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

Advances in Plant Breeding Strategies: Nut and Beverage Crops

Volume 4

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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 the 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 the 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, in addition to the development of crops for enhanced production of pharmaceuticals and innovative industrial uses. Three volumes of this book series were published in 2015, 2016, and 2018, respectively: Volume 1, *Breeding, Biotechnology and Molecular Tools*; Volume 2, *Agronomic, Abiotic and Biotic Stress Traits*; and Volume 3, *Fruits*. In 2019, the following four volumes are concurrently being published: Volume 4, *Nut and Beverage Crops*; Volume 5, *Cereals*; Volume 6, *Industrial and Food Crops*; and Volume 7, *Legumes*.

This Volume 4, subtitled *Nut and Beverage Crops*, focuses on the advances in breeding strategies using both traditional and modern approaches for the improvement of individual plantation crops. Included in Part I are 11 important nut species recognized for their economical and nutritional importance including almond, argan, Brazil nut, cashew nut, chestnut, hazelnut, macadamia, peanut, pine nut, pistachio, and walnut. Part II covers two popular beverage species, coffee and tea.

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. This volume contains a total of 108 figures and 55 tables to illustrate presented concepts. The chapter concludes with an overview of the current status of breeding and recommendations for future research directions as well as appendixes listing research institutes and genetic resources relevant to the topic crop. 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 and policy-makers.

We are greatly appreciative of all the chapter authors for their contributions towards the success and quality of this book. We are proud of this diverse collaborative undertaking, especially since this volume represents the efforts of 53 scientists from 13 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
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Contents

Part I Nut Crops

1 Almond [*Prunus dulcis* (Miller) D.A. Webb] Breeding 3
Pedro J. Martínez-García, Angela S. Prudencio, Raquel Sánchez-Pérez, Felipe Pérez De Los Cobos, Ossama Kodad, Hassouna Gouta, Manuel Rubio, and Pedro Martínez-Gómez

2 Genetic Diversity and Breeding of Argan Tree (*Argania spinosa* L. Skeels). 31
Naima Ait Aabd, Rachid Bouharroud, Abdelghani Tahiri, Ahmed Wifaya, Abdelaziz Mimouni, and Abdelhamid El Mousadik

3 Brazil Nut (*Bertholletia excelsa* Bonpl.) Breeding 57
Aisy Botega Baldoni, Lúcia Helena de Oliveira Wadt, and Cássia Ângela Pedrozo

4 Cashew Nut (*Anacardium occidentale* L.) Breeding Strategies. 77
Suvalaxmi Palei, Rasheswari Dasmohapatra, Sunil Samal, and Gyana Ranjan Rout

5 Chestnut (*Castanea* spp. Miller) Breeding 105
J. Hill Craddock and M. Taylor Perkins

6 Hazelnut (*Corylus* spp.) Breeding 157
Roberto Botta, Thomas J. Molnar, Veli Erdogan, Nadia Valentini, Daniela Torello Marinoni, and Shawn A. Mehlenbacher

7 Macadamia (*Macadamia* spp.) Breeding. 221
Bruce L. Topp, Catherine J. Nock, Craig M. Hardner, Mobashwer Alam, and Katie M. O’Connor

8 Peanut (*Arachis hypogaea* L.) Breeding 253
Saikat Gantait, Jitendriya Panigrahi, Illa C. Patel, Catherine Labrooy, Arulthambi Luke Rathnakumar, and Jeshima Khan Yasin

9 Advances in Edible Pine Nut Trees (*Pinus* spp.) Breeding Strategies 301
Hanguo Zhang and Zhen Zhang

10 Pistachio (*Pistacia* spp.) Breeding 353
Abdollatif Sheikhi, Mohammad M. Arab, Patrick J. Brown, Louise Ferguson, and Mohammad Akbari

11 Advances in Persian Walnut (*Juglans regia* L.) Breeding Strategies . 401
Kourosh Vahdati, Mohammad M. Arab, Saadat Sarikhani, Mohammad Sadat-Hosseini, Charles A. Leslie, and Patrick J. Brown

Part II Beverage Crops

12 Genetic Resources and Breeding of Coffee (*Coffea* spp.) 475
Manoj K. Mishra

13 Advances in Tea [*Camellia sinensis* (L.) O. Kuntze] Breeding 517
Mahasen A. B. Ranatunga

Index 567

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Jameel M. Al-Khayri is a Professor of Plant Biotechnology affiliated with the Department of Agricultural Biotechnology, King Faisal University, Saudi Arabia. He received his B.S. in Biology in 1984 from the University of Toledo and his M.S. in Agronomy in 1988 and Ph.D. in Plant Science in 1991 both 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 60 research articles in refereed international journals and 25 review chapters and edited 7 journal special issues. In addition, he edited 5 reference books on date palm biotechnology and utilization of genetic resources and 7 volumes of the book series Advances in Plant Breeding Strategies. He has been involved in organizing international scientific conferences and contributed numerous research presentations. In addition to teaching, student advising, 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 the Development and Quality Assurance. He served as a Member of Majlis-ash-Shura (Saudi

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Shri Mohan Jain is a Consultant and Plant Biotechnologist, Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland; he received his M.Phil. in 1973 and Ph.D. in 1978 at Jawaharlal Nehru University, New Delhi, India. He was a Postdoctoral Fellow in Israel and the USA; a Visiting Scientist/Professor in Japan, Malaysia, Germany, and Italy; and a Technical Officer, Plant Breeding and Genetics, International Atomic Energy Agency (IAEA), in Vienna, Austria, 1999–2005. He is a Member of the International Association for Plant Tissue Culture and Biotechnology and Editorial Board Member of *Euphytica*, *In Vivo*, *Propagation of Ornamental Plants*, *Emirates J. Food and Agriculture*, and the series Forest Health and Biotechnology. His publications are more than 160 in peer reviewed journals, book chapters, and conference proceedings. He edited 55 books and acted as a Chair Person in several international conferences worldwide and has been an Invited Speaker. He was awarded Nobel Peace Prize in 2005 in commemoration of the awarding to the IAEA of the Nobel Peace Prize for 2005. In addition, he was Former Consultant to the IAEA, the European Union, the Government of Grenada, Iranian Private Company, and the Egyptian Government. Currently, his research interests are on 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.

Dennis V. Johnson is a Consultant and Former University Professor. 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 the 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 the 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 nontraditional areas such as Spain, North and South America, and Australia. Moreover, he coauthored a book on date growing in the USA, has made presentations at five international date palm conferences, and coedited books on date palm, sago palm, and plant breeding.

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Part I
Nut Crops

Chapter 1
Almond [*Prunus dulcis* (Miller) D.A. Webb] Breeding

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Abstract Almond [*Prunus dulcis* (Miller) D.A. Webb] presents clear challenges within plant breeding given its plurennial woody character, long juvenile period and multiplication by grafting. These challenges make the improvement process generally long and tedious. Therefore, it is necessary to have the most current information on developing new cultivars that currently take 12 years. Additionally, the breeder has to consider diverse internal (genetic background of the existing material, actual and the new methodologies) and external factors (consumer preferences, biotic and abiotic factors) to ensure the success of a new cultivar. The degree of knowledge of these aspects determines the quality of the prediction and success in the design of new almond cultivars. Although the size of the breeding population can be unlimited, the management, phenotyping and selection of these seedlings are major limiting factors. High-throughput phenotyping methods, genomic (DNA), transcriptomic (RNA) and epigenetic studies can help to develop better selection strategies particularly useful to deal with complex target traits in tree crops such as almond, with a long juvenile periods and a high degree of heterozygosity. Recently, epigenetic marks have been developed associated to dormancy in flower buds.

Keywords Almond · Breeding · Epigenetic marks · Genomics · Molecular markers · *Prunus* · Transcriptomics

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1.1 Introduction

The cultivated almond [*Prunus dulcis* (Miller) Webb, syn. *P. amygdalus* (Batsch)], of the Rosaceae family, is an ancient domesticated nut crop that has coevolved with human civilization. The edible and tasty kernel of this nut crop species is the main reason for its production and use (Gradziel and Martínez-Gómez 2013).

Almond is the only *Prunus* species cultivated exclusively for its kernel (Fig. 1.1). Today, almonds are cultivated in more than 50 countries (http://faostat.fao.org), with 95% grown in California, Australia and the Mediterranean Basin. Historically, almonds were consumed as fresh and processed food, and are actually considered as a functional food with both nutritional and purported medical properties including purported anti-inflammatory and hypocholesterolemia properties (Musa-Velasco et al. 2016; Poonan et al. 2011).

The objective of this review is to assess new breeding perspectives including nutraceutical values and drought resistance together with the development of the more recent technologies applied to the breeding programs including DNA, RNA and epigenetic marks.

Fig. 1.1 (**a**) Cultivated almond tree, (**b**) Almond flowers, (**c**) Fruit and kernel morphology. (Source: Figure created by Raquel Sánchez-Pérez)

1.2 Origin of Cultivation

A wide dissemination of cultivated almond is notable today; several authors have distinguished four different stages: (a) Asiatic, (b) Mediterranean, (c) Californian and (d) Southern Hemisphere stages. Gradziel (2011) described each stage in detail. In each of the different stages, there was particular reference to almonds in several aspects of human civilization including in literature, medicine and food. However, although the ancestry and domestication history of cultivated almond has been studied extensively (Gradziel 2011; Grasselly 1976; Kester et al. 1991; Ladizinky 1999; Velasco et al. 2016; Watkins 1979), many facts still remain unknown.

Central and western Asia are believed to be the origin of the wild almond relatives (Browicz 1972; Browicz and Zohary 1996; Denisov 1988; Grasselly and Crossa-Reynaud 1980; Grasselly 1976; Kester et al. 1991; Komorov et al. 1941). Primarily, *Amygdalus communis* L. (syn. *Prunus communis* Archang.) was proposed as the origin of cultivated almond (Kovalyov and Kostina 1935). A later study by Ladizinsky (1999) proposed *A. fenziliana* Fiitsch as the ancestral species of the cultivated almond. This origin propositions have led to at least three different theories being suggested, which seem to have some degree of communality. The first states that the cultivated almond derived from wild Near East *P. dulcis* populations (Levant theory), the second hypothesizes that cultivated almonds are stabilized hybrids among south-western Asian species (hybrid theory) and the third theory is based on coalescence-based models, which partly supports both scenarios and suggests that most almond lineages differentiated during the Holocene (within the past 10,000 years), concomitant with the origins and expansion of agricultural practices in the Mediterranean Basin (Delplancke et al. 2012).

Another unknown in the history of almond cultivation, is the place of origin of domestication itself. Some evidence suggests that the most plausible spatio-temporal origin of *Prunus dulcis* domestication occurred in the Fertile Crescent, and dispersed across present day Israel, Palestine and Lebanon up through Syria and eastern Turkey and across to Iraq (Albala 2009), during the first half of the Holocene, ~5000 years ago (Browicz and Zohary 1996; Delplancke 2011; Velasco et al. 2016; Willcox et al. 2009; Zohary et al. 2012).

1.3 Almond Production

With a world value approaching USD 2.30 billion in 2016, almond has become the largest specialty crop export in the USA and the largest agricultural export by value (USD 4532 million) of California (ABC 2016).

California accounted for over 80% of the global production of 929,864 mt in 2017 on over 380,405 ha. Australia is now the second largest producer of almond kernels with 79,461 mt and only 39,662 ha (29,358 ha bearing, 6758 ha non-bearing, 3546 ha new plantings). Spain, is the third largest producer but with the

largest area, at over 544,518 ha, under cultivation. The rest of the world's almond production comes from about 20 countries, mainly Tunisia, Morocco, Italy, Turkey, Chile and Iran. The Balkan Peninsula including areas of Bulgaria and Romania; Mexico and Hungary also have limited almond production. Additional areas exist in central and southwestern Asia including Syria, Iraq, Israel, Ukraine, Tajikistan, Uzbekistan, Afghanistan and Pakistan, extending into western China (ABA 2017) (Table 1.1).

The main grown cultivar in California is Nonpareil, with a cultivation area of 131,622 ha, representing 38% of the total area cultivated and producing 40.7% of the total production. Other important cultivars are Monterey, Butte and Carmel with cultivation areas of 46,085 ha, 32,810 ha and 30,495 ha, respectively. In addition, the Spanish cultivar Marcona is cultivated in only 60 ha in California. This cultivar is the best-evaluated traditional cultivar in Spain recognized by growers and market for its excellent kernel quality.

Almond kernels can be eaten in their natural state or processed. They have an excellent taste, crunchy texture and good visual appeal and support diverse food uses. Kernels may be roasted dry or in oil followed by salting with various seasonings and used as an ingredient in many manufactured food products. The processed kernel is usually blanched, which removes the pellicle (skin) using hot water or steam. For pastry, ice cream, breakfast cereals and vegetable mixtures use, almond kernels can be sliced or diced. The kernels are important ingredients in bakery products and, ground into a paste, in the production of marzipan. Several factors such as cultivar, moisture content, processing and handling procedures can impact the flavor and texture of almonds (Kester and Gradziel 1996).

Almond cultivation in the Mediterranean countries is mostly non-irrigated, with kernel yields around 400–500 kg/ha, much lower than the 2300 kg/ha of the USA, where the production is much more intensive and exclusively irrigated (http://faostat.fao.org). The Mediterranean almond-producing areas play an important role in social strengthening and retention of families in the area, thus contributing to reduce

Table 1.1 Global almond kernel production in 2017

Country	Production (mt)
California (USA)	929,864
Australia	79,461
Spain	50,954
Tunisia	15,000
Iran	15,000
Turkey	13,000
Chile	14,000
Morocco	11,000
Italy	7500
Greece	3000
Others	30,000

Source: ABA (2017)

emigration. However, in spite of the higher oil quality and nutraceutical content found in the cultivars grown in the Mediterranean area, almond culture is affected by the low rainfall and drought-limited production.

The Spanish Agriculture Services reported that the summer 2014 drought affected 72,829 ha (18% of cultivated area) in non-irrigated areas, considering only the Murcia Region. Damage was estimated by agricultural organizations at around EUR 123.5 million and more than 2 million trees died. The situation was even more severe in Morocco and Tunisia. Data reported from Tunisia indicated that during the last major drought period, 2000 to 2003, more than 20 million trees and 200,000 ha (80% of the cultivated area) were seriously affected. In these areas of the Mediterranean Basin, water has become a scarce resource due to high consumption and the high level of overall pollution. Additionally, the increasing vulnerability to inclement weather and to long periods of drought has led to depopulation of many rural areas. Therefore, the implementation of appropriate measures is absolutely necessary to protect affected farms, their trade and income, as well as that of associations, to ensure their independence through collective management. Beyond the ethical imperative of doing research on how to increase production and to strengthen the adaptation of plants to drought on a global basis, there is also a need to provide a beneficial solution to the Mediterranean countries affected by spatial and temporal complexity of regional droughts (Gouveia et al. 2017). Moreover, climate change conditions accentuate drought since a reduction of 10% in precipitation translates into 25% loss of soil water. In almond growing, water availability is a major constraint in the cultivation of the species.

1.4 Genetic Diversity and Conservation

Although, the wild almond progenitor is unknown, a significant reduction in genetic diversity relative to wild progenitor species (once the wild progenitor is confirmed) should be expected as a consequence of the process of domestication, which is attributed to a genetic bottleneck. However, in almond this *domestication bottleneck* and the following expansion in population size seems unlikely (Meyer and Purugganan 2013; Velasco et al. 2016). With the use of population genetics approaches and after the evaluation of genome-wide diversity patterns, results have shown no evidence of a bottleneck during the domestication of almond (Velasco et al. 2016). Additionally, the authors observed that some regions showed signatures of selection during domestication and also candidate regions presented some overlap between almond and peach (having a divergence time ~8 million years ago). The lack of a genetic bottleneck has also been observed in other perennial crops such as apple and grape (Gross et al. 2014; Zhou et al. 2017), and was associated with the life history of perennials exploited for domestication (Gaut et al. 2015). Interestingly, Velasco et al. (2016) observed that fruit traits were not preferentially targeted during domestication but likely selected much earlier during species (peach and almond) divergence.

The outcrossing nature of *Prunus dulcis*, due to its self-incompatible origin, has contributed to maintain a high level of genetic variability in cultivated almond. Early studies observed a limited genetic base in commercial cultivars; the authors considered the use of a few founder genotypes selected for their desirable regional value could be the main reason of the low variability (Socias i Company and Felipe 1992). This can be the case of most commercially-important California cultivars originated from crosses between only two parents: cvs. Nonpareil and Mission (Bartolozzi et al. 1998; Hauagge et al. 1987; Kester and Gradziel 1996). Also, in Europe the main source of self-compatibility used by breeders has been the almond cv. Tuono, which could limit the genetic base present in new commercial cultivars. In addition, only a limited number of accessions are currently maintained (Appendix II). However, more recently, Szikriszt et al. (2011) found no correlations between the origin of the germplasm and the level of diversity. According to these authors, the actual germplasm has not suffered any genetic erosion after inbreeding, since the self-compatibility is a new trait only present in almond breeding for the last few decades.

