchi* or *li-zhi* in Chinese Pinyin, refers to the rapid deterioration that the fruit undergoes after harvesting and longan literally means *the eye of the dragon* because of the similarity of the fruit to an eye showing white pulp and a round, bright black seed (Huang et al. 2005). In this review, we discuss several aspects of genetics and breeding of litchi and longan.

23.2 Origin and Botany

23.2.1 Origin, Production and Dissemination

Litchi and longan are closely related taxonomically (Harrington et al. 2005). In fact, although they are currently classified in different genera, they were initially both considered to belong to the genus *Dimocarpus* by Loureiro (1790). Cultivated litchi originated in southern China and northern Vietnam. The origin of longan has also

been reported in the mountain chain extending from Myanmar to southern China; other authors have extended this range to southwestern India and Sri Lanka (Choo 2000; Tindall 2010). Ke et al. (1994) suggested Yunnan as the primary center of origin of longan and Guangdong, Guangxi and Hainan provinces as a secondary center based on palynological studies. China is the largest litchi and longan producer and consumer country.

The limited environmental adaptation of litchi and longan to the warm subtropics has restricted their production to countries with subtropical climates and most production is located in southern China. Production data for litchi and longan are not included in the FAOSTAT database. World annual production of litchi is estimated at 2.6–2.8 mt and about 70% is produced in China with 1.45 mt in 2005, which increased to 1.9 mt in 2011 (Chen and Huang 2014). Chinese production is mainly concentrated in Guangdong, Guangxi, Fujian, Hainan and Taiwan, undergoing a dramatic expansion during the 1990s and with most litchi production (98%) remaining in the domestic market (Huang et al. 2005). A substantial expansion has also taken place in India over the past 60 years.

Litchi propagation was by seed before the tenth century, but due to its long juvenile period, requiring 3–5 years to enter into production and the variation in performance observed in seedlings, air layering and grafting became a common practice among growers (Huang et al. 2005). From China, litchi was introduced into Myanmar by the end of seventeenth century and later into Bangladesh and Nepal (Huang et al. 2005). Litchi reached India and Thailand 100 years later. In 1870 it was introduced to Madagascar and Mauritius and three years later to Hawaii. Probably Chinese migrants brought litchi to Australia around 1854 during the gold rushes. Litchi was introduced in Florida from India between 1870 and 1880 and reached California in 1897. Litchi reached Israel between 1930 and 1940 (Huang et al. 2005; Papademetriou and Dent 2002). Spain is the only country in continental Europe with a significant litchi production and its introduction took place in the 1990s.

Although longan is less popular outside Asia than litchi, it followed a rapid expansion since 1980 in China in parallel with litchi (Huang et al. 2005). Longan also has a long juvenile period of 7–8 years and, consequently, vegetative propagation is recommended; air layering is also the most popular propagation method (Choo 2000). In 2010, longan reached a cultivation area close to 400,000 ha in China, which represents 70% of longan world cultivation area, producing 60% of the world production with about 1300 mt (Qiu 2014). However, China is still a net importer of longan, while Thailand is the biggest exporter with over 500 mt in 2010 (FAO 2011). In fact, although litchi is commercially more important than longan in most producing countries, in Thailand longan contributes more to the local economy (Choo 2000). Vietnam also has a large longan industry yielding 500 mt in 2010 (FAO 2011); a minor but increasing longan producer is Australia (Nicholls 2001). As in litchi, Spain is the only country in continental Europe where longan can be produced commercially but production is still incipient.

23.2.2 Description

Both litchi and longan are diploid species ($x = 15$ and $2n = 30$) and share many similarities in their botany and flowering; in fact, some intergeneric hybrids have been reported (McConchie et al. 1994). Both are slow growing medium to large evergreen trees. Litchis can grow up to 10–12 m (Fig. 23.1a) or even 20 m in very old specimens (Morton 1987; Subhadrabandhu and Stern 2005). Litchi trees older than 1200 years can still flower and set fruit (Subhadrabandhu and Stern 2005). Longan trees can also reach 20 m (Fig. 23.1c) but, while the trunk of litchi tree is smooth, it is rough in longan. Litchi trees develop deep roots but the absorbing roots are superficial at 20–30 cm in depth and need 50% soil moisture or above (Sarin et al. 2009). The leaves of both species are pinnately compound with 4–7 leaflets in litchi and 6–9 in longan, arranged oppositely along the rachis. The fruits are borne in clusters outside the leaf crown (Subhadrabandhu and Stern 2005) with similar fruit development in litchi and longan (Stern and Gazit 2010). Litchi fruit has a rough, red exocarp (skin) (Fig. 23.1b) while longan fruit has a bark-like, light brown exocarp (Fig. 23.1d).