A complete genetic characterization of the whole almond germplasm should be considered by the research community to increase information about the historical gene pool and to assure its useful protection and utilization for future breeding. Advances in DNA sequencing technology now permits a more precise analysis of large collections. Traditionally, markers such as simple-sequence repeats (SSRs) have been used to address all these questions (Fernández í Martí et al. 2009; Martínez-Gómez et al. 2003; Xie et al. 2006). More recently, different markers such as inter-retrotransposon amplified polymorphism (IRAP), sequence-specific amplification polymorphism (S-SAP), retrotransposon-microsatellite amplified polymorphism (REMAP) and inter simple sequence repeats (ISSR), have been used to genotype large number of accessions of several species. In addition, high resolution melting (HRM) analysis was developed to detect SNPs in wild almond species based on publicly available *Prunus* ESTs and next-generation sequencing (NGS) data (Sorkheh et al. 2017). An increment of the deployment of genome-wide genetic diversity using high-throughput technologies and NGS data will provide new tools to add significant value to germplasm collections.

1.5 Classical Almond Breeding

Classical almond breeding can be described as a long and tedious process, similar to the improvement processes of the majority of fruit trees. Specifically, its plurennial woody character, long juvenile period and multiplication by grafting as a standard has clearly controlled the selection process. In this sense, breeders must have a rigorous and extensive set of data for the specific design of new cultivars (Fig. 1.2).

New cultivars will have an impact in the agricultural system around 12 years from the time they were first selected, which is the average time to release a new almond variety (Fig. 1.2). One of the fundamental keys of this process is the

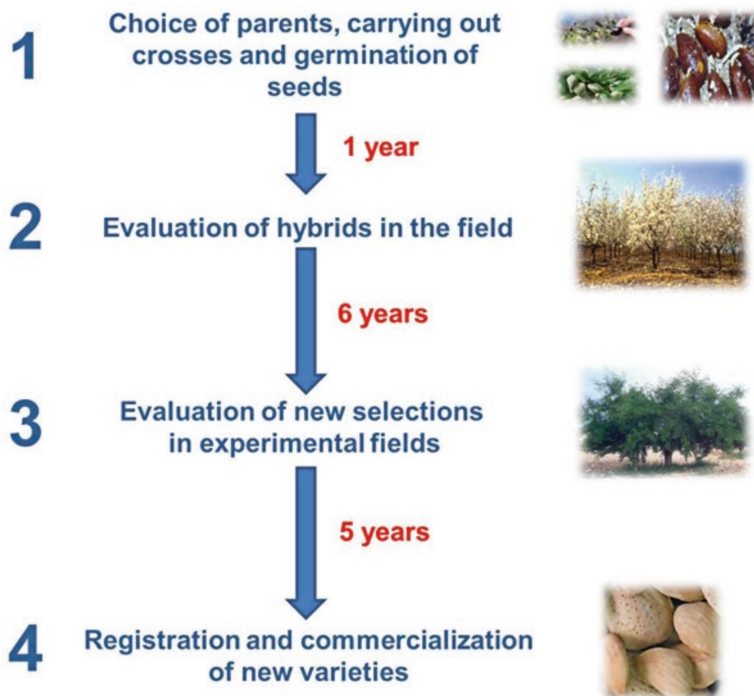

Fig. 1.2 Scheme of an almond breeding program based on the realization of crosses and later selection. (Source: Figure created by Pedro Martínez-Gómez)

selection of the genotypes that should be used as genitors. The basis of the design is the realization of crosses and, therefore, it is necessary to choose well which cultivars must be crossed to generate new cultivars. These crosses can be of the complementary type, when two cultivars are crossed with complementary characteristics to obtain a new cultivar that assembles the interesting phenotypic traits of both genitors, or of the transgressive type, where two cultivars with good phenotypic traits are crossed to obtain offspring exhibiting a more extreme phenotype than either parent (Martínez-Gómez et al. 2003).

One important issue, from the economic point of view, is the cost of maintaining a large number of offspring populations, something that must be considered by the breeder at each crossing period. This population number is directly related to the cost in the process of evaluation of the hybrids.

In addition, to develop suitable new cultivars, we need to know the relevant variables that influence our knowledge of the possible future in the success of new cultivars. It is thus necessary to examine the endogenous (internal) or exogenous (external) variables influencing the phenomenon in question. Both types of variables must be subjected to scientific evaluation (Fig. 1.3).

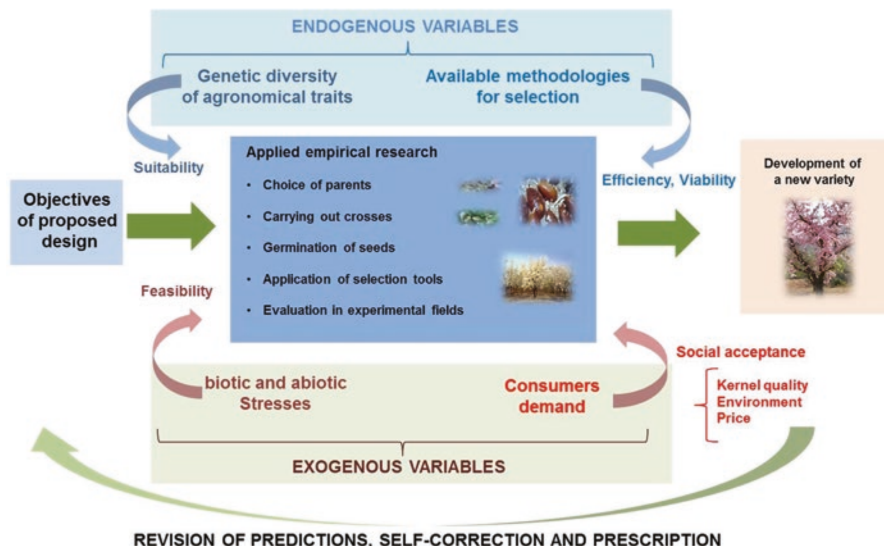

Fig. 1.3 Internal and external variables affecting the viability and establishment of a new almond cultivar obtained through classical breeding. (Source: Figure created by Pedro Martínez-Gómez)

The first internal variables to consider in any almond breeding program are derived from plant traits that are considered as objectives. The most important characteristics in these breeding programs are as follows (Martínez-Gómez et al. 2003):

(a) Tree: Floral self-compatibility, time of flowering and maturation, productivity and resistance to pests and diseases (Ortega and Dicenta 2003; Sánchez-Pérez et al. 2004).
(b) Seed (kernel) quality: Organoleptic quality of the seed size, shape, flavor, or hardness of the shell (Socias i Company et al. 2007).

The knowledge of these internal variables of genetic type will direct their suitability with respect to the proposed design objectives. *Prunus* species, such as almond, are characterized by great interspecific compatibility, meaning it is relatively easy to perform interspecific crosses. In addition, one of the main handicaps of *Prunus* breeding is the reduced variability of most species derived from their mating system, being less polymorphic self-compatible species such as peach or apricot in comparison with self-incompatible such as almond and cherry (Graziel and Martínez-Gómez 2013). In this context of reduced genetic variability, the realization of interspecific crosses is very useful for the development of new cultivars (Gradziel et al. 2001; Martínez-Gómez et al. 2004; Sorkheh et al. 2009).

Secondly, internal variables also include the current methodologies available for use in the selection of individuals. These methodologies are closely related to the level of knowledge available at the meso- and micro-levels, especially in relation to the development of molecular markers for the selection of individuals (Gradziel et al. 2001; Martínez-Gómez et al. 2003) and will provide an idea of the efficacy and fea-

sibility of new designs or cultivars. Finally, we can include various economic factors within the production framework, i.e. the goals, processes and outcomes. The effectiveness and viability of the improvement program will depend on these methodological developments as applied to the evaluation. These two terms (effectiveness and viability) are related in the economic context to the relationship between science and economics. The evaluation of this feasibility, effectiveness and feasibility can lead to the abandonment of the new design if it is not feasible or to its production. We would have to proceed with the design of a new variety with new crosses and new variables to evaluate within the program. In any case, the evaluation process is continuous.

On the other hand, in almond breeding, it is necessary to consider a number of external variables including interactions with the environment over a period of several years. Many characteristics such as flowering time and floral compatibility must be evaluated for at least 3 years, when trees produce the first flowers. Due to drawbacks, the use of molecular marker-assisted selection methods is of particular interest in breeding programs. Environmental conditions will determine the feasibility of the objectives and the new cultivar in a given location. The adaptation of new designs or new cultivars to specific climatic conditions is fundamentally conditioned by their winter cold needs for dormancy (Campoy et al. 2011) and by their adaptation to different soil conditions in the case of rootstocks. Their capacity for production depends largely on this character and on their interaction with the environment considered as an external variable.

Almond bloom in response to an established pattern of low (cold needs) and high (heat needs) temperatures after the breaking of winter dormancy (Sánchez-Pérez et al. 2014). These requirements of cold and heat temperatures guarantee that in each zone the flowering will take place at a favorable time for pollination. Pollination will be viable in a context of floral self-compatibility (fertilization of the ovule by pollen from the same variety occurs) (Gradziel et al. 2002; Gradziel and Martínez-Gómez 2013), whereas in the context of floral self-incompatibility flowering will require pollen of another variety and an isolated variety will not produce fruit (Sánchez-Pérez et al. 2004). Therefore, late flowering cultivars are sought to avoid frost but are self-compatible in the case of the almond tree (Sánchez-Pérez et al. 2014). In other fruit trees such as peach or apricot trees where flowering is not as early, what is sought is the precocity in flowering and fruiting to obtain better market prices.

The knowledge of these external variables related to possible stresses will indicate their feasibility with respect to the proposed design objectives. The feasibility of developments is another important issue to consider. The technological feasibility will also depend on the existence of germplasm with the desired genes for resistance to different biotic or abiotic stresses.

Satisfying consumer demands is also an important external variable. New designs must have the appropriate characteristics to meet such demands. In addition, the availability of different techniques for analysis and selection is another important external variable. In addition, the social acceptance of these new cultivars will depend on criteria of environmental sustainability. On the other hand, we can include various factors of an economic nature, provided that they are considered within the framework of production: the phase of objectives, processes and results.

This affects external economic variables, which are those that affect their market value. Because the result of a new genetic cultivar presents a durability (a life cycle) and involves a market price, which conditions the creative transformation carried out by technological innovation.

The degree of knowledge of the variables determines the quality of the prediction in the design of new almond cultivars, in addition to their effectiveness, feasibility, suitability and feasibility. New designs must provide the appropriate characteristics to meet those requirements. External variables include the availability of the different analysis and the selection contemporary techniques, since they have varied throughout history.

Making predictions in *Prunus* breeding also involves human activity that is geared towards reaching feasible goals in the field. The processes to be carried out must have a reasonable cost, in terms of the level of effort for the realization of the design and the means to be used. The result is to create a new cultivar, which requires scientific knowledge and scientific processes. However, the real impact for society comes with the technology that fosters this innovation through objectives and a task, which originate in a new cultivar that arises from transforming one's own reality and making it have a value in the market. Thus, the scientific, technological and economic rationalities interact. Economic mediation directly affects the human activity deployed.

Under new climatic and consumer conditions, an integrated approach to evaluating more drought resistant and productive cultivars with enhanced nutraceutical quality, is of great interest in almond breeding.

1.6 Mutation Breeding

Almond mutation breeding is not common as far as we know. However the use of spontaneous mutants in almond breeding has been performed. One example is the development of a late-blooming almond through classical breeding. Martínez-Gómez et al. (2017) concluded that the adaptation of almonds from the Mediterranean Basin to colder regions in northern Europe and North America has been mainly achieved through delayed flowering. These adapted late-flowering cultivars have usually been developed by selecting desired quantitative genes within each region. The use of molecular markers for the early selection of genes conferring late flowering has also facilitated the development of additional late cultivars including ultra-late cultivars, flowering as late as April, together with the combination of material from different gene pools. These ultra-late flowering cultivars also include genetic material from spontaneous late-flowering mutations.

1.7 Drought Resistance

Climate variability and water availability require the rapid development of production systems able to cope with risk and uncertainty. In this sense, drought resistance in almond, linked to the efficient water use (Yadollahi et al. 2011), together with the ability of the root system to access water, are important breeding targets.

Rusticity and flexibility of the different components of the production systems (including cultivars and rootstocks) should be improved. Clearly, the plantation and management of new sustainable agro-systems in the Mediterranean Basin for almond production, designed to last for long periods, must consider the impact of climatic conditions when selecting new cultivars and rootstocks. In this sense, drought is one of the biggest problems for non-irrigated culture across the Mediterranean countries. Studies about drought resistance have been conducted on wild and cultivated almonds (Camposeo et al. 2011; Palasciano et al. 2014) but these are scarce in the case of native germplasm from other areas. Some of these studies conducted by different research groups in Morocco, Tunisia and Spain provided preliminary results on drought resistance of almond cultivars and hybrid rootstocks (Alarcón et al. 2002; Esmaeli et al. 2017) and also the discovery of new genetic diversity from these regions (Gouta et al. 2012; Kodad and Socias i Company 2008).

Under drought conditions, plant develop strategies to cope with stress (accelerating the life cycle) or to avoid it (controlling stomatal conductance, investing in the development of the root system, reducing canopy, etc.) or activate osmotic adjustments to increase tolerance to low tissue water potential (for instance accumulating compatible solutes). The efficiency of photosynthetic carbon gain relative to the rate of water loss can be used as an indicator (Ennaheli and Earl 2005; Jiménez et al. 2013). In the seasonally dry and variable environment of the Mediterranean region, the ability of species like almond to cope with water scarcity is not only dependent on the cultivar, but also very dependent on the rootstock on which it is grafted.

The characterization of drought resistance in almond cultivars and other fruit tree crops is linked to efficient water use (Yadollahi et al. 2011), together with the ability of the root system to access water. Collecting breeding material for future research provides helpful tools to reduce drought losses, but this is a lengthy process (Neale et al. 2017). However, development of improved production systems using drought resistant almonds may be possible utilizing native germplasm. These materials allow a more sustainable production, particularly in the marginal areas of harsh climate conditions found around the Mediterranean Basin (Gouta et al. 2010, 2011, 2019). This preliminary material has proven to be more efficient and resilient than wild species that, moreover, show poorer agronomical behavior (Sorkheh et al. 2009). However, an integrated approach evaluating new materials, having good yield, better nutraceutical quality and highly resistant to drought has not yet been developed as far as we know.

1.8 Nutraceutical Values

Among cultivated nuts, almonds are one of the most nutritive (Kendall et al. 2003). They are considered a good source of essential fatty acids, vitamins and minerals (Table 1.2) (Saura-Calixto et al. 1981). Also, one of the best natural sources of vitamin E (Sabate and Haddad 2001) which is purported to play a role in preventing heart disease, certain kinds of cancer and cataract formation (Kodad et al. 2006).

One ounce of almonds (20–25 kernels) contains 15% of the recommended daily values of phosphorus, 37% of vitamin E and 21% of magnesium. Almonds also represent a convenient source of fiber and folic acid. Historical uses of sweet and/or bitter almond ointment include the treatment of asthma, male-pattern baldness and as a soothing salve for burns (Gradziel and Martínez-Gómez 2013).

Almond kernels are also a source of high-quality oil, representing over 50% of the kernel dry weight. This oil is primarily composed of the more stable oleic acid, making it desirable from ancient times to the present for use as a base for various ointments and pharmaceuticals. The high level of this monounsaturated fat in almond kernels may be partly responsible for the observed association between frequent nut consumption and purported reduced risk of coronary heart disease (Fulgoni et al. 2002; Lovejoy et al. 2002).

The almond is appreciated in the processed food industry and also as a functional food with both nutritional and medical (nutraceutical) properties including nutrients, vitamins, healthy blood lipids and purported anti-inflammatory and hypocholesterolemic properties (Kodad and Socias i Company 2008; Kodad

Table 1.2 Nutrient composition of the almond kernel per 100 g fresh weight of edible portion

Nutrient	Value
Energy	578 kcal
Protein	21.26 g
Carbohydrate	19.74 g
Fiber, total dietary	11.8 g
Glucose	4.54 g
Starch	0.73 g
Calcium	248 mg
Magnesium	275 mg
Phosphorus	474 mg
Potassium	728 mg
Sodium	1 mg
Folate, total	29 mcg
Vitamin E	25.87 mg
Saturated fatty acids	3.88 g
Monounsaturated fatty acids	32.16 g
Polyunsaturated fatty acid	12.21 g

Source: Adapted from Socias i Company et al. (2007)

et al. 2011; Musa-Velasco et al. 2016; Poonam et al. 2011). The implementation of methodologies to phenotype the nutraceutical properties of traditional almond cultivars, new cultivars and breeding populations are new goals in almond breeding programs. Understanding the influence of diverse stresses such as drought and heat that can modify the composition of the beneficial components in kernels is an important goal in almond breeding. New strategies combining food science knowledge and breeding approaches must be incorporated into the actual breeding programs to obtain better food for future generations (Kodad 2017).

1.9 Development of DNA Markers

The genetic mapping of populations to segregate characters of interest has been the principal approach to develop marker-assisted selection (MAS) strategies in almond. MAS allows early selection of a large number of plants, which then undergo final evaluation to certify the desirable traits using traditional methods. For instance, the accurate phenotyping of the parents and offspring is key to identifying the molecular markers linked to disease resistance.

The analysis of cosegregation among markers and characters allows isolating the map position of major genes and quantitative trait loci (QTL) responsible for their expression. Genetic linkage analysis was initially performed in almond by combining different molecular makers including RFLPs, CGs (candidate genes), SSRs and SNPs. Regarding the genetic structure of almond mapping populations, the typically assayed populations were of type F1 or F2. These different population types have advantages and disadvantages. The work developed in F1 populations is more extensive because the populations are easier to develop than F2, given the longer period of juvenile growth. F2 populations are common in interspecific crosses in the case of peach and almond species (Donoso et al. 2016).

During the last two decades, many QTL mapping studies have been carried out. The genetic dissection of the most important agronomic traits has resulted in the identification of several different QTLs across the almond genome (Ballester et al. 1998, 2001; Donoso et al. 2016; Fernández í Martí et al. 2011, 2013; Font i Forcada et al. 2012; Rasouli et al. 2014; Sánchez-Pérez et al. 2007, 2010, 2012) (Fig. 1.4; Table 1.3). Although, in general, the QTL are distributed in all the linkage groups (LGs), a high number of QTL can be observed in LG1 of almond, with LG8 having the lowest number of QTL.

The application of these results in marker-assisted selection has been developed for blooming time and kernel flavor (Ricciardi et al. 2018; Sánchez-Pérez et al. 2010). In the case of blooming date, different publications report the use of SSR markers in a F1 population between a seedling of cvs. Tardy Nonpareil (R1000) × Desmayo Largueta (R×D), and also confirmed the location of *late blooming gene* (*Lb*) in G4 while identifying other QTLs for flowering time in G1 and G6 (Rasouli et al. 2014; Sánchez-Pérez et al. 2007) (Fig. 1.4; Table 1.3). In these studies, the SSR UDP-96003 was located very close to the *Lb* gene in G4 linkage group. When QTL

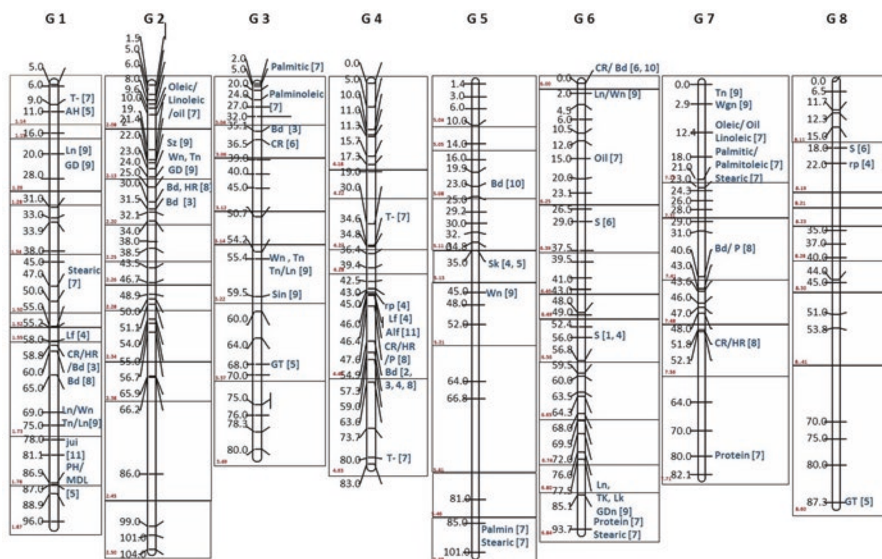

Fig. 1.4 Almond QTLs linked to traits of interest: blooming date (Bd), chilling requirements (CR), heat requirements (HR), productivity (P), self-incompatibility (S), double kernels (Dk), kernel length (Ln), kernel length/width (Ln/Wn), shell hardness (D), kernel size (Sz), spherical index (Sin), kernel thickness (Tn), kernel thickness/length (Tn/Ln), kernel weight (Wgn), kernel width (Wn), leafing date (Lf), ripening time (rp), kernel geometric diameter (GDn), amygdalin hydrolase (AH), Glucosyl transferase (GT), kernel taste (bitterness/sweet) (Sk), linoleic acid (Linoleic), mandelonitrile lyase (MDL), palmitic acid (palmitic), palmitoleic acid (palmitoleic), oil seed content (Oil), oleic acid (Oleic), prunasin hydrolase (PH), stearic acid (Stearic), tocopherol homologes (T-), total seed protein (Protein), germination date (GD), juiciness (jui) and almond fruit type (alf). A tentative scale of the map is performed in cM using as framework bin map of reference in *Prunus* indicating each bin in red. Between branches the references, 1: Ballester et al. (1998); 2: Ballester et al. (2001); 3: Silva et al. (2005); 4: Sánchez-Pérez et al. (2007); 5: Sánchez-Pérez et al. (2010); 6: Fernández í Martí et al. (2011); 7: Font i Forcada et al. (2012); 8: Sánchez-Pérez et al. (2012); 9: Fernández í Martí et al. (2013); 10: Rasouli et al. (2014); 11: Donoso et al. (2016). (Source: Figure created by Pedro Martínez-Gómez)

analysis was performed, this major QTL (*Lb*) in G4 was able to explain 50.0–86.3% of the variance. In addition, SSR markers PCeGA025, BPPCT037 and EPDCU2584 explained most of the observed variability in kernel flavor, being highly valuable for marker-assisted selection (Sánchez-Pérez et al. 2010). In addition, the markers linked to juiciness and almond-peach fruit type are also starting to be applied in breeding programs (Donoso et al. 2016). In addition, MAS for self-(in)compatibility is being performed in the routine basis in almond breeding programs using specific markers derived from transcriptomic studies, by sequencing RNAse genes associated to this trait (Sánchez-Pérez et al. 2004; Ushijima et al. 1998, 2003).

On the other hand, other traits including in-shell weight, leafing time, shell hardness, kernel length, nut width, nut length, kernel length, palmitoleic acid or tocopherol homologues were described as linked to specific molecular markers (Fig. 1.4), although until now the application of these results is still limited.

Table 1.3 Markers associated to main agronomic traits in almond

Trait	Symbol	Linkage group	Marker	References
Shell hardness	D	G2	SSR	Sánchez-Pérez et al. (2007)
Germination date	GD	G1	SSR	Fernández í Martí et al. (2013)
Blooming date	Bd	G4	RAPD	Ballester et al. (2001)
		G1, G4	SSR	Silva et al. (2005)
		G4	SSR	Sánchez-Pérez et al. (2007)
		G1, G4, G6, G7	SSR	Sánchez-Pérez et al. (2012)
		G5	SSR	Rasouli et al. (2014)
Chill requirements	CR	G1, G3, G4, G7	SSR	Sánchez-Pérez et al. (2012)
		G1, G3, G4, G7	SSR	Fernández í Martí et al. (2011)
Heat requirements	HR	G1, G3, G4, G7	SSR	Sánchez-Pérez et al. (2012)
Blooming density	Bd	G4	SSR	Sánchez-Pérez et al. (2007)
Leafing time	Lf	G1, G4	SSR	Sánchez-Pérez et al. (2007)
Reaping time	Rd	G4, G5	SSR	Sánchez-Pérez et al. (2007)
In-shell weight	Shw	G1, G2	SSR	Sánchez-Pérez et al. (2007)
Kernel weight	Kw	G1, G4	SSR	Sánchez-Pérez et al. (2007)
Kernel thickness	Tn	G4, G6	SSR	Fernández í Martí et al. (2013)
Kernel thickness/ length	Tn/L	G1, G6	SSR	Fernández í Martí et al. (2013)
Kernel width	Wn	G6	SSR	Fernández í Martí et al. (2013)
Kernel flavor	Sk	G5	SSR	Sánchez-Pérez et al. (2007)
Prunasin hidrolase	PH	G1	Candidate Gene	Sánchez-Pérez et al. (2007)
Amigdalin hidrolase	AH	G1	Candidate Gene	Sánchez-Pérez et al. (2007)
Glucosyl transferase	GT	G3	Candidate Gene	Sánchez-Pérez et al. (2007)

(continued)

Table 1.3 (continued)

Trait	Symbol	Linkage group	Marker	References
Mandelonitril liase	MDL	G1	Candidate Gene	Sánchez-Pérez et al. (2007)
Double kernel	Dk	G4	SSR	Sánchez-Pérez et al. (2007)
Kernel protein	Protein	G6, G7	SSR	Font i Forcada et al. (2012)
Kernel fatty acids	Oleic, linoleic, palmitic, palmiloleic, stearic	G2, G3, G5, G6, G7	SSR	Font i Forcada et al. (2012)
Kernel tocopherol	T	G1, G4		Font i Forcada et al. (2012)
Kernel oil	Oil	G6	SSR	Font i Forcada et al. (2012)
Compatibility	S	G6	RFLP	Ballester et al. (1998)
			SSR	Sánchez-Pérez et al. (2007)
			SSR	Fernández í Martí et al. (2011)
Juiciness	jui	G1	SSR	Donoso et al. (2016)
Almond fruit type	alph			

Also, the confirmation of the respective allelic association with phenotype must still be obtained using a large number of correctly phenotyped cultivars and progenies. This validation would indicate whether or not a marker could be used in routine screening for MAS (Boopathi 2013).

1.10 Development of RNA Markers

The transcriptome is represented by coding and non-coding RNAs, and is characterized by a huge capacity of adjusting to the developmental needs and environmental requirements of living organisms. RNA can easily change the number of copies (through increased expression or recycling) or generate diversity (by processing), thus having a tremendous impact on phenotype. RNA analysis techniques can be applied for gene functional characterization and development of new markers for specific traits. RNA markers have great potential as validation of DNA markers; they are also of great interest in monitoring process such as flowering (Prudencio et al. 2018a)

In the case of almond, targeted gene expression research has been conducted to study bud dormancy, flower development and cold acclimation (Barros et al. 2012; Prudencio et al. 2018a; Silva et al. 2007). Expression of two homeotic genes related to floral organ development, *PdMADS1* and *PdMADS3*, was found to gradually increase during ecodormancy break (Barros et al. 2012), particularly after the green-

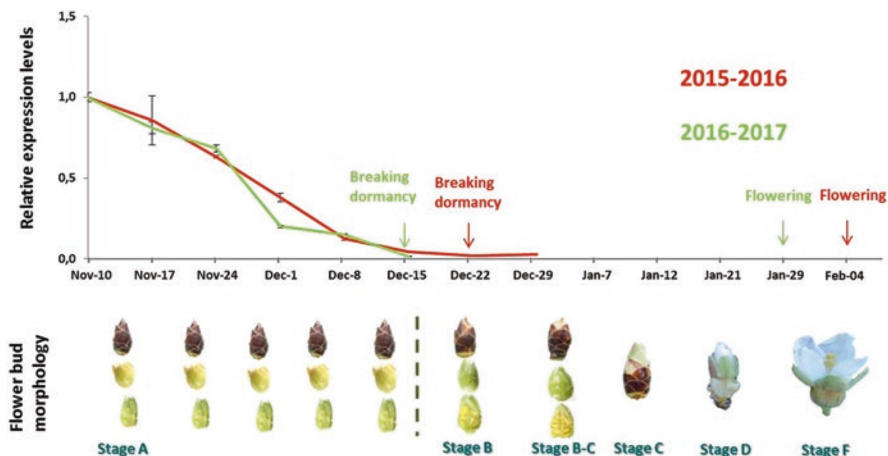

Fig. 1.5 Relative gene expression of *PdDAM6* gene evaluated by qPCR in Desmayo Largueta almond cultivar during the seasons 2015–2016 and 2016–2017 and flower morphology development. (Source: Adapted from Prudencio et al. 2018b)

tip stage (Fig. 1.5). In a previous study, *PdMADS1* expression showed to be carpel specific, agreeing with its predicted role in ovule development, whereas *PdMADS3* was expressed in all floral whorls (Silva et al. 2007).

Expression of an almond GA20-oxidase gene (*PdGA20ox*), with a predicted role in gibberellin (GA) biosynthesis was detected in floral buds before they enter winter dormancy (Silva et al. 2005), and was shown to decrease during growth resumption (Barros et al. 2012). The opposite pattern was found for almond GA2-oxidase (*PdGA2OX*), involved in GA catabolism, which increased during flower bud break. Based on these results Barros et al. (2012) proposed that a change in GA metabolism occurs after the late stages of almond flower bud break. Gibberellins are involved in the regulation of cell elongation in stamen filament as well as in cellular development in anthers, in the model plant *Arabidopsis thaliana* (Cheng et al. 2004).

Gene expression analysis of cold-responsive *PdCBF1*, *PdCBF2* and *PdDHN1* genes along seasonal development in flower buds and shoot internodes suggested a shut-down of the CBF-mediated regulatory pathway prior to blooming, in agreement with the decreased frost tolerance observed in flowers. Interestingly, CBF-specific CTR/DRE cis elements in promoters of peach *PpDAM5* and *PpDAM6* genes were also found, suggesting their association with a CBF regulon (Barros et al. 2012). Recently, Prudencio et al. (2018b) also studied the differential expression of other genes (*PdDREB2c*, *PdAWPM19, PdDAM5*) to determine dormancy break in two contrasting cultivars, finding a peak in expression correlating with bud break.

Finally, analysis of almond flower bud morphology allowed a preliminary association of anther maturation with the expression of *PdDAM6*, since transcript levels of this gene exhibited a decline when anthers were reaching full maturity (Prudencio et al. 2018b) (Fig. 1.5).

On the other hand, the understanding of transcription regulation linked to dormancy breaking is necessary for the development of new selection tools, as expres-

sion markers. Prudencio et al. (2018c), reported transcriptomes of flower buds in different stages from dormancy to active state with RNA-seq technology and informatics analysis. Experimental design included total RNA from flower bud pools of three almond cultivars with different chilling requirements for breaking dormancy and flowering time. A total of 22,833 transcript sequences were identified and 850–1710 transcripts were revealed as differentially expressed in comparisons between samples. Several candidate genes have been obtained and future validation by qPCR will help to confirm the observed expression results. Results will allow improving our knowledge about the transcriptional network of flower bud dormancy in fruit tree species, revealing differences in regulation of early and late almond cultivars. This information may be used to optimize agronomical production of consistently bred cultivars for new late flowering almonds.

1.11 Epigenetic Marks

Epigenetic modifications are heritable changes in gene expression not encoded by the DNA sequence. Actually, the epigenetic landscape has grown increasingly complicated, encompassing DNA methylation, the histone code, noncoding RNA and nucleosome positioning, along with DNA sequence (Jin et al. 2011). DNA methylation, a heritable epigenetic mark, catalyzed by the DNA methyltransferases (DNMTs), involving the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA, and is regarded as a key player in epigenetic silencing of transcription.

Genome-wide analysis of DNA methylation can be done through next-generation sequencing (NGS) technologies of bisulfite-treated DNA samples. The epigenotyping by sequencing (epiGBS) technique was developed and recently implemented to study bud dormancy in almond for the first time (Prudencio et al. 2018d). The strength of the technique is evident for the discovery of epigenetic variants, based on 5mC, that are genotype-dependent. In addition, the DNA methylation (5mC) pattern is generally genotype-dependent rather than dormancy state-dependent. Comparative DNA methylation studies of modern almond cultivars released from actual breeding programs and heirloom cultivars will surely contribute to the knowledge of methylation variants and provide candidate epialleles linked to agronomic traits. Such polymorphisms can be screened in large populations using NGS to confirm the locus methylation state associated with a given character of interest.

In this first study, results showed the identifications of different genes where DNA methylation state changed between the dormant and active state of the flower buds. This was possible in both the traditional early-flowering genotype Desmayo Largueta and the extra-late-flowering genotype Penta from the CEBAS-CSIC almond breeding program. Furthermore, common genes arose from the analysis (Prudencio et al. 2018d).

In the future, it would be interesting to improve the technique coverage to obtain a greater representation of the genome. Ultimately, the results will be an essential complement to RNAseq experiments in bud dormancy progression.

1.12 Genetic Transformation

The development of powerful new biotechnologies can also advance almond breeding efforts through the direct incorporation of genes using in vitro culture and genetic transformation. Genetic transformation can reduce the time and space required to improve fruit and tree characteristics compared with classical breeding methodologies. Genetic transformation can also increase the diversity of genes and germplasm available by allowing the stable integration of foreign DNA into the genome. In the case of fruit trees such as almond, genetic engineering represents an alternative to overcome obstacles of traditional breeding programs (long juvenility period, self-incompatibility, evaluation of agronomic traits in the field) (Archilletti et al. 1995). In addition, new genetic transformation approaches have the potential of success across species of *Prunus*; *P. domestica* has already been successfully transformed (Petri et al. 2018), and will serve a solid basis for the future implementation of genome editing of other *Prunus* species.