23.2.3 Flowering, Pollination and Fruit Set

The flowering period of litchi lasts for 20–25 days under the environmental conditions of northern Israel (Degani et al. 1995b) and that of longan continues for 35–45 days in southern Spain (Pham et al. 2015b). Both species have a compound dichasia inflorescence that exhibits duodichogamy, that is, the tree has three flower types: male flowers (M1) (Fig. 23.2a) that are staminate flowers with functional stamens but underdeveloped ovules, functionally female hermaphrodite flowers (F) and functional male hermaphrodite flowers (M2) (Fig. 23.2b). The three types of flowers appear in three waves in which the M1 flowers appear first, then F flowers, and, finally, M2 flowers. The waves of M1, F and M2 flowers are gradual and can overlap depending on the cultivar and the environmental conditions (Pham et al. 2015b; Subhadrabandhu and Stern 2005). In litchi, the first phase is often skipped (Mustard et al. 1953). Flowers of different types show little overlap in the same inflorescence preventing self-pollination, but the inflorescences are not synchronized within the same tree and even less so on different trees (Davenport and Stern 2005). Litchi appears to be self-compatible but fruits derived from outcrossing seem to be larger with larger seeds than selfed fruits (Degani et al. 1995b; Stern et al. 1993).

The study of the reproductive phase provides very useful information for breeding litchi and longan. In most crops, this is the stage most sensitive to environmental conditions and, specifically, to temperature (Hedhly et al. 2009). In fact, temperature is the main environmental factor affecting shoot induction and floral development (pollen viability, sex ratio and receptivity) in litchi and longan; cool temperatures promote flowering in both species (Davenport and Stern 2005; Menzel 1983). Nakata and Watanabe (1966) reported that low night temperatures of 22 °C during 62–70 days

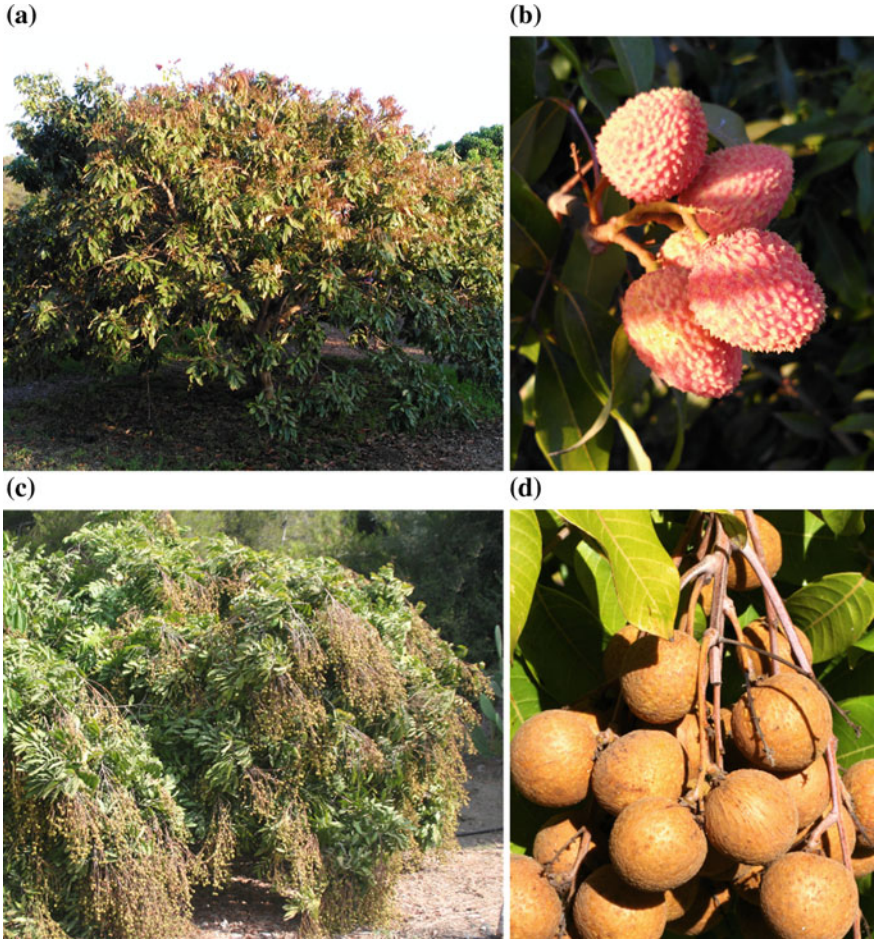

Fig. 23.1 **a** Litchi trees usually grows up to 10–12 m. **b** Pink-red fruit of litchi showing its rough texture. **c** Longan tree, which generally has a similar size to the litchi. **d** Longan fruits showing their bark-like exocarp

promote flowering in litchi but flowering is not influenced by photoperiod. Similar results were confirmed by Batten and Mcconchie (1995) and, in addition, they showed that high temperatures also promote leafy inflorescences. Thus, temperatures above 25 °C increase the proportion of leafy panicles and even reproductive buds can revert to vegetative growth. The sex ratio is also affected by temperature after panicle emergence in litchi. A recent study by Chen et al. (2016) reported a temperature model for litchi flowering. Water stress also decreases the percentage of female flowers in litchi (Menzel and Simpson 1988, 1991) and, on the other hand, oxidative stress created by potassium chlorate treatments favors flower induction in longan

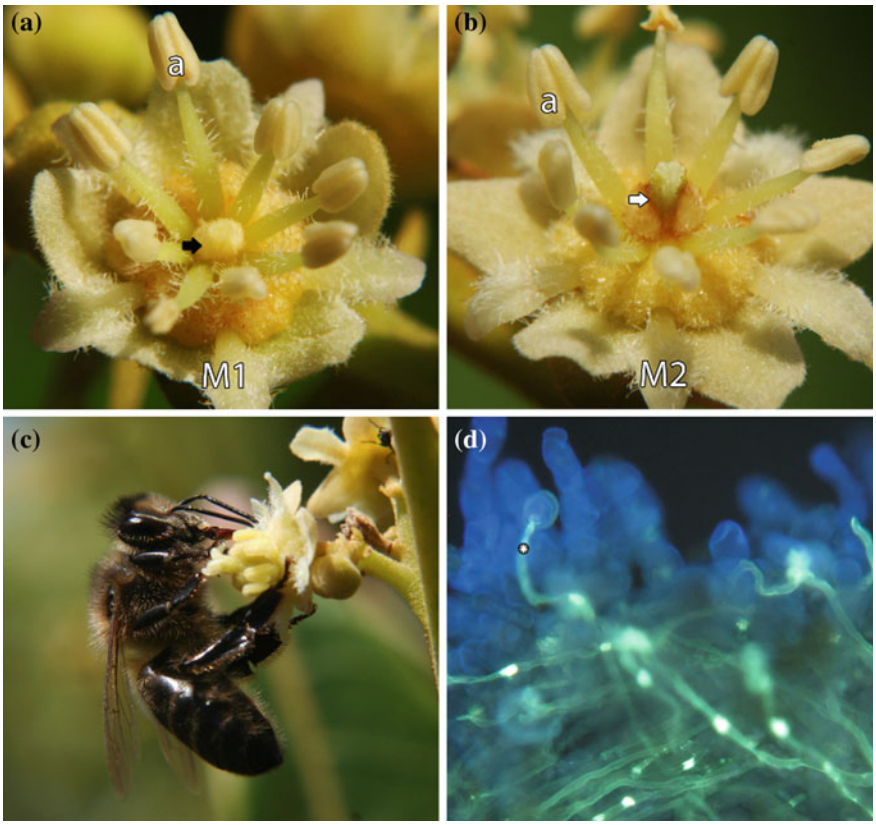

Fig. 23.2 The flower types and the stigmatic receptivity of longan. **a** M1 flower showing functional anthers (*a*) and an underdevelop pistil (black arrow). **b** M2 flower showing functional anthers (*a*) and pistil (white arrow). **c** Flower showing a bee removing nectar. **d** Pollen tube growth (asterisk) on the stigma

(Zhou et al. 2014). Litchi flower initiation in early spring was also promoted by ground mulching (Su et al. 2014).

Air temperature also has an effect on pollination, pollen tube growth (Fig. 23.2d) and fruit set. Thus, higher temperatures decrease the receptivity in litchi that lasts for 5 days at 20/17 or 22/17 °C, but less than 2 days at 33/27 °C (Davenport and Stern 2005; Stern et al. 1997). In longan, stigmatic receptivity is higher during the second day after pollination of the three receptive days (Pham et al. 2016).

Studies on pollen performance have reported that pollen from M2 flowers show a better pollen germination than pollen from M1 flowers in both litchi (Mustard et al. 1953; Stern and Gazit 1998) and longan (Pham et al. 2015a) with best pollen performance at 30 °C in litchi (Stern and Gazit 1998) and 23/24 °C in longan (Pham et al. 2015a). Matsuda and Higuchi (2015) also reported differences between two litchi cultivars showing a high pollen germination at 25–30 °C in Bengal and at

25–35 °C in Chakrapat. Pollen tube growth from pollination to fertilization takes 2 days in litchi (Stern and Gazit 1998) and 3 days in longan (Pham et al. 2016), although different studies of longan reported fertilization ranging from 3 h to 3 days (Li 1984; Liu et al. 1996; Xu et al. 2012), probably due to different environmental conditions, mainly temperature, during pollen tube growth.

Litchi and longan are usually pollinated by insects, chiefly honey bees (Fig. 23.2c), and generally in the morning (Davenport and Stern 2005) when nectar was observed on the litchi flowers (Butcher 1970; Stern and Gazit 1996). Interestingly, while the nectar composition is similar in the three flower types of litchi, the nectar volume is highest in F flowers, medium in M2 flowers and low in M1 flowers. Consequently, flower visits of honey bees are also higher in F flowers, intermediate in M2 flowers and low in M1 flowers (Stern and Gazit 1996). A study performed in India showed the need for an abundance of insect pollinators and the European honey bee (*Apis mellifera*) seems to be the most efficient (Kumar 2014).