Almond transgenic plants have been created via *Agrobacterium*-mediated transformation because of its efficacy (Archilletti et al. 1995, Costa et al. 2007; Miguel and Oliveira 1999). Much of this research involved the introduction of reporter genes in seedling tissue. The gus gene, which codes for b-glucuronidase, has been successfully utilized as a marker gene in genetic transformation of almond (Archilletti et al. 1995; Miguel and Oliveira 1999). In addition, the *nptII* (neomycin phosphotranspherase) gene, which confers kanamycin resistance, has been used in this species (Archilletti et al. 1995). While almond seedlings are readily transformed using *Agrobacterium*-mediated approaches, the regeneration of plantlets from established cultivar cells has proven very difficult (Archilletti et al. 1995; Miguel and Oliveira 1999). This difficulty is believed to be due to the recalcitrance of cultivar cells to initiate the required organogenesis, presumably because they have lost their juvenility with their advanced clonal age (Costa et al. 2007; Santos et al. 2009). In addition, while new genetic engineering techniques offer significant advantages for the discrete addition of new genes to commercially- established cultivars, the current dearth of transgenes useful to tree crop breeding limits their present application. A final barrier is social: the reluctance of the economically-important European and Asian markets to accept transgenic fruits and nuts.

1.13 Conclusions and Prospects

Originating from Central and Southwest Asia, almond was easily dispersed widely by seed, becoming an important crop worldwide but being mostly grown commercially in California, the Mediterranean Basin, and other countries such as Australia. Due to the natural high self-incompatibility and the fact that almond was, until recently, mostly multiplied by seed, the crop exhibits very high variability for many traits. Variability is obviously an advantage for the species and for the breeders; however, it is not appreciated by farmers who want the best traits in their production

orchards. Breeding for improved traits is always difficult without an easy system to monitor the process, and proper phenotyping often requires extended and difficult analyses. In this sense, the ability of breeders to generate large populations is almost unlimited; the management, phenotyping (genetic studies) and selection of these seedlings are the main factors limiting the creation of new cultivars. The development of molecular markers associated with traits of interest (such as self-incompatibility, blooming time, kernel flavor) has been a major benefit to breeders, but there is still much to be done, as there are not enough good molecular markers available to meet all of the targeted-breeding goals. Genomic (DNA) and transcriptomic (RNA) and genetic engineering studies for the development of marker-assisted selection (MAS) strategies are particularly useful when the evaluation of the character is expensive or time-consuming or, in a tree crop such as almond, which has a long juvenile period. Recent progress has been made in epigenetic marks for dormancy in flower buds. These strategies will help to accelerate development of new cultivars with desired traits with improved adaptation to, or mitigate the effects of, global climate change. Adaptation to extreme weather conditions, such as high temperatures or alternative period of droughts and floods, new pathogens and pests are some of the goals of these new strategies to increase production in a scenario of global climate change. One such strategy is called *breeding by design*, based on the combination of precise tools (MAS, MAB) and the development of extensive phenotyping.

New genetic transformation approaches have the potential of success across species of *Prunus*. Currently, only *P. domestica* has been successfully transformed (Petri et al. 2018). This is a prerequisite for future implementation of genome editing in *Prunus* species. Genome editing technology has been satisfactorily used to edit the genome of several plants, animals, bacteria and yeast; however, its application in woody species is scarce and limited to those having whole genome sequences and efficient transformation systems (Fernández í Marti and Dodd 2018). Belonging to this group of species are Chinese white poplar, *Populus tomentosa* Carr. (Fan et al. 2015), orange, *Citrus* × *sinensis* (L.) Osbeck (Jia and Wang 2014), Duncan grapefruit *C. paradisi* Macf. (Jia et al. 2016) and apple, *Malus* × *domestica* Borkh. (Nishitani et al. 2016). Precise knowledge about the genetic basic of all agronomic traits should enable breeders to deploy more rational and refined strategies in the near future of almond breeding. Philosophically, the future of science by design in *Prunus* breeding will be based on maximizing the prediction of the different internal and external variables and in a subsequent prescription of better solutions for the different breeding approaches. The success of this philosophical theory will promote effectiveness, viability and suitability in the future design of new cultivars with improved traits more rapidly than ever.

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Appendices

Appendix I: Research Institutes Dedicated to Almond Breeding

Institution	Specialization	Contact information and website
Center for Edaphology and Applied Biology of the River Segura (CEBAS-CSIC), Murcia, Spain	Molecular Biology and Breeding	Dr. Federico Dicenta Email: fdicenta@cebas.csic.es ww.cebas.csic.es
Agrifood Research and Technology Centre of Aragón (CITA), Zaragoza, Spain	Molecular Biology and Breeding	Dr. Maria J Rubio Email: mjrubioc@aragon.es www.cita-aragon.es/
Institute of Agrifood Research and Technology (IRTA-Mas Bove), Reus, Spain	Breeding	Dr. Ignasi Batlle Email: ignasi.batlle@irta.es www.irta.cat/es/centre/irta-mas-bove/
University of California-Davis (UC Davis), California, USA	Breeding	Dr. Thomas M Gradziel Email: tmgradziel@ucdavis.edu www.ucdavis.edu
University of Adelaide, Australia	Molecular Biology and Breeding	Dr. Michelle Wirthensohn Email: michelle.wirthensohn@adelaide.edu.au www.adelaide.edu.au/
University of Bari, Italy	Breeding	Dr. Marino Palasciano Email: marino.palasciano@uniba.it www.uniba.it
Ecole National d'Agriculture Meknès, Morocco	Breeding	Dr. Ossama Kodad Email: osama.kodad@yahoo.es www.enameknes.ac.ma/
Olive Tree Institute, Tunisia	Breeding	Dr. Hassouna Gouta Email: zallaouz@yahoo.fr www.iosfax.agrinet.tn/

Appendix II: Almond Genetic Resources Available at Germplasm Banks

Country	Research center	Cultivation area	Accessions
Europe			
France	INRA-Avignon	Avignon	180
Italy	University of Udine	Udine	65
Spain	CEBAS-CSIC	Murcia	70
	CITA	Zaragoza	80
	IRTA-Mas Bove	Reus	83
Asia			
Iran	National Plant Gene Bank	Karaj	67
Syria	Centre for Studies of Arid Zones	Sednaya	130
Turkey	Ege University	Izmir	51
America			
USA	USDA-UC Davis	Davis	165
Africa			
Morocco	INRA-Rabat	Rabat	120
Oceania			
Australia	University of Adelaide	Adelaide	45

Source: http://www.fao.org/genetic-resources/

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Chapter 2
Genetic Diversity and Breeding of Argan Tree (*Argania spinosa* L. Skeels)

Naima Ait Aabd, Rachid Bouharroud, Abdelghani Tahiri, Ahmed Wifaya, Abdelaziz Mimouni, and Abdelhamid El Mousadik

Abstract *Argania spinosa* (L.) Skeels is an endemic woody species in Morocco with important ecological and socioeconomic interest. The argan tree constitutes an essential element of the biodiversity of agroforestry ecosystems. To alleviate the pressure on the species and to satisfy the growing demand for its oil, the preservation and characterization of the high diversity of endangered populations of argan trees and its cultivation (in their natural habitat), constitute a crucial step toward their conservation. Thus, the analysis of the structure and distribution patterns of genetic diversity is paramount for the management and development of conservation strategies. Thereby, various strategies have been employed to evaluate the degree of genetic diversity based on morphological, chemical, biochemical and molecular markers. The status of different morphotypes and molecular markers were examined and investigated to evaluate the measure of genetic diversity. The application of molecular biotechnologies (DNA markers) to practical breeding and selection is a novel strategy and a powerful methodology for plant breeding. This chapter summarizes current knowledge and progresses made in argan tree, and discusses their limitations and perspectives related to the genetic aspects of this ecological and socioeconomically important tree crop.

Keywords Argan oil · Biotechnology · Cultivation · Germplasm · Molecular markers · Socioeconomic

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2.1 Introduction

Argan, or argane, tree (*Argania spinosa* (L.) Skeels) is a forest species of arid and semiarid areas of southwestern Morocco where it covers approximately 952,200 ha (FAO 2014). It belongs to the monospecific genus *Argania*, a unique representative of the Sapotaceae family in Morocco (Pennington 1991) where it grows mostly in the dry lowlands of the Souss Valley and three small relict populations north of Oued Grou (near Rabat), northeast of the Beni Snassen Mountains (northern Oujda) and south of Goulimime (Ehrig 1974; Prendergast and Walker 1992). In Algeria, an area of natural argan forest is represented by more than 90,000 ha in the Tindouf Region (Kechairi 2009; Kechairi and Abdoun 2013).

This species is multipurpose in nature with all the plant parts used for oil and agro-sylvo-pastoral use and constitutes an essential element of the biodiversity of forest ecosystems and the income of the indigenous population (Msanda et al. 2005). The argan tree occurs in its ecosystem in the form of trees and shrubs with different architectural aspects (upright form, weeping form, very or less spiny branched) and various forms of fruits and seeds (oval, rounded and fusiform) (Ait Aabd et al. 2011, 2012; Bani Aameur 2004). The seeds contains kernels used to extract oil, with a yield range of 60–70%, suitable for food and cosmetic purposes, with nutritional and dietary value confirmed by its richness in polyunsaturated fatty acids of more than 80% (Charrouf and Guillaume 1999; Lizard et al. 2017; Schmid and Zülli 2012). Khallouki et al. (2003) concluded that argan oil can enhance the cancer prevention effects of the Moroccan diet because of its content of tocopherol, squalene and oleic acid are high compared to other food oils.

Argan contributes to the socioeconomic development of the local population. However, productivity shows a great inconsistency in time and space adding to its current exploitation in the wild. Flowering begins with the first rains in December and may continue until April and into the summer season in some cases (Benlahbil et al. 2015).This diploid species (2n = 24) (Majourhat et al. 2007) has a predominantly allogamous reproduction, where anemophilous (80%) and entomophilous (20%) pollination occurs (M'hirit et al. 1998).

In fact, the cumulative effects of overgrazing, the dry climate, the weak natural regeneration and anthropomorphic actions have accentuated the degradation of the argan forests. In addition, overexploitation of the argan tree is irrational, especially given the currently high demand for argan oil from the international market. As a result, the conservation of argan ecosystems becomes a priority, especially since UNESCO declared the argan ecosystem as the Arganeraie Biosphere Reserve in 1998. In this context, there is an urgent need for applied research to better understand the quantitative and qualitative potential of the argan tree in order to know and reproduce the performant trees and provenance. Genetic diversity research is necessary for adaptation to climate change and for long-term survival of this species.The analysis of the structure and distribution patterns of genetic diversity is accordingly important for the management and development of conservation strategies (Chakhchar et al. 2017; Hamrick and Godt 1996). For that reason, various strategies have been employed to evaluate the degree of genetic diversity based on

morphological, chemical, biochemical and molecular markers. Thus, its phenotypical variability using agro-morphological charactershas been importantly focused on by several researchers (Ait Aabd et al. 2011, 2012, 2013; Bani Aameur and Ferradous 2001; Benlahbil 2003; Ferradous 1995; Metougui et al. 2017; Zahidi et al. 2013). The authors have consequently used several phenological traits related to trees, flowers, leaves, fruits, seeds and kernels of the argan tree originating from various geographic areas. These studies have showed a remarkable phenotypic variation in their results, attributable to the diverse natural conditions of the studied populations. These research results were influenced by plant adaptations to different climatic and ecological environments rather than genetics. Biochemical characterization studies have been mainly focused on oil content and fatty acids. Thus, a broad variation was found for most of the studied parameters of argan oil in their agroclimatic conditions (Ait Aabd et al. 2013, 2014; Alaoui et al. 1998; Bani Aameur et al. 1999; Charrouf and Guillaume 1999; Charrouf et al. 2002; Harhar et al. 2014; Sparg et al. 2004). Even so, these characters can be useful and exploited in breeding programs. Ait Aabd et al. (2014) also drew attention to breeding the species for oil production; studies using the agronomic performance of accessions established in the collection of elite genotypes. To assess the extent of genetic diversity and to establish conservation strategies for argan germplasm requires gauging phenotypic diversity using the markers related to agronomic performance and oil yield; however, they have limited application. This is due to the influence of environmental factors along with simple Mendelian inheritance of morphological traits in the argan forest. Regarding all aforementioned elements, phenotyping characteristics cannot be sufficient to assess the levels of genetic variability, but it is recommended as a first step before starting DNA-based studies (Hoogendijk and Williams 2001). Therefore, it is also prudent to combine phenotypic analysis with molecular markers because of their neutrality to environmental factors (Bernatsky and Tanksley 1986; Gepts 1993).

The assessment of genetic diversity using DNA markers has proved to be more accurate and reliable. The first investigations studied the isozymes markers and the chloroplast DNA using PCR-restriction fragment length polymorphism (RFLP) (El Mousadik and Petit 1996a, b) followed by studying the random amplified polymorphic DNA (RAPD) (Bani Aameur and Benlahbil 2004; Majourhat et al. 2008). Recent studies use the single sequence repeats (SSR) or microsatellites (El Bahloul et al. 2014). The inter-simple sequence repeats (ISSR) markers have often been successfully used to evaluate the genetic diversity of many species (Ait Aabd et al. 2015; Mouhaddab et al. 2017; Yatrib et al. 2015). Molecular genetic have now been widely used to address ecological and conservation issue for several forest species. Many molecular markers have been developed within species (specific markers) and other transferred into different populations for genetic analysis. Currently, there are several molecular methods (SSR, ISSR, sequence characterized amplified region (SCAR) and single nucleotide polymorphism (SNP)) used to analyze the genetic variability of the argan tree.

The aim of this work is to collect, analyze and discuss the available data about the argan tree addressing several aspects including selection, cultivation, molecular breeding as well as potential applications such as propagation and biodiversity conservation.

2.2 Natural Selection and Cultivation

A forest is an ecosystem from which rural populations meet a large part of their food and energy needs, especially in low-income countries. All implemented strategies to conserve forest would be more beneficial if we have the maximum amount of data regarding the multipurpose trees species and how they impact social and economic aspects of these classes of population.

The argan tree is the most original woody species with multiple uses in North Africa, thanks to its botanical and ecological interest and its social value. Its wood gives an excellent charcoal but its main interest lies in the extracted oil from the seeds which is consumed as food. The foliage is used by to feed goats and sheep during most of the year.

The unique quality of the argan tree is also expressed in its high indifference to the lithological nature of soils; this species can grow in all types of soil such as sand, dolomite, clay and shale.

The argan tree can be regenerated naturally by seeds and shoots. However, regeneration by seeds becomes more and more impossible because the fruits are regularly harvested by the local populations and any seedlings (if there are any) are grazed by livestock, especially goats. Fortunately, the Moroccan government enacted laws to prohibit hunting the Barbary ground squirrel (*Atlantoxerus getulus*). The squirrel has special feeding habits characterized by the collection of argan fruits and storing them in *safe* places (buried). When trying to break fruits, the squirrel leaves some fruits that may be difficult to open, and these seeds germinate in the protected area of the forest. The squirrel's actions qualify as seed scarification.

For many centuries, the argan forest has undergone two kinds of natural selection: environmental and anthropomorphic. The first one leads to the interactions of soil and climate, but the second is more destructive than constructive. Depending on the area, the argan trees has been grazed and cultivated or simply exploited as a forest species. In regard to the environmental selection, some populations become individualized. In fact, two types of argan ecosystems can be distinguished: orchard and forest. In the orchard system, the tree density is very low in order to allow intercropping for conventional agriculture. The forest ecosystem is found in uncultivated lands, especially in coastal and hilly mountainous areas.

In order to domesticate *Argania spinosa* in the Negev desert, Nerd et al. (1998) studied the phenology and pollination parameters of argan trees. In this study, the trees were fertigated which showed a unique flowering period during spring compared to other studies carried out in Morocco (Ferradous et al. 1995) where the flowering period was observed across the crop season. The argan tree flowers were protogynous and 4 pollination treatments performed (auto-self, hand-self, hand cross, open pollination) showed that pollinator vectors (insects, wind) were necessary to complete the pollination process. This observation was confirmed during fruit set which was more important for cross- and open pollination (6–9%). The fruits were ripe 9 months after anthesis, exhibiting a bisigmoidal growth curve. The

insect pollinators identified during this study belonged to 3 families (Syrphidae, Alleculidae and Calliphoridae).

2.3 Argan Tree Propagation

The argan tree is a multipurpose species endemic to the southwestern Morocco and represents the basis of a traditional agroforestry system. However, the area of natural occurrence in Morocco has been reduced by half due to heavy animal and anthropomorphic pressure, deforestation, uncontrolled urbanization and more recently climate change (Charrouf and Guillaume 2008; Lybbert et al. 2011; Martin 2012). The restoration of this ecosystem requires the implementation of in-situ and ex-situ conservation of the genetic resources of the argan tree and the creation of high-performing varieties in terms of yield and quality product through the selection and mass propagation of genotypes with the capability to grow rapidly and produce a quality oil in sufficient quantity. Several conventional vegetative multiplication trials have been carried out to accelerate the mass production of trees and to conserve this species.

2.3.1 Conventional Propagation

Propagation of the argan tree by seed is a classic method of sexual reproduction that remains very limited by the risk of variability within the population, due to its allogamous reproduction mode, and also the extension of the juvenile phase before reaching maturity and flowering, which are strongly affected by environmental conditions, such as light intensity, temperature and soil moisture (Alouani and Bani-Aameur 2004; Benaouf et al. 2016; Msanda 1993; Nouaim et al. 2002). Seed germination is a very complex biological phenomenon which requires a good understanding and control of the underlying factors. According to the literature, argan seed germination is difficult because of the combined effects of low seed viability and dormancy, considered limiting factors in the propagation of argan (Al-Menaie et al. 2007; Alouani and Bani-Aameur 2004; Zahidi and Bani-Aameur 1997). Both germination and the first stages of seminal development are critical periods in the establishment of argan, and the few trials of seed production in the nursery are very heterogeneous in their success (Alouni and Bani-Aameur 2004). Different tests have been carried out and it has been observed that the germination of the argan seed requires nocturnal temperatures above 21 °C (Zahidi and Bani-Aameur 1997). In addition, a combination of cold storage, scarification of the seed, soaking in water at different temperatures, gibberellic acid and fungicide treatments seem to reduce dormancy and increase the rate of germination (Al-Menaie et al. 2007; Alouani and Bani-Aameur 1999; Nouaim et al. 2002). The produced seedlings exhibited a tap root system with a fast and powerful development but the

emission of rootlets along the main axis is limited. These seedlings require acclimation before placing them under natural conditions. Currently, the natural regeneration of argansis very scarce because its seeds are collected for oil.

Through selection and mass production of trees with desirable characteristics, biotechnology could improve argan tree productivity, as well as overall production. Currently, several techniques of vegetative propagation of argan plants are in use, including herbaceous cuttings and grafting techniques (Bellefontaine et al. 2010; Ferradous et al. 2011). These techniques offer the opportunity to multiply selected genotypes and to provide a significant step toward domestication (Nouaim et al. 2002).

The first vegetative propagation trials reported on the argan tree date back to the 1970s and focused on cuttings (Platteborze 1976). Cuttings have been used to multiply many argan trees and work on this technique has shown that the lignification of stem cuttings from mature trees are still difficult to root (Fig. 2.1a). Therefore, cutting is usually restricted to juvenile material because ageing reduces the ability of cuttings to root (Nouaim et al. 2002). These traditional propagation methods remain time consuming, difficult, laborintensive and limited by low rooting success (30%) and growth rate of the root system (Fig. 2.1b). Its success remains dependent on several parameters such as: the genotypic effect, the age of the mother plant, the nature of the cutting, and the nature and concentration of usedauxins (Bousselmame et al. 2001; Mokhtari and Kerbernes 2008; Mokhtari and Zakri 1998). Most of the roots obtained by cutting in argancome from the periphery of the cutting (at the callus) and are therefore fragile. Also, seedlings from cuttings under controlled conditions will dry out once transferred to field conditions which leads to a search for other safe techniques (Mokhtari 2002).

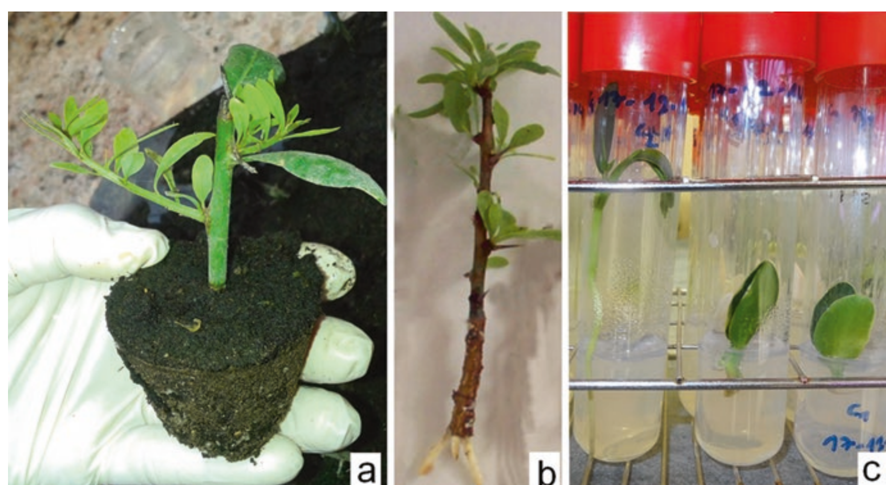

Fig. 2.1 Argan tree (*Argania spinosa* L. Skeels) vegetative propagation. (**a**) Sprout elongation of mature semi-hardwood stem cuttings. (**b**) Cutting root primordia initiation and adventitious root initiation. (**c**) In vitro germination of argan seeds on MS medium

Grafting of the argan tree consists of bringing together the performances of the scion and the benefits of the rootstock (Fig. 2.1c). This technique is the most adapted to the multiplication of the argan tree and is considered successful when contact between the vascular tissues is established (Mokhtari 2002). In addition, this technique offers the possibility of maintaining the performance of grafts (selected argan clones) and allows keeping the advantages of sowing (long roots that draw deep water) conveyed by the rootstock. Different types of grafts have been tested in the argan tree (budding, lateral and apical perforation, apical cleft). The simple apical cleft graft and the lateral or apical perforation graft are the easiest and give better results. However, the other forms of grafting dry out or become detached (Mokhtari 2002). Therefore, recent studies demonstrated that the graft/rootstock interaction is still a limitation to the technique (Mokhtari et al. 2011; Taoufiq et al. 2011).

2.3.2 Micropropagation and Microcutting

Facing the limits of conventional multiplication methods, in vitro culture could be a useful technique for the mass production and preservation of selected genetic material under controlled and pathogen-free conditions (Justamante et al. 2017). This technique seems to have the greatest potential in the micropropagation of argan, minimizing the influence of the environment and the seed covering and facilitating rooting (Nouaim et al. 2002).

The integration of in vitro culture techniques within a program that aims to propagate the argan tree is recent and also rare. In vitro regeneration potential of *Argania spinosa* plants has been studied. Initial cultures were established using different explant types and culture media that vary in their mineral and growth regulators compositions. Thus, in vitro germination of argan seeds has been tested previously using different media based on the MS medium (Fig. 2.1d) (Murashige and Skoog 1962) together with auxin-type hormones such as 1-naphthalene acetic acid (NAA) or indolebutyric acid (IBA), with varying success (Bousselmame et al. 2001; Justamante et al. 2017; Martínez-Gómez et al. 2007).

Microcutting seems to be more successful for *Argania spinosa* than organogenesis. Studies of cuttings (herbaceous and semi-woody) lead to various types of responses depending on the auxin/cytokinin concentration. The results obtained for the induction and proliferation phase are encouraging, but efforts are still needed to ensure rooting and a good plant survival rate. Indeed, the results reported by some authors have shown that growth of microcutting from juvenile plant material stopped at the early stages of culture (Aaouine and Bazagra 1990). However, microcutting from mature trees is characterized by a low rooting rate of not more than 30% and adventitious root formation seems to be difficult and strongly genotype-dependent (Bakkali 1997). In a study by Nouaim et al. (2002), the rooting rate of microcutting was better when Terra Green (calcinated clay) was used as a substrate instead of agar. This indicates that the rooting phase of the argan tree is very sensitive to the physical properties of the substrate rather than its chemical composition. Auxiliary

bud growth of argan was obtained on MS medium, supplemented with 0.5 mg/LBA and the shoot elongation took place on MS containing GA (0.1 mg/L). However, rooting (small and very fragile) was reached, at a very low level, on an induction medium (MS) containing 5 mg/L of NAA and IBA each (Naas and Ziani 2008).

For organogenesis, most works undertaken have led mainly to obtaining calli and/or roots without new bud induction or somatic embryo formation (Chakeur and Yousfi 2007). Thus, additional research is needed to improve this technique that represents a fundamental step towards ex-situ conservation of selected germplasm of this endangered species.

2.4 Germplasm Biodiversity

Genetic diversity within species reflects its evolutionary potential. This diversity is best appreciated when studied in its natural ecosystem. The genetic diversity of the argan tree has been studied for many years ago using agro-morphological traits to analyze the status of different morphotypes and molecular markers to evaluate the organization of genetic diversity. Recently, Ait Aabd et al. (2011, 2012, 2013, 2014), evaluated argan germplasm represented by 150 genotypes from 5 different ecogeographic areas by measuring 6 qualitative and 16 quantitative traits over 3 years to determine if morphological traits could be useful for genotypic identification. Likewise, most of the traits studied have an economic interest especially those related to tree, fruit, seed, kernel and the oil yield and its chemical composition. The high degree of variation between genotypes could be useful and helpful in the identification and evaluation of interesting genotypes for selection and breeding programs. Therefore, targeted selection for these traits and elite selections in particular for oil production would not be difficult.

In fact, this variability appears at first sight at the landscape-scale of the argan at different aspects: tree shape, ramification, density of thorns, leaf polymorphism, alternation in phenology and fruiting (Fig. 2.2). Based on the phenotypic characters of tree appearance, the shape of the argan trees has been split into three groups: a weeping type with flexible drooping non-spiny branches (Ehrig 1974; Rieuf 1962; Sauvage and Vindt 1952), upright type highly spiny (Miege 1952) and an intermediate type. For various agro-morphological traits studied (Ait Aabd et al. 2011, 2012), 29 combinations from 150 genotypes studied among tree shape (upright, weeping, intermediate), fruit form (rounded, oval, fusiform), seed form (rounded, oval, fusiform); the results related to the morphotypes are summarized in Table 2.1. It is possible to define several combinations for each genotype group and to highlight the candidate trees for each desired criteria. This variability seems to be distributed randomly among populations. The spiny upright form appears to be related to ecogeographic conditions (abiotic stress) unlike the non-spiny weeping form. However, in the same population, the coexistence of the two forms can be observed, which reinforces the hypothesis of the existence of fixed forms genetically adapted to the same local conditions. In addition, vegetative multiplication trials of these

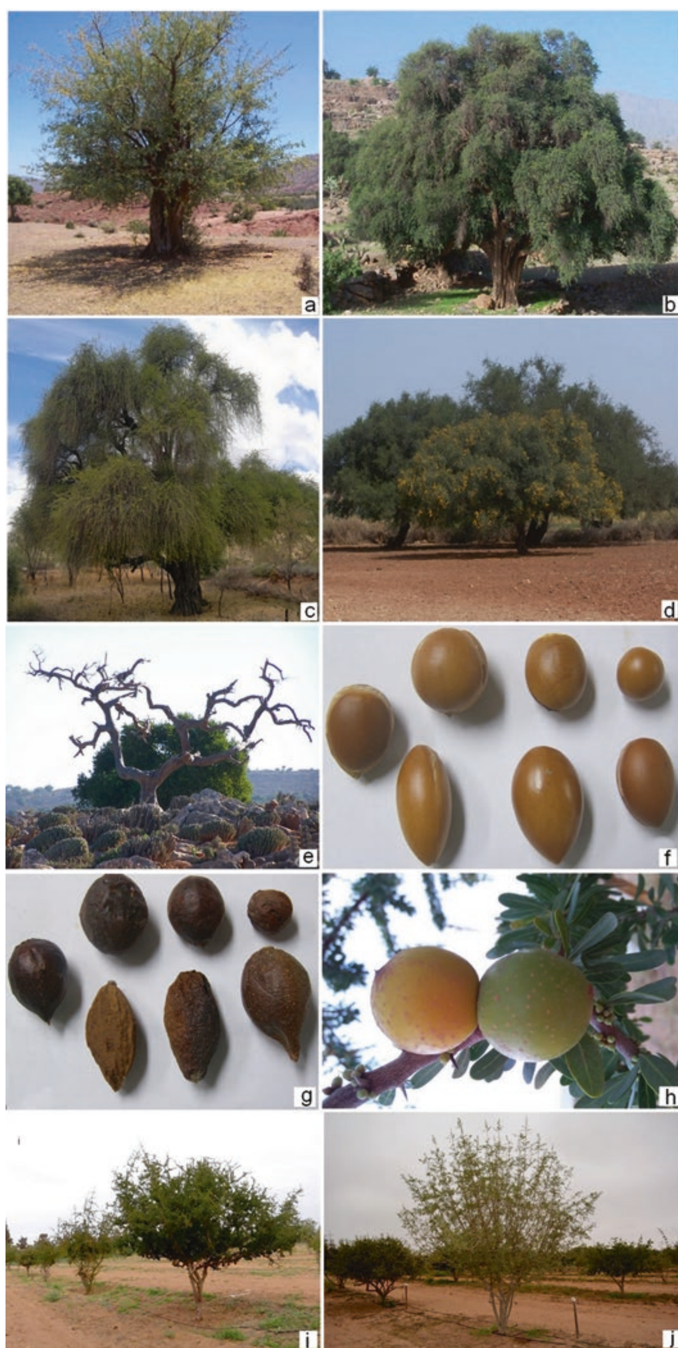

Fig. 2.2 Habitat and morphological variability of tree and seeds of *Argania spinosa*. (**a**–**e**) Naturally growing trees in forest ecosystem, (**f**) Seed form diversity, (**g**–**h**) Fruits of argan tree with distinct morphological variations, (**i**–**j**) Orchard of domestication test of the argan tree

Table 2.1 Morphotypes of argan (*Argania spinosa*) tree observed in five populations studied in Souss-Massa (pop. 1,3,4, 5) and Essaouira (pop. 2) regions, Morocco

Morphotype	Possible combinations				Number of morphotypes/ population					% of morphotype existing in 150 evaluated argan trees
	Tree form	Fruit form	Seed form	Seed crushing	Pop 1[a]	Pop 2	Pop 3	Pop 4	Pop 5	
M1	Upright	Oval	Oval	Easy	13	2	5	5	6	20.67
M2	Upright	Oval	Oval	Medium	–	–	2	1	–	02.00
M3	Upright	Oval	Oval	Difficult	3	1	–	1	–	03.33
M4	Upright	Oval	Rounded	Easy	–	–	–	–	1	00.67
M5	Upright	Oval	Rounded	Medium	–	–	1	–	–	00.67
M6	Upright	Oval	Rounded	Difficult	–	1	–	–	–	00.67
M7	Upright	Rounded	Oval	Easy	1	7	–	4	3	10.00
M8	Upright	Rounded	Oval	Medium	–	–	2	–	–	01.33
M9	Upright	Rounded	Oval	Difficult	2	1	1	1	–	03.33
M10	Upright	Rounded	Rounded	Easy	3	3	11	3	11	20.67
M11	Upright	Rounded	Rounded	Difficult	1	1	2	1	1	04.00
M12	Upright	Fusiform	Oval	Difficult	–	–	–	2	–	01.33
M13	Upright	Fusiform	Fusiform	Easy	2	2	2	1	2	06.00
M14	Upright	Fusiform	Fusiform	Medium	–	–	–	3	–	02.00
M15	Upright	Fusiform	Fusiform	Difficult	1	–	–	2	–	02.00
M16	Weeping	Oval	Oval	Easy	1	1	–	2	1	03.33
M17	Weeping	Oval	Oval	Difficult	–	2	–	–	–	01.33
M18	Weeping	Rounded	Oval	Easy	–	2	–	1	1	02.67
M19	Weeping	Rounded	Rounded	Easy	–	2	–	–	2	02.67
M20	Weeping	Rounded	Rounded	Difficult	–	–	–	–	1	00.67
M21	Weeping	Rounded	Fusiform	Easy	–	1	–	–	–	00.67
M22	Weeping	Fusiform	Fusiform	Easy	–	1	–	–	1	01.33
M23	Weeping	Fusiform	Oval	Easy	–	–	–	1	–	00.67
M24	Semi-weeping	Oval	Oval	Easy	1	–	–	–	–	00.67
M25	Semi-weeping	Oval	Oval	Medium	–	3	–	–	–	20.00
M26	Semi-weeping	Rounded	Oval	Easy	–		2	1	–	02.00
M27	Semi-weeping	Rounded	Oval	Difficult	1	–	–	–	–	00.67
M28	Semi-weeping	Rounded	Rounded	Easy	–	–	2	1	–	02.00
M29	Semi-weeping	Fusiform	Fusiform	Easy	1	–	–	–	–	00.67

(−): absent of morphotype

[a]pop. 1, pop. 3, pop. 4 and pop. 5 described as populations from the Souss-Massa region and pop. 2 from Essaouira Region

morphotypes and controlled hybridizations between the spiny and weepy non-spiny upright form are needed to elucidate the genetic determinism of this trait.

In relation to fruit traits, several studies have shown that the effect of a season, locality, genotypes and their interactions were very highly significant (Ait Aabd et al. 2011, 2012; Bani Aameur and Ferradous 2001; Ferradous 1995). Six typological forms of fruit (fusiform, oval, rounded, globose, drop, ovate apiculate) and three seed forms (fusiform, oval, rounded) were determined. The most abundant and distinct forms at the ripe stage are oval, rounded and fusiform. Moreover, these different forms (fruits) appear to be independent of environmental conditions and have been used as tools for several studies looking for a possible correlation with oil yield and chemical composition (Ait Aabd et al. 2013; Belcadi et al. 2008; Gharby 2012; Mallah et al. 1995; Nouaim et al. 2007). The results of Ait Aabd et al. (2013) showed that there is a very significant effect of tree (genotype) on oil yield which makes selection more focusable on tree rather than region or population. The shape of the tree, fruit and seed forms did not have a direct link with the oil yield or its chemical composition as reported by Gharby (2012). The variation of oil yield range 38–63% at the level of 150 trees (Ait Aabd et al. 2013) and these extreme values have never been cited in the literature. These findings encourage continued searching to identify elite trees. As for the factors that influence this yield, this study showed that the oil yield is a strongly heritable trait ($H^2 > 93\%$) (Ait Aabd et al. 2011, 2012). The differences observed in oil yield at intra- and inter-population levels have highly significant genetic effect. Indeed, many studies have shown that oil yield is an inherited trait influenced by the additive effects of genes (Kaushik et al. 2007; Mudassar et al. 2009). In addition, the principal component analysis showed that fruit weight, seed and kernel weight and size are correlated with oil content and there is no clear correlation between oil content and geographic origin. The uni- and multivariate analyzes used has shown that the geographical origin was not related to oil yield. Highly productive genotypes can be found either in the plains or in the mountains, and this variation does not follow any elevation gradient and the independent relationship between oil content and the shape of trees, fruits and seeds were noted. For all evaluated genotypes, it can be noted that at least 25% exceed the average in oil yield after over 3 years of evaluation, which corresponds to the preselection of 31 genotypes (elites). Opening up new avenues for future multi-clonal comparative trials comes as a result (Table 2.2, Appendix II).