The edible fruit (in fact a fruit-like structure) in litchi, longan and other species in the Sapindaceae is the fleshy aril, which is an outgrowth of the ovule that envelops the seed (Endress 1973; Pham et al. 2016). Usually only one of the two locules present in the ovary develops into a fruit while the other aborts, although in some cases both locules can develop. Litchi and longan show a similar pattern of fruit development but fruit maturation and harvest take 2–3 months in litchi and 4–5 months in longan (Pham et al. 2016; Stern 2005). Up to 60–80 fruits can be harvested per inflorescence in longan resulting in about 7% final fruit set under the environmental conditions of southern Spain (Pham et al. 2016) similar to the 8% fruit set reported in Israel by Stern and Gazit (2010). This low fruit set compared to the initial number of flowers produced is mainly due to a massive abscission of flowers after pollination and is also common to litchi (Mitra et al. 2005). Fruits can show aborted seeds, known as *chicken tongues*. The presence of small or aborted seed is an important commercial characteristic that varies with the cultivar and the environment.

23.3 Genetic Resources

Due to the long tradition of cultivation and being the center of origin, China has the richest germplasm diversity of litchi and longan. The first record of 4 litchi cultivars was made 1800 years ago, including a cultivar with shriveled seed (Huang et al. 2005). A total of 32 cultivars of litchi were described in the year 1059 (Papademetriou and Dent 2002) but most of them are currently lost (Huang et al. 2005). Chen Ding recorded 43 cultivars around the year 1600 and 58 cultivars of Guangdong were recorded in 1826 (Huang et al. 2005). Currently, there are hundreds of cultivars of litchi and longan that are mostly collected and preserved in China.

The National Litchi Germplasm Genebank at the Institute of Fruit Tree Research, Guangdong Academy of Agricultural Science in Guangzhou, which is the largest litchi germplasm collection in the world, contains more than 500 litchi cultivars (Liu et al. 2015). However, only a few cultivars are used for production. World

litchi production is mainly based on the cv. Tai So (Mauritius) although China litchi production is multi-cultivar, where more than 50% of litchi production is based on Heiye and Huaizhi cvs (Chen and Huang 2014).

In 2014, about 400 cultivars of longan were reported in China. Most of them (68%) are mid-season, 14% are early season and 16% late season cultivars. The main cultivars are Fuyan, Wulongling, Songfengben and Lidongben in Fujian province, Shixia and Chuliang in Guangdong province and Dawuyuan and Guangyan in Guangxi province. Several germplasm collections have been established in the different longan-growing countries. The Fujian Academy of Agricultural Sciences was established in 1981 and has collected and preserved 202 cultivars. Other germplasm collections are in Guangdong province and Taiwan (Qiu 2014).

23.4 Plant Improvement and Breeding Efforts

Litchi and longan usually have low yields with an average of 2–5 mt/ha or less, but yields up to 19.5 mt/ha have been achieved in Israel; there is, therefore, considerable margin for production improvements, which depend on specific ambient conditions and/or appropriate crop management practices (Huang and Chen 2014; Menzel 1984). The information on reproductive biology mentioned above is key to optimizing litchi and longan production. Thus, cool temperatures around 15 °C or lower are required for flower induction and appropriate litchi flowering and, consequently, this crop is restricted to regions with subtropical climates. However, there are exceptions such as in Taiwan where litchi cultivars that can flower at higher temperatures are cultivated, although some of these cultivars show lower fruit quality. Although litchi and longan are non-climacteric fruits, the short shelf life due to decay and browning make it difficult to commercialize the fruits in world markets. Chemical treatments based on the application of SO_2-related techniques are commonly used to prolong shelf life, especially in litchi (Mitra et al. 2014). The availability of a high proportion of small seeds or aborted seeds (chicken tongues) is also a valuable feature in litchi and longan such as the litchi cvs. Nuomici and Guiwei (Menzel et al. 2005).

The diversity of germplasm resources available in both litchi and longan is a guarantee that adequate breeding programs can be developed to produce new varieties adapted to different environmental conditions and with a superior fruit quality and higher yield. Although the percentage of fruit set is low and, consequently conventional breeding methods are difficult in both species, recent studies on pollen storage report that litchi pollen can be stored at −86 °C (Wang et al. 2015b) and −40 °C (Matsuda and Higuchi 2015), and longan at −20 °C (Pham et al. 2015a) for long-term preservation. The use of these pollen storage methods will be very useful for breeding programs and to overcome differences in flowering time between cultivars of interest.

Several litchi and longan breeding programs have been developed worldwide, mainly in China. A seedless litchi variety was obtained by conventional breeding

in Hainan (Sarin et al. 2009). Five strains of longan with aborted seeds and, among them, especially Minjiao No. 4, with a high proportion of aborted seeds, were selected in Fujian province after a 30-year breeding program (Huang et al. 2001). Recently, the Litchi Selection and Breeding Group of the South China Agricultural University selected a litchi strain, 9919, that shows earlier maturity (Hu et al. 2014).

In South Africa production is mainly based on two cultivars, Mauritius (Tai So) and McLean's Red (Bengal). A litchi breeding project focusing on obtaining cultivars better adapted to local environmental conditions, as well as to extend the short harvesting period and the improvement of tree and fruit quality, is currently ongoing (Froneman et al. 2014). There are also breeding programs in other areas, such as Hawaii, where a small selection results in the cvs. Kaimana and Groff with good performance and less chilling requirement for flower initiation (Zee et al. 1999), India with the Saharanpur selection (Lal and Nirwan 1980) and, more recently, in Australia (Dixon et al. 2005).

23.5 Biotechnology

23.5.1 Micropropagation and Genetic Transformation

Due to the long juvenile period and the high heterozygosity, propagation by seed is usually not recommended for these crops and the most common method of propagation is air layering in both litchi (Menzel 1985) and longan (Choo 2000). For a faster, yet efficient alternative, clonal propagation using in vitro techniques can potentially be used. In this sense, several studies have reported clonal propagation using embryo culture (Kantharajah et al. 1992), anther or pollen culture to obtain haploid plants and protoplast culture for basic research (Lai et al. 2001; Zheng et al. 2001).

The added benefit of in vitro culture is that it allows a transgenic approach and accelerating breeding programs. Transformation using *Agrobacterium* is a powerful tool for genetic improvement that has been used in several fruit trees (Gambino and Gribaudo 2012). Green fluorescent protein (GFP) was introgressed in litchi using *Agrobacterium* and in vitro grown leaf tissues. GFP was observed in the regenerated callus and the leaves (Puchooa 2004). Transgenic plants of longan were also obtained using strains of *Agrobacterium rhizogenes*, but without introducing a gene with agronomic interest (Zeng et al. 2001). In vitro culture may also provide a rapid mutagenesis using gamma ray and ethyl-methano sulfonate (EMS) that are commonly used in the model plant thale cress, *Arabidopsis thaliana* (Kim et al. 2006) and can increase variability in litchi and longan (Sarin et al. 2009).

23.5.2 Germplasm Diversity and Conservation

As in most fruit tree crops, litchi and longan cultivars traditionally have been identified by morphological characters (Wu et al. 2007) which are still being used (Wu et al. 2016). However, those traits are often influenced by environmental factors that create confusion in cultivar identification. Thus, homonymies (cultivars under the same name with different genetic profiles) and synonymies (cultivars under different names with the same genetic profile) are frequent in both crops (Madhou et al. 2013). Part of this confusion results from mere misidentification using morphological traits, but an additional explanation is the use of varying names for the same cultivar in different Chinese dialects (Degani et al. 2003) and their translation into English and other languages (Anuntalabhochai et al. 2002; Aradhya et al. 1995; Degani et al. 2003; Liu and Mei 2005; Viruel and Hormaza 2004). To obtain a more precise identification, isozyme analysis and DNA markers can be used for cultivar characterization. As in other crops, isozyme fingerprinting was one of the first molecular identification methods used in litchi (Aradhya et al. 1995; Degani et al. 1995a, 2003; Stern et al. 1993) and longan (Arias et al. 2012) characterization and it has been useful to reveal different cases of apparent synonymy or mislabeling of cultivar names (Aradhya et al. 1995). For example, the litchi cv. Kwai Mi in Hawaii was found to be identical to Mauritius and Kim Jee in Hawaii and to Tai So in Australia, but different from Kwai Mi from China and Taiwan although mismatches have been found in some cases using this method (Anuntalabhochai et al. 2002; Degani et al. 2003; Madhou et al. 2013; Viruel and Hormaza 2004).

Since the discovery of the polymerase chain reaction (PCR) by Mullis et al. (1986), numerous molecular marker systems based on PCR have been developed for detection of genetic variation and have been widely applied in different plant species (Semagn et al. 2006). Among them, randomly amplified polymorphic DNA (RAPD) was first used for the identification of 34 Chinese litchi cultivars (Ding et al. 2000) and continued in the following years (Chen et al. 2004, 2005; Liu and Mei 2005; Wang et al. 2006). Later studies using amplified fragment length polymorphisms (AFLP) and restriction fragment length polymorphism (RFLP) were performed by Tongpamnak et al. (2002) and showed low genetic variation in 47 selected accessions conserved in Thailand. Yi et al. (2003) used AFLPs to analyze 39 cultivars. RAPD markers were also used to study Indian litchi accessions (Kumar et al. 2006; Misra et al. 2014) and AFLP markers for the study of genetic diversity of 83 litchi accessions from Florida, Hawaii, Thailand, and three areas of China, conserved at the USDA-ARS germplasm collection at the Pacific Area Basin Research Center (PBARC) in Hilo, Hawaii (Jones et al. 2006). More recently, AFLP markers were used in the identification of 23 popular commercial cultivars of litchi and revealed five markers associated with small-seeded cultivars (Pathak et al. 2014). A more stable marker derived from RAPD, sequence-characterized amplified region (SCAR) markers, which are based in PCR using specific primers designed from cloned RAPD fragments, were recently developed for the identification of litchi cultivars (Cheng et al. 2015).