The estimation of heritability in the broad sense of the quantitative traits related to fruit, seed and oil yield show that the latter is highly heritable (Ait Aabd et al. 2011, 2012). The genetic parameters that allow making a choice of highly heritable characters are very useful in the process of selection. The analysis of fatty acid proportions by chromatography was carried out on 20 elite trees from 4 populations. The research by Ait Aabd et al. (2013) aims to understand the variability of chemical composition in fatty acids which is organized according to different hierarchical levels (genotype origin, season and their interactions). This study has also investigated for a correlation between the proportions of fatty acids and morphological characters (shape of trees, fruits, seeds) on the one hand and the geographical location of trees on the other hand. Nine fatty acids were therefore detected and selected

Table 2.2 Thirty one elite Moroccan genotypes preselected from 4 provenances over 3 years

Cultivar (or germplasm resources)	Important traits							Geographical location	
	Morphotype	Seed crushing	Thorns	Bloom period[a]	Kernel weight/90 fruits (g)	Fruit production/ tree (kg)	Oil yield (%)	Population	Province
Ao-4V	M1	Easy	Thorny	M	19	100	55.03	Aoulouz	Taroudant
Ao-7V	M1	Easy	Thorny	E	20	110	53.92	Aoulouz	Taroudant
Ao-11V	M13	Easy	Very thorny	M	22	60	53.42	Aoulouz	Taroudant
Ao-4R	M1	Easy	Slightly thorny	M	28	60	53.90	Aoulouz	Taroudant
Ao-7R	M1	Easy	Very thorny	L	26	60	55.07	Aoulouz	Taroudant
Ao-11R	M15	Difficult	Thorny	M	28	200	54.33	Aoulouz	Taroudant
Ao-12R	M1	Easy	Thorny	M	22	200	56.22	Had Dra	Essaouira
Hd-1V	M25	Medium	Thorny	M	17	100	54.71	Had Dra	Essaouira
Hd-2V	M10	Easy	Thorny	E	28	100	54.10	Had Dra	Essaouira
Hd-3V	M7	Easy	Thorny	M	18	40	57.01	Had Dra	Essaouira
Hd-4V	M7	Easy	Slightly thorny	M	27	150	54.19	Had Dra	Essaouira
Hd-3R	M26	Easy	Thorny	M	24	45	56.20	Had Dra	Essaouira
Hd-7R	M16	Easy	Slightly thorny	E	21	70	56.19	Had Dra	Essaouira
Hd-8R	M7	Easy	Thorny	M	22	200	58.40	Had Dra	Essaouira
Hd-9R	M13	Easy	Thorny	M	14	20	57.87	Had Dra	Essaouira
Hd-13R	M7	Easy	Thorny	E	27	50	55.85	Had Dra	Essaouira
Bi-8V	M10	Easy	Slightly thorny	E	20	120	54.20	Biougra	Chtouka Ait Baha
Bi-10V	M12	Difficult	Thorny	E	24	70	52.29	Biougra	Chtouka Ait Baha
Bi-12V	M7	Easy	Thorny	L	17	40	55.55	Biougra	Chtouka Ait Baha
Bi-15V	M23	Easy	Slightly thorny	M	28	200	53.11	Biougra	Chtouka Ait Baha

Bi-4R	M1	Easy	Thorny	E	17	50	54.95	Biougra	Chtouka Ait Baha
Bi-8R	M14	Medium	Thorny	E	31	80	53.54	Biougra	Chtouka Ait Baha
Bi-10R	M1	Easy	Very thorny	E	19	60	55.32	Biougra	Chtouka Ait Baha
Bi-13R	M10	Easy	Slightly thorny	M	29	160	53.31	Biougra	Chtouka Ait Baha
La-1V	M10	Easy	Slightly thorny	L	22	120	56.33	Lakhsas	Tiznit
La-2V	M1	Easy	Slightly thorny	L	14	120	55.58	Lakhsas	Tiznit
La-3V	M20	Difficult	Not thorny	E	22	220	58.17	Lakhsas	Tiznit
La-6V	M1	Easy	Thorny	M	33	80	55.23	Lakhsas	Tiznit
La-11V	M10	Easy	Thorny	M	24	240	54.43	Lakhsas	Tiznit
La-7R	M13	Easy	Thorny	M	20	130	54.52	Lakhsas	Tiznit
La-9R	M1	Easy	Thorny	M	25	100	55.06	Lakhsas	Tiznit

[a]Early E (March–May), Medium M (June–August), Late L (October–December)

for study including 4 saturated fatty acids (myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and arachidic acid (C20:0), 3 monounsaturated fatty acids (palmitoleic acid (C16:1), oleic acids (C18:1) and eicosenoic acid (C20:1)) and 2 polyunsaturated fatty acids (linoleic acid (C18:2) and linoleic acid (C18:3)). Two fatty acids were very predominant (oleic acid (45.40%) and linoleic acid (34.05%) and represent 79.45% of the total fatty acids contained in the analyzed oils. Palmitic acid and stearic acid were ranked second with 13.12% and 6.23%, respectively. Palmitoleic acids (0.11%), linolenic (0.11%) myristic (0.15%), eicosenoic (0.32%) and arachidic (0.37%) were very minor and are not representative of fatty acids in argan oil (Ait Aabd et al. 2013). Other research reported relatively the same findings for other geographic areas (Charrouf 1984; Charrouf and Guillaume 1999, 2008; Gharby et al. 2011; Nerd et al. 1994). The high variability observed was related to the tree effect in the population. To our knowledge, no study has investigated the factors promoting the differentiation of chemical constituents in argan trees. Moreover, no significant correlation was noted between the morphotypes studied and the oil yield or with the percentage of fatty acids. However, a significant correlation was stated between the longitude and the variations in monounsaturated fatty acids [(C18: 1), (C18: 2), (C18: 3) and (C20: 1)] and the elevation gradient with polyunsaturated fatty acids [(C18: 2), (C18: 3)].

2.5 Molecular Breeding

The initialresearch using PCR-RFLP chloroplast DNA to identify haplotypes of this endangered species was done by El-Mousadik and Petit (1996b). Another analysis used random amplified polymorphic DNA (RAPD) markers (Bani Aameur and Benlahbil 2004), and simple sequence repeats (SSR) markers (Majourhat et al. 2008) to characterize the most common morphotypes. However, the sample sizes used were a limiting factor in the analysis of genetic diversity for these kinds of studies. Recently, ISSR, SCAR and SNP have been used to analyze the genetic diversity in plant species. These molecular markers are known to be neutral, dominant or codominant, and detect different levels of polymorphism according to their specificity.

In 2014, microsatellite loci were developed and characterized using an ultra-high-throughput sequencing-based approach which provided the first codominant markers specific to *Argania spinosa* (El Bahloul et al. 2014). In this study, 150 argan trees were genotyped with 11 SSR markers;which were selected and showed a high ability to detect polymorphism between or among analyzed genotypes. Therefore, developed markers constitute valuable tools for evaluation of genetic diversity and to conserve the germplasm insituorexsitu. Furthermore, Ait Aabd et al. (2015) studied the genetic diversity of argan tree using ISSR markers (Fig. 2.3). This work is the first application of the ISSR markers to assess the genetic diversity in argan accessions from 5 different ecogeographic populations in Southwest Morocco. In this study, ISSR analysis using 9 primers generated higher polymorphism compared

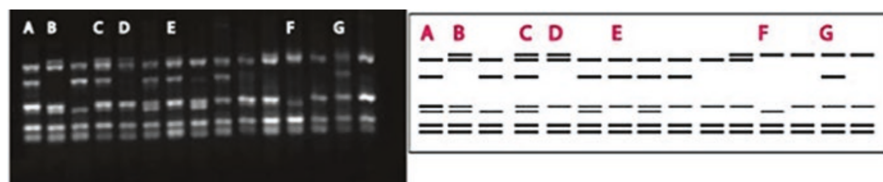

Fig. 2.3 ISSR amplification profiles using primer 3/8 (**a**), detailed of ISSR profile of 15 genotypes of pop 5 (**b**). (Source: Ait Aabd et al. 2015)

to other molecular markers such as isozymes (El Mousadik and Petit 1996a) and RAPD (Bani Aameur and Benlahbil 2004; Majourhat et al. 2008).

The genetic diversity of *Argania spinosa* at the population level was considered high when compared to various endangered and endemic forest species using various ISSR markers. Analysis of molecular variance (AMOVA) provides high levels of genetic diversity within argan populations, where 28% of the genetic variation existed among the populations and 72% within the populations. Gst coefficient of genetic differentiation was high (0.22) and the level of gene flow (Nm) was 1.78, indicating a low or limited rate of gene exchange among provenances. ISSR, SSR and AFLP markers were used also to analyze the genetic diversity and population structure of *A. spinosa* in its natural area and a larger number of samples are required to expand this study. Thus, this study assesses the genetic diversity and population structure of 480 trees of *A. spinosa* growing in 24 different ecogeographic regions of Morocco.

The objective of this research was to draw a map of genetic diversity and population structure and to develop a core collection for the conservation of the argan tree. Therefore, this study used 7 ISSR markers to amplify a total of 340 individuals and the samples were collected from 17 argan populations distributed in 3 major bioclimatic regions under arid and semiarid climate (Yatrib et al. 2015). A relatively high and low level of genetic diversity was revealed, respectively, at the species and population level, and the relatively low level of genetic differentiation (Gst = 0.39) revealed was in agreement with the results obtained by the AMOVA analysis which was further confirmed by a limited gene flow (*Nm* = 0.781) among populations. The Mantel test showed no significant correlation between genetic and geographic distance, indicating that the geographic distribution is not the major factor determining the present population genetic structure (Yatrib et al. 2015). These researchers reported that the genetic diversity within and among 13 populations (130 genotypes) of argan tree was studied using AFLP markers with 2 sets of primer combinations, one specific for small genome and the other specific for regular genome (Pakhrou et al. 2016). In fact, the regular genome primer combinations have generated 477 AFLP loci. AMOVA showed that 19% of the genetic variation was partitioned among populations and 81% of the genetic variation was within populations. This was confirmed by the coefficient of gene differentiation between populations (Gst = 0.22), and gene flow was estimated to 1.709.

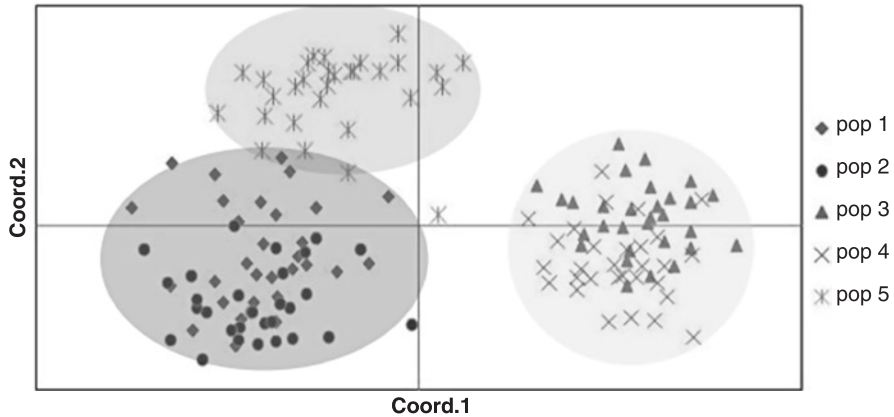

Fig. 2.4 Principle Coordinate analysis based on Nei's (1978) genetic distances. (Source: Ait Aabd et al. 2015)

The principal coordinate analysis (PCoA), unweighted pair group method with arithmetic mean (UPGMA) and STRUCTURE analysis revealed that populations of *Argania spinosa* were clustered into 3 genetic groups (Fig. 2.4). These results can be explored in the design of in-situ and ex-situ conservation and management programs (Pakhrou et al. 2016). To achieve the ultimate aim of the argan project, Mouhaddab et al. (2017) assessed the genetic diversity of 480 wild *A. spinosa* populations from 24 different geographical regions at elevations of 58 to 1250 m, using 4 polymorphic simple sequence repeat (SSR) markers and 10 inter simple sequence repeat markers (ISSR). The results showed a high level of genetic differentiation among populations (Gst = 0.44). Cluster analysis based on UPGMA, PCoA, and Bayesian-based analyses, showed that the entire populations were divided into 2 main groups for both markers used. Based on genotyping data of ISSRs and SSRs, the core collections were established by 13 and 96 genotypes representing, respectively, 2.7% and 20% of the entire *A. spinosa* collection (Mouhaddab et al. 2017). Indeed, these results were obtained from mass collection for each population.

The genetic variation of the argan tree is not only caused by environmental factors, but also by individual genetic differences. This implication was supported by the result of the PCoA, UPGMA and Bayesian-based approach (Ait Aabd et al. 2015; Mouhaddab et al. 2017; Pakhrou et al. 2016; Yatrib et al. 2015)and also showed that there was no clear geographical trend among wild populations of *Argania spinosa*. Moreover, the Mantel test between the diversity matrix based on genetic and geographic isolation revealed a nonsignificant relationship ($r = 0.063$; $p < 0.05$), indicating that geographic isolation was not the main factor inducing genetic diversity. Likewise, the genetic structure of plant populations is influenced by various evolutionary process interactions including the long-term evolutionary history of the species such as habitat fragmentation, population isolation, mutation, genetic drift, gene flow, breeding system and selection (Gabrielsen et al. 1997; Hamrick and Godt 1996; Schaal et al. 1998; Slatkin 1987).

According to Hogbin and Peakall (1999), in a breeding system, genetic drift or genetic isolation of populations can cause a high level of genetic differentiation among plant species populations. In general, the genetic structure of argan tree could be attributed to population isolation and breeding system. Thus, the argan tree is outcrossing and its pollen dispersed mostly by insects and wind (Bani Aameur et al. 1999). However, gene flow via seed dispersal shows the importance of pollen dissemination in the argan tree, when comparing the coefficients of genetic nuclear differentiation (Gstn) and cytoplasmic (Gstcp) (El Mousadik and Petit 1996b), dissemination via animals (ruminants) and some rodents would be also determining the space genetic structuring of argan. Moreover, these results, which show that the argan tree has a highly differentiated genetic structure in space, lead to consideration of the cost and effort to be likely for any preservation and/or conservation strategy of genetic diversity in situ. In addition, it can be inferred that a more detailed analysis of several other populations is reasonable because more than 20% of the diversity is distributed among populations.

To the best of our knowledge, this is the first report about the application of a combination between the molecular-marker technique and agro-morphological evaluation, to select elite genotypes according to the desired criteria for argan species domestication. In general, most of the selected accessions are clustered in a way that is consistent with the traditional classification and the morphological characteristics (Fig. 2.5). This report is designed to serve as a model for describing and assessing the variability of certain socioeconomic traits for a potential program for domestication and management of the genetic resources diversity of the argan tree. Related to the existence of a possible relationship between the molecular profiles and certain agro-morphometric characteristics, it is clear that this relationship is independent of the shape of the trees, ranging from upright to weeping, or fruits and seeds according to rounded, oval or fusiform forms, or the level of ease of crushing, and for oil yield (Fig. 2.5). Indeed, all combinations are possible and are present in all sources, but in different proportions. Characterization of interesting genotypes that are above average for oil yield values, such as ease of seed crushing, encourages further study. It is obvious there is a need to widen the genetic base of departure for the selection or for the domestication of argan.

Until now, the only published work on the typology of fruits in relation to molecular markers (SSR and RAPD) is that of Majourhat et al. (2008), noting that the lack of any correlation between the three forms of fruit (rounded, oval, fusiform) with the genotypic profiles observed. In the light of the results obtained, the evaluation of genetic variability for adaptive traits with socioeconomic interest should be conducted separately from genetic markers. The latter are especially useful for managing the diversity still available in an in-situ conservation strategy of the most representative genetic structures. The combination of its two types of morpho-biometric and molecular (genetic) markers could be exploited in selection and crossbreeding programs aimed to try the useful of mineral fertilization and for cultivar identification. This strategy could benefit from the existence of very polymorphic markers allowing the study of segregations between parents and descendants.