Among the different types of molecular markers recently used in litchi, microsatellites or simple sequence repeats (SSRs) have been for several years the marker of choice for genotype identification due to their high polymorphism and reproducibility (Larrañaga and Hormaza 2016). Microsatellites are short regions of repeated DNA sequences in tandem with 1 to 6 nucleotides that are ubiquitous in eukaryotic genomes (Fig. 23.3). A first set of 12 SSRs was developed by Viruel and Hormaza (2004) and were initially used to fingerprint 21 litchi accessions conserved in Spain. Moreover, those markers proved to be transferable to longan, thus avoiding the need to develop specific markers in this crop. In addition, SSR markers have also been found to be transferable to other species in the Sapindaceae such as pulasan (Sim et al. 2005) and ackee (Ekué et al. 2009). This set of markers was useful in evaluating 88 litchi accessions of three different litchi germplasm collections from Mauritius, Réunion and Spain (Madhou et al. 2013) showing several synonymies and homonymies in litchi cultivar nomenclature both within and across geographical regions providing the basis for the standardization of litchi cultivar nomenclature. Later studies have also reported 22 (Li and Zheng 2004) and 16 (Li et al. 2006) additional litchi SSR markers and, more recently, 384 SSR markers were developed using the SSR-enrichment method of Techen et al. (2010) and pyrosequencing (Arias et al. 2012). Additional work using SSR markers has been performed in recent years such as the construction of a core collection of 38 litchi accessions based on 127 litchi genotypes from the National Lychee Germplasm Repository (Guangzhou, China) and the Lychee/Longan Germplasm Repository of the College of Horticulture (South China Agricultural University) (Wang et al. 2014). Another core collection based on 96 accessions from the National Litchi Germplasm Gene Bank, located in the Institute of Fruit Tree Research, Academy of Guangdong Agricultural Science (China) was performed using expressed sequence tags SSRs (EST-SSR) that were derived from transcribed DNA (Sun et al. 2012).

A different microsatellite-based method, which does not require previous genome information, inter simple sequence repeats (ISSR) was used to characterize litchi germplasm conserved in Israel (Degani et al. 2003). High genetic diversity was also observed among 83 early-maturing litchi cultivars from southwest Guangxi using ISSRs (Shen et al. 2013). Recently, 6 litchi genotypes from different provinces of China were characterized by combining RAPD and ISSR markers (Long et al. 2015).

More recently, simple nucleotide polymorphisms (SNPs), which are variations of single nucleotides which do not change the overall length of the DNA sequence in the region and occur throughout the genome, are being increasingly used for crop fingerprinting and other uses, especially due to the increasingly more economic availability of next-generation sequencing methods (Larrañaga and Hormaza 2016). SNPs have been used for the evaluation of the genetic diversity of 96 litchi accessions from China (Liu et al. 2015). This study reported a high genetic variation among the litchi accessions that were grouped in extremely early, early, middle and late-maturing, indicating that the fruit-maturation period may be a useful criteria for litchi identification (Liu et al. 2015).

A high-density linkage map of litchi combining AFLPs and RAPDs using 73 individuals from a cross of late ripening Maguili and early ripening Jiaohesanyuehong

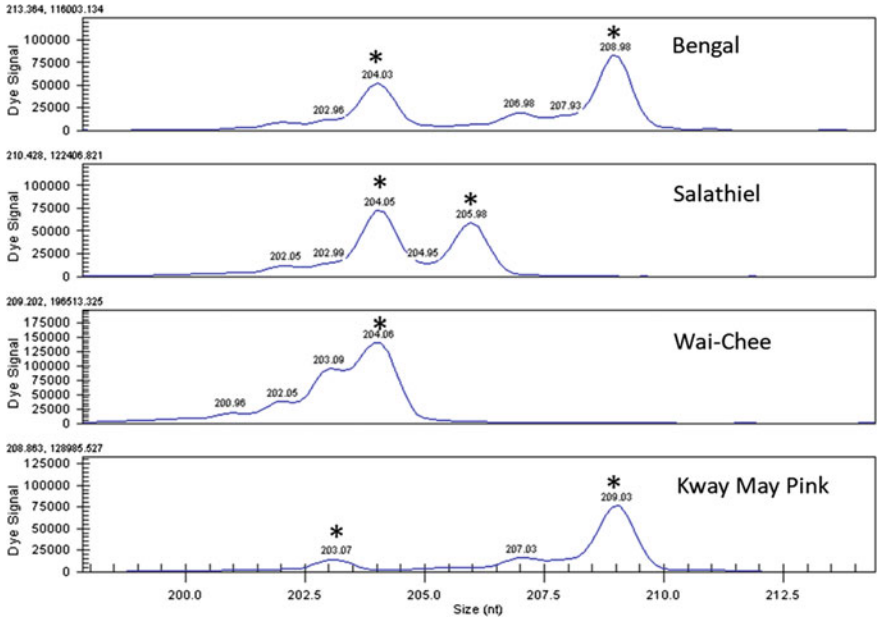

Fig. 23.3 Example of microsatellite analysis in litchi. The microsatellite LMLY12 (Viruel and Hormaza 2004) analyzed with an automatic fragment analyzing system in four cultivars, Bengal (204 and 209 bp), Salathiel (204 and 206 bp), Wai-Chee (204 bp) and Kway May Pink (203 and 209 bp). The alleles are indicated by an asterisk

cultivars has been constructed by Liu et al. (2010) identifying 16 linkage groups, whereas in a later work Zhou et al. (2012) used RAPDs, AFLPs and SRAPs using 83 individuals derived from the same cross with 18 linkage groups for Maguili and 16 for Jiaohesanyuehong.

Longan germplasm has also been evaluated using DNA markers. RAPDs were first used for molecular characterization of longan accessions (Lin et al. 1998). In a later study (Lin et al. 2005), AFLP markers and a partial sequence of the rbcL gene were used for the molecular characterization of 41 longan accessions. AFLP markers were also used for the identification of 48 longan cultivars from China (Ganjun et al. 2003). A selection of 22 genotypes of longan from Southeast Asian countries was analyzed using RAPDs (Yonemoto et al. 2006) allowing the separation between genotypes from Taiwan, China and Malaysia. Yang et al. (2013) cloned fragments of RAPDs in order to obtain stable and co-dominant sequence characterized amplified regions (SCARs) markers for longan identification. Start codon targeted (SCoT) markers, that uses single primers designed to anneal to the flanking regions of the ATG initiation codon on both DNA strands, have been used to analyze the genetic diversity of 24 longan accessions (Chen et al. 2010). ISSR markers were also used to study the genetic diversity of longan cultivars (Chiang et al. 2010). The most recent study on longan identification evaluated 50 longan accessions using 60 single

nucleotide polymorphism (SNP) markers that were developed based on transcriptome sequences (Wang et al. 2015a).

A longan linkage map using RAPDs, ISSRs, SRAPs and AFLPs was developed by Guo et al. (2010) using 94 genotypes from a cross between the cultivars Fengliduo and Dawuyuan identifying 21 linkage groups in Fengliduo and 22 in Dawuyuan.

23.5.3 Genomics

Over the past 10 years the so-called next generation sequencing (NGS) technologies have been developed. These technologies provide several advantages over previous *first generation* sequencing technologies and have revolutionized many areas in biology since they allow genome-wide characterization and profiling of mRNAs, small RNAs, transcription factor regions, structure of chromatin and DNA methylation patterns, microbiology and metagenomics (Ansorge 2009). NGS technologies provide fast, inexpensive and accurate genome information (Metzker 2010) making this information more available for non-model plant species, such as litchi and longan, in which genomic data are limited. Thus, NGS technology has allowed studying numerous genomes that represent a revolution in our understanding of genome variation among species. A genome project for litchi using whole genome shotgun strategy was initiated in 2010 (http://litchidb.genomics.cn/page/species/index.jsp) (Liu et al. 2015) and a first de novo assembly of a fruit transcriptome in litchi was developed using high-throughput RNA sequencing (RNA-seq) (Li et al. 2013). This transcriptome work revealed than more than 1000 genes could be involved in fruit abscission in litchi (Li et al. 2013) and was followed by more recent works on the same subject (Li et al. 2015a, b). An additional transcriptome study was also performed in litchi focused on floral initiation (Zhang et al. 2014); the results showed the involvement of hormones and flowering-related genes in the regulation of floral initiation. Lu et al. (2014) analyzed the transcriptome to understand the molecular function of the ROS-induced abortion of rudimentary leaves in litchi, whereas Lai et al. (2015) and Zhang et al. (2016a) analyzed the transcriptomics of litchi pericarp and genes expressed during bud development. Recently, a new study has compared the transcriptomes of two genetically closely related litchi genotypes that clearly differ in seed size (Pathak et al. 2016); most of the differences in expression were found before anthesis, suggesting the role of maternal factors in seed development. The genome information makes possible studies on genetic diversity and high-density linkage maps, and the transcriptome information will be very useful to perform functional studies and develop additional DNA markers such as the abovementioned SNPs (Wang et al. 2015a).

Although the level of genomic work on longan is low compared to litchi, some transcriptomic works have also been carried out in this fruit crop. Thus, Lai et al. (2013) generated a global transcriptome from longan embryogenic callus whereas Jia et al. (2014) used RNA-seq to analyze genes associated with continuous flowering in cv. Sijimi. Recently, a transcriptome analysis, focused especially on flowering, has

been performed in cv. Sijihua that shows a unique flowering pattern (Zhang et al. 2016b).

NGS methods will likely replace most molecular markers used so far since they provide a larger amount of information at a steadily decreasing price. However, it is important to stress the fact that the huge amount of molecular data that are becoming available will still need phenotypic studies. An understanding of the association between nucleotide sequences and functional traits is needed for agronomic, genetic mapping, functional diversity, breeding, or evolutionary studies, but thorough phenotypic characterization of litchi and longan germplasm will be necessary for such studies.

23.6 Conclusions and Prospects

Litchi and longan are the most popular crops in the Sapindaceae but it is also worthwhile to advance knowledge of other species in the family, such as rambutan and pulasan, which could be of interest in countries with tropical climates. In spite of the increasing production of litchi and longan in countries with subtropical climates, there is still a lack of knowledge of the production limitations in these crops and a confusion of cultivar identification with frequent homonymies and synonymies. A global molecular characterization effort of the main cultivars conserved in different germplasm collections will contribute to resolving these problems. Several researchers have used molecular markers for cultivar fingerprinting, diversity analyses or breeding programs. As in other crops, in the future, next generation sequencing which can generate hundreds to thousands of molecular markers, will surely make qualitative changes in litchi and longan breeding. Although currently the genomic resources for litchi and longan are scarce, the main future limitation will be the availability of thorough and reliable phenotypic information. This highlights the urgent need for curation, analysis and exchange of litchi and longan germplasm among collections worldwide to ensure the preservation of genetically and phenotypically diverse material for future generations.