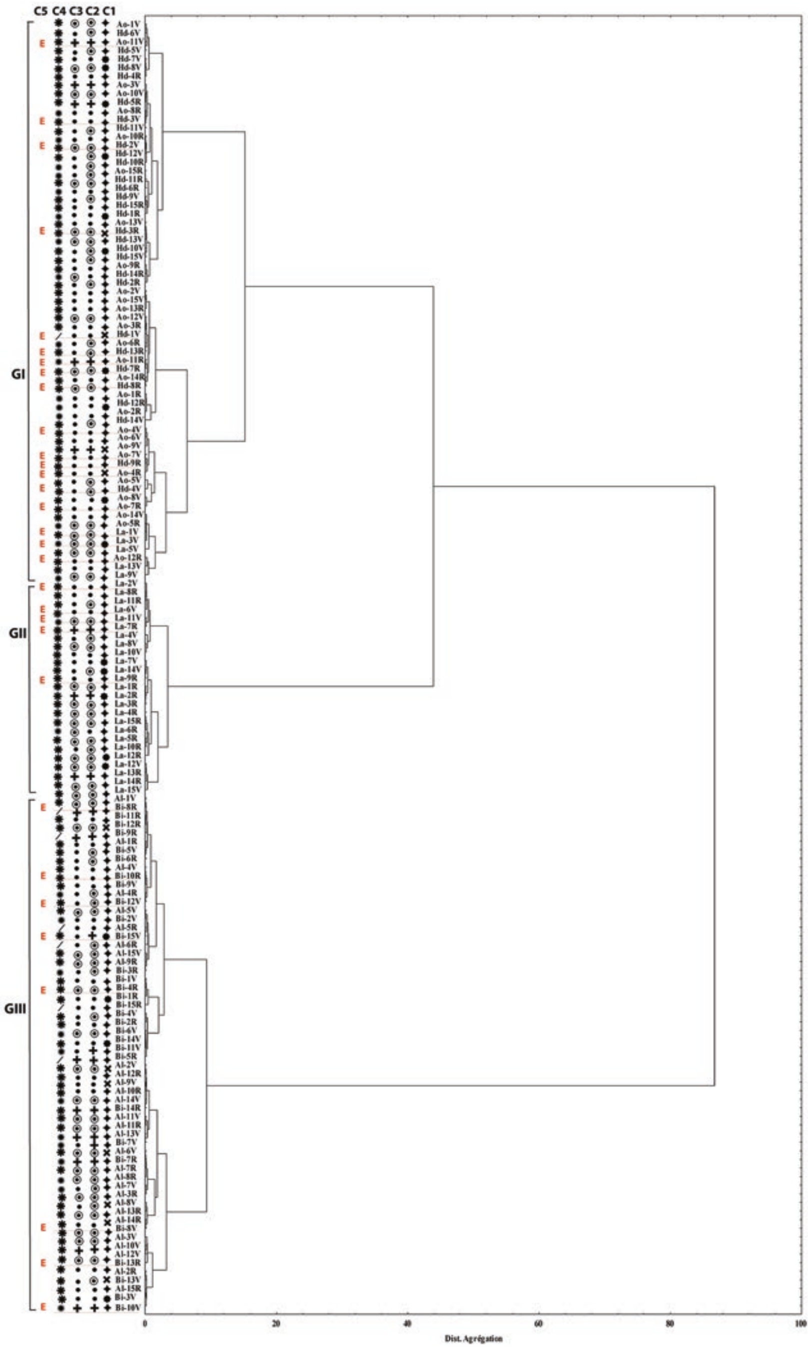

Fig. 2.5 UPGMA dendrogram of 150 argan trees belonging to 5 populations based on ISSR markers. C1: shape of tree (✦ upright; ✹ weeping; ✕ Semi-weeping);**C2–C3**: Form of fruit and seed (● Oval; ⦿ rounded; ✚ Fusiform); **C4**: Crushing of the seed (❋ Easy; / Way; ✷ Difficult). **C5**: Elite trees selected with higher oil content over 3 years

2.6 Conclusions and Prospects

The high diversity which exists and has been studied of the natural distribution of argan should be explored in order to design in-situ and ex-situ conservation strategies and management programs. There is hence a great challenge for conservation of *Argania spinosa* as a wild forest species, especially in the source of regeneration plants using vegetative propagation (cuttings and grafting) or seedlings. Some breeding specialists are against vegetative multiplication using unselected genotypes. Thus, the regeneration of the argan tree from seed produces a heterogeneous orchard where the potential of each tree is unknown. The creation of intensive orchards of fruit trees requires the planting of homogeneous varieties, which are stable and have high production potential. At present, these conditions are not yet met in the argan tree. Nevertheless, some research work has been done on various programs related to the creation of argan varieties.

Anthropogenic pressure and climate change have reduced natural argan ecosystems. The development of an argan crop (arganiculture) is the ultimate goal anticipated by the strategies of the Morocco Green Plan with different national decision-makers and the scientific community to preserve this genetic patrimony. Currently, the breeding research program is undertaking at the National Institute of Agronomic Research and elite trees are growing at the experimental farm of INRA of Agadir (Belfaa locality). The elite trees are cultivated under greenhouses and in open fields (Fig. 2.6). In addition, the selection component based on good production (fruit and quality) and stress tolerance has been well developed. Also, safeguarding and reforestation measures have been implemented. On the one hand, agronomic characterization and interaction analysis help to distinguish genotypes with forest potential and, on the other hand, to preselect genotypic structures that could constitute a genetic basis favorable to the selection of stable and highly-productive cultivars. Likewise, the first place of associations with mainly discontinuous characters (absence of thorns, regularity of interannual production, shape of seeds and ease of seed crushing) remains more attractive for stability and control homogeneity tests in comparative tests of progeny or clones from vegetative propagation and can deal with this diversity using good conservation strategies.

The argan tree has been designated as part of a protected geographic area by a national law published in the Official Bulletin 5806, January 21, 2010. By law the new common nomenclature of argan was replaced by *argane*.

Fig. 2.6 Orchard of domestication testing of argan trees. (**a**) test of the effect of plant density on growth and development. (**b–d**) Elite trees cultivated in open fields at the experimental farm of the National Institute of Agronomic Research (INRA) Agadir, Belfaa Locality

Appendices

Appendix I: Institutwes Involved in Argan Research

Institution	Specialization research activities	Contact information and website
INRA, Morocco	Genetic diversity, breeding, propagation, pollination and cultural practices	www.inra.org.ma Address: Avenue Ennasser, Rabat, Morocco
Ibn Zohr University, Agadir, Morocco	Genetic diversity	www.uiz.ac.ma Address: BP32/S, 80000, Agadir, Morocco
Agronomic and Veterinary Institute HASSAN II, Morocco	Propagation and cultural practices	www.iav.ac.ma Address: Madinat Al Irfane, B.P. 6202. Rabat, Morocco

(continued)

Institution	Specialization research activities	Contact information and website
Mohamed V University, Rabat, Morocco	Genetic diversity	www.um5.ac.ma Address: Avenue des Nations Unies, Agdal, Rabat, Morocco
Cadi Ayyad, University, Marrakesh, Morocco	Genetic diversity	www.uca.ma Address: Av Abdelkrim Khattabi, BP 511, 40000, Marrakesh, Morocco
National Agency for the Development of Oasis Areas and Argan	Promoting all research activities related to argan tree	www.andzoa.ma Address: Nouveau quartier administratif, Haut Founty, Agadir, Morocco
National Forestry School of Engineers, Salé, Morocco	Management and conservation of the biosphere reserve of argan ecosystem	www.etudiant.ma/etablissements/enfi Address: ENFI, BP. 511 Tabrikt, 11000 Salé, Morocco
Forest Research Center	Management and conservation of the biosphere reserve of argan ecosystem	www.eauxetforets.gov.ma Address: 25, bd Brahim Roudani, Parc de la Ligue Arabe, Casablanca, Morocco
French Agricultural Research Centre for International Development (Cirad)	Forest genetics	www.cirad.fr/en Address: Av. Agropolis, 34398 Montpellier Cedex 5, France
Kuwait Institute for Scientific Research	Aridland agriculture and greenery	www.kisr.edu.kw Address: P.O. box 24885 Safat, 13109, Kuwait
Abou BakerBelkaid University	Forest resources	www.univ-tlemcen.dz Address: 22, Rue Abi Ayad Abdelkrim Fg Pasteur B.P 119 13000, Tlemcen, Algeria
Hassiba Benbouali, Chlef University	Agronomy	www.univ-chlef.dz Address: B.P 78C , Ouled Fares Chlef 02180, Algeria
Instituto de Bioingeniería, Universidad Miguel Hernández	Bio-energy	www.bioingenieria.umh.es Address: Av. de la Universidad s/n.03202 Elche, Spain
Ben-Gurion University of the Negev	Life sciences	http://in.bgu.ac.il Address: P.O.B. 653 Beer-Sheva, Israel
Faculty of biological sciences, University of Science and Technology Houari Boumediene	Plant ecology	www.usthb.dz Address: University of Science and Technology Houari Boumediene, BP 32 El-Alia 16111 Alger, Algeria
Institut d'Études Politiques de Lyon	Economics of sustainable development	www.sciencespo-lyon.fr Address: 14 Avenue Berthelot, 69007 Lyon, France

Appendix II Genetic Resources of Argan in Morocco

Cultivar[a]	Important traits			Cultivation location
	Seed crushing	Fruit production (kg)	Oil yield (%)	
Ao-4V	Easy	100	55.03	Aoulouz
Ao-7V	Easy	110	53.92	Aoulouz
Ao-12R	Easy	200	56.22	Had Dra
Hd-2V	Easy	100	54.10	Had Dra
Hd-4V	Easy	150	54.19	Had Dra
Hd-8R	Easy	200	58.40	Had Dra
Bi-8V	Easy	120	54.20	Biougra
Bi-15V	Easy	200	53.11	Biougra
Bi-13R	Easy	160	53.31	Biougra
La-1V	Easy	120	56.33	Lakhsas
La-2V	Easy	120	55.58	Lakhsas
La-11V	Easy	240	54.43	Lakhsas
La-7R	Easy	130	54.52	Lakhsas
La-9R	Easy	100	55.06	Lakhsas

[a]Unregistered cultivar in the official catalog

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Chapter 3
Brazil Nut (*Bertholletia excelsa* Bonpl.) Breeding

Aisy Botega Baldoni, Lúcia Helena de Oliveira Wadt, and Cássia Ângela Pedrozo

Abstract Brazil nut (*Bertholletia excelsa* Bonpl.), member of the Lecythidaceae family, is a native monotypic tree species which occurs in the Amazon Region and is important for its economic, social and environmental values. The tree bears fruit containing seeds known as Brazil nuts, Pará nuts or Amazon nuts. Most production comes from the natural forest with the yield of fruits and seeds highly variable from year to year. Brazil nut cultivation has yet to reach significant levels, due to propagation problems and to the lengthy period of time before the tree reaches maximum production levels; in addition, until now low productivity has been experienced in cultivated trees. Studies to increase overall production, precocious production and improvement of nut qualities are prime objectives of Brazil nut cultivation. For a perennial species, predominantly cross-pollinating and dependent upon specific pollinators, information about genetic diversity is the first step on which to base future programs of improvement. Genetic studies of molecular markers are contributing to basic knowledge of the tree and to its conservation, which are important to guarantee the economic value and perpetuation of the species.

Keywords *Bertholletia excelsa* · Breeding · Conservation · Genetic diversity · Molecular markers · Production

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3.1 Introduction

3.1.1 Botanical Classification and Distribution

The Brazil nut (*Bertholletia excelsa* Bonpl.) is a tree species embedded in the culture of the indigenous communities of the Amazon Region. It is an endemic species distinguished by its economic, social and environmental values. The tree bears large fruits containing multiple seeds which are the Brazil nuts (castanhas do Brasil) of commerce; also referred to as Pará nuts or Amazon nuts. The Portuguese gave their term for *chestnut*, *castanha*, to the common name of the tree and its nuts. *Bertholletia excelsa* belongs to the botanical class Angiospermae, subclass Magnoliopsida, order Lecythidales and family Lecythidaceae (Ribeiro et al. 1999). The genus *Bertholletia* was considered to be within the Myrtaceae family until 1825, when the Lecythidaceae was given familial status and *Bertholletia* placed in it. The Brazil nut tree is monotypic, with only a single species, although in the past botanists disagreed about its classification (Mori and Prance 1990a). Souza (1963) referred to this divergence and commented that botanically Brazil nut is classified as *Bertholletia excelsa*; although Miers in 1874 described another species collected in Amazonas State which he named *B. nobilis*. Nevertheless, Mori and Prance (1990a) argued that such a classification was based only on characteristics that are not valid to differentiate species of Lecythidaceae, and considered *B. nobilis* to be a synonym of *B. excelsa*.

According to Corrêa (1931), *Bertholletia excelsa* is described as follows: *trunk cylindrical, smooth and unbranched to the crown; bark dark and fissured; branches curved at their ends; leaves sparse, alternate, petiolated (the petiole cylindrical-canaliculated), oblong or oblong-ovate, short acuminate, undulating, dark green, glabrous on the upper surface and pale beneath, glaucous, reticulate-veined and with a medial vein slightly velvety on the upper part.*

The tree occurs in non-flooding (*terra firme*) forests throughout the Amazon Basin (Prance 1990). The environmental conditions where this species naturally grows are limited to areas of annual rainfall of 1400–2800 mm year^{-1} and an annual water balance deficit for 2–7 months (Diniz and Bastos 1974). In the Eastern Amazon, studies report that *Bertholletia excelsa* is clumped in *groves* of 50–100 adults (Mori and Prance 1990a; Müller et al. 1980; Peres and Baider 1997). More recently Scoles et al. (2011) reported average adult densities of 6.3–9.2 trees ha^{-1}. In Acre State, Brazil and the Madre de Dios Region, Peru, in the western Amazon, populations are distributed irregularly with densities of 1.01 adult trees per ha^{-1} (Wadt et al. 2008); 0.50 adult trees ha^{-1} (Thomas et al. 2017) and 0.54–0.75 trees ha^{-1} (Rockwell et al. 2015).

At maturity, Brazil nut trees are canopy emergent, with an estimated lifespan of 500–1000 years and can reach a height of 50 m (Camargo et al. 1994; Mori and Prance 1990a; Vieira et al. 2005). The tree is monoecious and self-incompatible (O'Malley et al. 1988; Wadt et al. 2015).

Brazil nut tree flowers are arranged in erect terminal panicles, measuring about 3 cm in diameter, and are zygomorphic pedunculate with 6 fleshy petals, white,

grayish white or ocherous white. They have 80–130 stamens; the stigma may be long or short (Corrêa 1931; Moritz 1984). Each flower has 5–6 locules, with a total of 20–25 ovules (Maués and Oliveira 1999).

The fruit is a type of seed capsule (a pyxidium) globose-depressed, nearly spherical, the remainder of the calyx being visible on the upper portion; the shell is thick, woody, hard, brown in color and replete with resinous cells (Corrêa 1931). Fruits measure 10–12.5 cm × 10–12.5 cm, but can reach 16 × 14 cm (Mori and Prance 1990a), and weight 1–2 kg, each containing approximately 10–25 seeds (Mori and Prance 1990a; Zuidema 2003). They reach maturity and synchronously fall on average 12–14 months after successful pollination (Maués 2002; Reis 2015). Fruit production is highly variable among individuals (Kainer et al. 2007), and variable from year to year (Kainer et al. 2014). Seeds remain inside the fallen fruits until extraction by humans or animals.

3.1.2 Importance

The extracted nuts (botanically, the seeds) from natural populations are collected by families living in or near tropical forests in Bolivia, Brazil and Peru, constituting almost the sole supply to satisfy the over USD $166 million international market in unshelled Brazil nuts (FAOSTAT 2013). Uniquely, Brazil has increasingly diverged from its historical and nearly sole focus on international markets; almost three-fourths of the 2016 Brazilian harvest, valued at USD $30 million (IBGE 2016), went to internal domestic markets (Toledo et al. 2016). For harvesters, who almost exclusively extract nuts from sustainable sources, protected areas or indigenous lands, Brazil nut income can represent as much as 43% of total annual household income (Duchelle et al. 2011). This long-lived forest giant is often a key component of conservation strategies, particularly in modern-day protected areas across the Amazon Basin (Guariguata et al. 2017).

According to the Brazilian Institute of Geography and Statistics (IBGE 2016), Brazil nuts rank second in importance among commercial nonwood forest product in Northern Brazil, only exceeded by açaí (*Euterpe* spp.) fruits. Around the world, Brazil nuts are known for their high nutritional value, being rich in protein, fiber, vitamins, lipids, minerals and elements which act as antioxidants, most notably selenium (Kornsteiner et al. 2006; Prance and Mori 1979). This trace element is recommended in the prevention of cancer and cardiovascular diseases, but there are instances that eating a single Brazil nut may exceed the recommended daily intake of selenium, according to studies conducted at the Federal University of Lavras (Embrapa 2016).

Brazil nuts are eaten directly as a snack food or consumed as an ingredient in processed products such as chocolate bars, cakes, bisquits, sweet deserts and in mixed nuts (Collinson et al. 2000; Ferberg et al. 2002). The nuts are also utilized to extract oil and in the production of *Brazil nut milk*, the latter a component in certain regional dishes (MMA/SUFRAMA/SEBRAE/GTA 1998).