Appendix 1

List of major Institutes engaged in research on litchi and longan (Sapindaceae)

Institute	Location
Department of Agriculture and Fisheries	80 Meiers Road, Indooroopilly, Queensland 4068, Australia
Department of Agriculture and Fisheries	PO Box 5083, SCMC, Nambour, Queensland 4560, Australia
Department of Agriculture and Fisheries	Centre for Tropical Agriculture, Mareeba 4880 Queensland, Australia
Fujian Agriculture and Forestry University	College of Food Science and Technology, Fuzhou 350002, China
Guangxi University	Horticultural Department, Agricultural College 530005 Nanning, China
Institute of Fruit Tree Research	Guangdong Academy of Agricultural Sciences. 510640 Guangzhou, China
Institute of Tropical Fruit Trees	Hainan Academy of Agricultural Sciences, 571100 Haikou, China
Key Laboratory of South Tropical Fruit Biology and Genetic Resource Utilization	510640 Guangzhou, China
Kunming University	School of Agriculture, Kunming 650000, China
South China Agricultural University	College of Horticulture, Guangzhou 510642, China
South Subtropical Crops Research Institute. Chinese Academy of Tropical Agricultural Sciences	Zhanjiang 524091, China
CIRAD	Station de Bassin-Plat, 97455 Siant Pierre, Réunion Island, France
Faculty of Horticulture	Bidhan Chandra Krishi Viswasvidyalaya, Mohanpur 741252, Nadia, West Bengal, India
Mizoram University	796004 Aizawul, India
National Research Centre for Litchi (ICAR)	842002 Muzaffarpur, India
Rajendra Agricultural University	Horticulture Department, Pusa 848125, Samastipur, India
Agricultural Research Organization (ARO)	Volcani Center, Israel
University of Pretoria	Department of Agricultural Economics, Extension and Rural Development. Pretoria 0002, South Africa
Institute for Tropical and Subtropical Crops	Agricultural Research Council. 1200 Nelspruit, South Africa
Institute for Subtropical and Mediterranean Horticulture la Mayora (IHSM la Mayora—CSIC—UMA)	29750 Algarrobo-Costa, Málaga, Spain

Institute	Location
Rajamangala University of Technology Lanna	Agricultural Technology Research Institute, Lampang, Thailand
Maejo University	Faculty of Agricultural Production, 50290 Chiang Mai, Thailand
Phetchaburi Rajabjat University	Faculty of Agricultural Technology, 76000 Phetchaburi, Thailand
Kasetsart University	Department of Horticulture, Chatuchak, Bangkok 10900, Thailand
Chiang Mai University	Chiang Mai 50200, Thailand
Mae Jo University	Department of Horticulture, Chiang Mai, Thailand
Kasetsart University	Faculty of Agriculture, Chatuchak, Bangkok 10900, Thailand
University of Florida	18905 SW 280 Street, Homestead, FL 33031-3314, USA

Appendix 2: Genetic Resources

List of the main litchi cultivars in different countries (Cheng and Huang 2014; Menzel et al. 2005)

Country	Main cultivars
China	Sum Yee Hong, Baitangying, Bah Lup, Souey Tung, Fay Zee Siu, Tai So (Mauritius), Haak Yip, No Mai Chee, Kwai May Red, Wai Chee, Lanzhu, Brewster
Vietnam	Thieuthauhha
Thailand	Tai So (Mauritius), Chapacat, Wai Chee, Haak Yip, Kom, Hong Huay
India	Muzaffarpur, China, Calcuttia, Bedana, Longia
Nepal	Mujafpuri, Raha Saheb, Deharaduni, China, Calcuttia
Bangladesh	Bombai, Muzaffarpur, Bedana
Philippines	Sinco, Tai So (Mauritius)
Israel	Tai So (Mauritius), Floridian
South Africa	Tai So (Mauritius), McLean's Red, Fay Zee Siu, Wai Chee
Madagascar	Tai So (Mauritius)
Mauritius	Tai So (Mauritius)
Australia	Kwai May Pink, Tai So (Mauritius), Souey Tung, Fay Zee Siu, Salathiel, Wai Chee

List of the main longan cultivars in different countries (Cheng and Huang 2014; Menzel et al. 2005)

Country	Main cultivars
China	Fuyan, Wulongling, Chike, Shixia, Dawuyuan, Guangyan, Chulian, Gushan 2, Fengko
Vietnam	Longnhan, Tieuhue, Xuongcomvang, Longhungyen
Thailand	Daw, Chompoo, Haew, Biew Kiew, Phetsakon
Australia	Kohala, Chompoo, Haew, Biew Kiew
USA (Florida, Hawaii)	Kohala

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