3.2 Cultivation and Traditional Breeding

3.2.1 Current Agricultural Challenges

Production of Brazil nuts in Brazil in 2016 amounted to about 35,000 mt (IBGE 2016), the majority of this production coming from forest extraction (*extractivismo*) carried out by families in the Amazon Region, an activity that serves as an important income source for thousands of families of that region (Angelo et al. 2013; CIFOR 2009; Enriquez 2008). Nevertheless, sustainable forest extraction faces serious challenges, not only with respect to social and ecological aspects of the activity, but also in economic terms (Aguiar 2014).

Brazil has been losing world market share in the production and quality of Brazil nuts produced, compared to Peru and Bolivia. The latter country is the world's current leader in both production and exports of Brazil nuts (FAO 2016), although Peru is gaining with respect to the export market (Figs. 3.1 and 3.2).

Since the early 1990s in Brazil, production of Brazil nuts has experienced periods of decline and recovery, but with a general downward trend up to the 2000s, when the production curve began to ascend (Fig. 3.1).

The principal bottleneck being experienced in the Brazil nut market has been and continues to be, in part, unsanitary processing conditions, principally leading to aflatoxin caused by fungi and fecal coliform contamination. It is necessary to make significant efforts to modify and improve all of the production steps. This represents not only a local issue but also to meet international export standards which threaten the economic survival of the activity.

In 2003, the European Union (EU) banned the import of unshelled Brazil nuts from Brazil because of contamination as a result of unsanitary conditions of the production process. In reaction to this, the Ministry of Agriculture, Livestock and

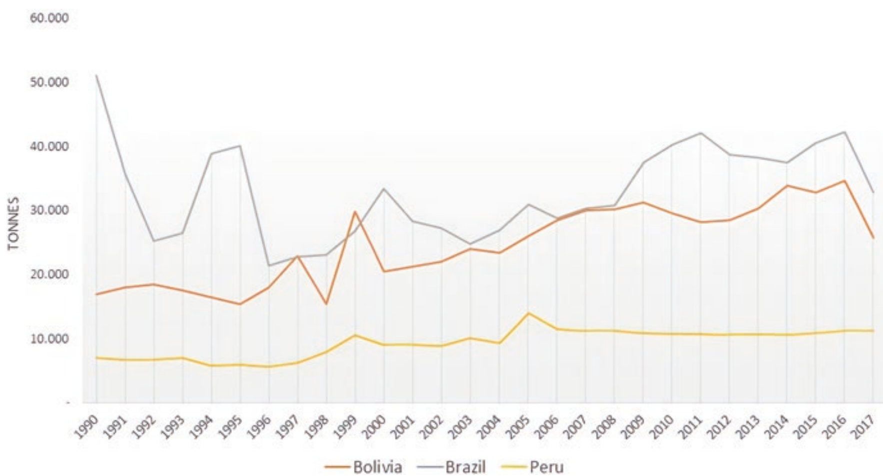

Fig. 3.1 History of unshelled Brazil nut production by Brazil, Peru and Bolivia 1990–2016. (Source: FAO 2016)

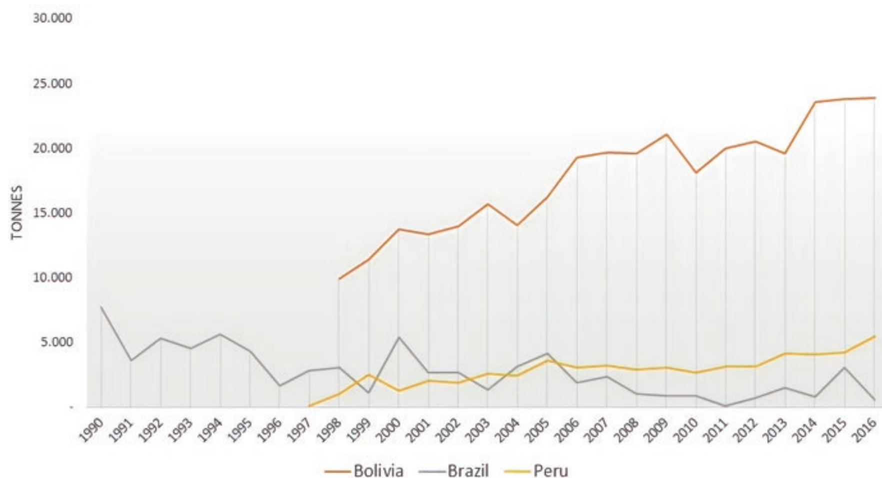

Fig. 3.2 History of unshelled Brazil nut exports by Brazil, Peru and Bolivia 1990–2013. (Source: FAO 2016)

Food (MAPA) issued a series of processing standards with the objective of regulating sanitary certification of the product, which included detailed procedures for each stage of processing and export shipment (Wadt and Kainer 2009).

Studies of Brazil nut mycotoxins by Calderari et al. (2013) and Taniwaki et al. (2017) provided basic information to develop strategies for contamination control of the product. This led to the establishment of national and international regulations regarding the maximum acceptable limits of mycotoxin in the Brazil nuts. Based on these results, Brazil succeeded in having the Codex Alimentarius FAO-WHO (www.fao.org/fao-who-codexalimentarius/about-codex/en/) modified to increase the maximum levels of total aflatoxins to 15 μg per kg of product before processing, and to 10 μg of total aflatoxins per kg of the retail product. The EU then revised its regulations accordingly permitting the resumption of Brazil nut imports (Alisson 2017).

Within the scope of the National Project for Public and Private Actions for Biodiversity (PROBIO II), a set of directives and technical recommendations were promulgated for best management practices in the organic and sustainable extraction of Brazil nuts (MAPA/ACS 2012). This initiative complemented public policies to foster more productive forest extractives in Brazil. These objectives are reflected in the National Plan for the Promotion of Product Chains and Socio-biodiversity (PNPSB), the Program of Food Acquisition (PAA), the Policy of Guaranteed Minimum Prices (PGPM), the Federal Program for Community and Family Forestry (MFCF) and standard legislation dealing with products originating from sustainable organic extractives.

In the scenario of reduced production, rigorous sanitary requirements and increased demand on the part of consumers, the cultivation of Brazil nuts has stirred interest among the agro-extrativists and businessmen. However, the lack of selected and recommended genetic material creates a bottleneck to the expansion of plantation cultivation.

3.2.2 Cultivation Methods

Studies envisioning commercial Brazil nut cultivation were initiated in the 1970s by various research institutions in northern Brazil, particularly Embrapa Eastern Amazonia (CPATU), in Belém, Pará. The focus of these studies can be summarized as: improvement of the germination rate of seeds, cultivation techniques and grafting to create short and precocious plants (Locatelli and Souza 1990; Moreira 1994; Müller et al. 1995; Nascimento et al. 2010).

The production of Brazil nut seedlings is carried out when the objective is to plant trees for timber or for grafting stock. Nevertheless, the emergence of plantlets is slow and ununiform, and under normal conditions can take 12–18 months (Müller 1982). According to Reis et al. (1979), this limitation may be explained by the presence of undifferentiated embryos. In addition, the Brazil nut shell or integument is tough, and has low water permeability, which impedes the seed development of the embryonic axis. Thus, removal of the integument is a necessary practice, and one recommended by Müller (1982), to make germination uniform and speed up emergence. Using this process, plantlet emergence begins about the third week after seeding (Müller 1982; Müller and Freire 1979; Pedrozo et al. 2017).

Grafting techniques should be utilized when the objective is fruit production, as it provides, among other advantages, precocity of production because under conditions of cultivation initial fruiting requires 8–10 years (Müller et al. 1995). More time is required before the trees reach a stable level of fruit production. In the case of grafted plants, fruiting can begin at around 4 years of age and provide regular production from age 7 (Müller et al. 1980, 1995).

The grafting method commonly used for Brazil nut is bud graft (Fig. 3.3), which when carried out in the field, has various limitations that increase the time and cost

Fig. 3.3 (**a**) Brazil nut tree recently grafted, (**b**) Budding at 45 days after grafting in the field, (**c**) A grafted Brazil nut about 18 months after grafting in the field. (Photos by Cássia Ângela Pedrozo)

of the process to obtain grafted seedlings. Carvalho and Nascimento (2016) envisioned the development of an alternative method to produce grafted seedlings in the nursery, looked into the viability of using cleft graft. This method has realized about 75% successful grafts.

Despite past research efforts and current investigations, the productivity of cultivated trees is low, possibly because of problems of limited genetic variability and incompatibility of clones, as well as the long period of time required for the plants to reach their maximum production potential, suggests that Brazil nut tree cultivation be restricted to agroforestry systems (Homma 1989; Homma et al. 2014; Nascimento et al. 2010). Brazil nut monoculture may not have the productivity to sustain itself economically.

In Brazil, thus far only a few commercial plantings of Brazil nut exist. The oldest are in Pará and Amazonas; in Mato Grosso and Rondônia it is also possible to find a few plantings. A majority of the more recent Brazil nut plantings have as their main objective forest restoration; nut production is considered of secondary importance. Fazenda Agropecuária Aruanã, in the municipality of Itacoatiara AM, has the largest planting of Brazil nut trees, with 318,000 grafted trees for nut production, along with 939,000 trees for timber production (Agropecuária Aruanã S/A 2018).

3.2.3 Propagation by Tissue Culture

Given the difficulties of Brazil nut propagation, by producing plantlets from seed or by vegetative propagation using grafting, tissue culture can be considered an important tool to be studied in the improvement of the species. Nevertheless, tissue culture studies are scarce and still in initial stages for Brazil nut; they have basically consisted of research to establish suitable protocols for in vitro regeneration by micropropagation and somatic embryogenesis (Santos et al. 2013; Serra et al. 2000; Veltilari and Quisen 2012; Vieira et al. 2008, 2009). In general, it has not yet been possible to develop an efficient protocol for in vitro propagation of Brazil nut because of the great difficulties encountered with asepsis of the tissue used. The results reported by Carvalho (2012) are the most promising. Low contamination of immature seeds was achieved by imersion in 70% alcohol for 1 min, followed by imersion in 5% calcium hypochlorite for 30 min. In the study, a higher percentage of callogenesis was realized by using a culture medium containing 2 mg/L 2,4-D and 3.2 mg/L TDZ.

3.2.4 Strategies for Improvement

The Brazil nut tree is a predominantly cross-pollinating species (O'Malley et al. 1988), which depends upon insects for fruit set (Maués 2002). Cultivation has not reached significant levels due, in part, to difficulties of propagation and the

relatively long time period for the trees to achieve their maximum nut production level, along with the low productivity of cultivated trees up to now (Homma et al. 2014; Nascimento et al. 2010). Propagative material currently used to plant Brazil nut is principally sourced from native mother trees whose genetic origin is unknown. However, the difficulties previously mentioned can be minimized, among other factors, by training workers in seedling production and by the availability of productive and precocious varieties, which also produce good quality nuts. Plant improvement actions are extremely necessary so that the propagative material is of the highest genetic quality available and also suitable for growth under different systems of cultivation. Nevertheless, up to now, there do not exist clones of varieties recommended for the Brazil nut tree. Currently, MAPA has identified and registered only a single cultivar, without having reliable data about its productivity and plant characteristics and, moreover, no information about the process used in its selection.

Recommendations of varieties and/or clones, derived from genetic improvement, will allow good future economic viability in the commercial cultivation of Brazil nuts (Pimentel et al. 2007), whether as monoculture or in mixed systems. Besides productivity and juvenility, there must also be considered, during the selection process, morphological characteristics of fruits and seeds, as well as the physical and chemical properties of the seeds.

Moritz (1984) demonstrated the occurrence of self-incompatibility in Brazil nut, finding that pollination between trees from the same clone resulted in low fruit production. In terms of grafting practices, Müller et al. (1980) recommended that scions from at least 5 different mother trees be used to guarantee good fruit production. However, there is no scientific evidence to support such a recommendation and, in studies recently published by Embrapa, they have utilized at least 20 mother trees for the establishment of clonal gardens and clonal seed orchards.

Due to the fact that Brazil nut is a perennial species, and fruit and seed production is quite variable from year to year (Kainer et al. 2007; 2014; Tonini and Pedrozo 2014), the selection of superior genotypes is a laborious task, requiring that trees be evaluated over several harvests to confirm long-term productive capability (Pedrozo et al. 2015). This factor discourages effective participation of the private sector in the development of Brazil nut tree cultivars.

According to a study by Tonini and Pedrozo (2014) of an area of native Brazil nut trees in Roraima, the production of both fruits and seeds varies between years, populations and trees. Pedrozo et al. (2015), Assis (2016) and Baldoni et al. (2017a) demonstrated that, depending upon the population, it may be necessary to wait until the trees are 9 years old before they can be evaluated for genotypes linked to high fruit production. Brazil nut trees that produce nuts of good quality, and are high producers consistently over the years are of great interest for the production of seedlings of high genetic quality.

Bringhurst (1983) proposed four steps to achieve improvement of Brazil nut tree production: (1) identification of superior phenotypes within native populations; (2) field propagation of selected plants; (3) development of cultivation practices to increase the performance of the selected plants; (4) crossing between selected plants, followed by the selection of superior progenies. Mother trees should be

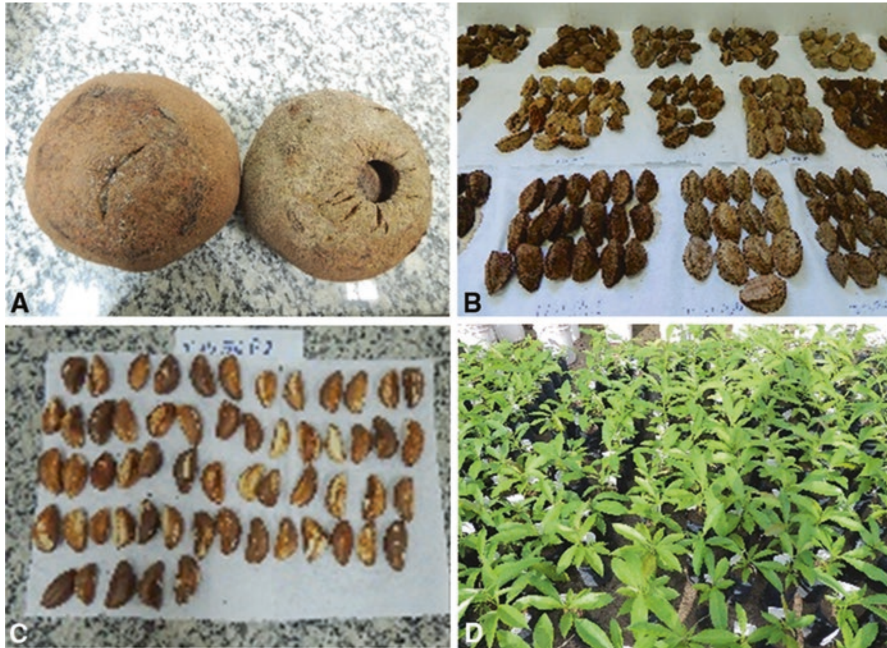

Fig. 3.4 Brazil nut. (**a**) Diversity of pore size in fruits, (**b**) Unshelled nuts, (**c**) Kernels and (**d**) Seedlings. (Photos by Cássia Ângela Pedrozo)

selected on the basis of overall health, high productivity and good morphological characteristics of both fruits and nuts (Corvera and Suri 2008; Peña 2008).

The first initiative to improve Brazil nut began in the 1960s, by Embrapa Eastern Amazon, formerly the Institute of Research and Agriculture and Livestock Experimentation of the North (IPEAN), (Pinheiro 1970), in which selections were made of 28 native Brazil nut trees which exhibited high production. Some of the mother trees were planted in clonal gardens to supply propagative materials, but no information was published with respect to the outcome of this initiative. In the 2000s, a program of Brazil nut improvement was initiated by the Peruvian Institute for Peruvian Amazon Research (IIAP), Iquitos. Similarly, in 2012, Brazil established Embrapa Brazil nut research units in the states of Amapá, Acre and Roraima; later adding Mato Grosso. The first activities of these programs consisted of pre-improvement studies, where mother trees were characterized, evaluated and selected on the basis of fruit production, which initiated the creation of clonal gardens and seed gardens in several Amazonian states. The objective was to provide selected genotypes as a source of propagative material. After determining the superiority of the selected mother trees, they would be used in crossbreeding experiments.

Besides data on production, researchers at IIAP and Embrapa gathered information on molecular characterization, morphology of the tree, fruit and nut, and the physical and chemical properties of the seeds of selected trees. Morphological variability (Fig. 3.4) has been observed in Brazil nut fruits and seeds, such as size and

weight, as well as variation relative to rates of germination and seedling growth in the nursery (Camargo et al. 2010; Giustina et al. 2017; Pedrozo et al. 2017; Peña 2008; Rocha et al. 2016; Teixeira et al. 2015), These results suggest the possibility of gains for these characters by means of genetic improvement.

3.3 Germplasm Biodiversity and Conservation

3.3.1 Germplasm Diversity

Brazil nut trees occur naturally in almost the entire Amazon Region, being found in Bolivia, Peru, Brazil, the three Guianas and southern Venezuela in the Rio Negro area (Corrêa 1931; Mori and Prance 1990a). Given this broad distribution, Brazil nut is adapted to a wide gradient of rainfall amounts and seasonality (Davidson et al. 2012), probably in response to natural selection and its historical geography. Species adapted to different environments, generally exhibit wide genetic variability.

In the case of Brazil nut, there are two schools of thought with regard to its wide distribution. One is based on natural seed dispersal of the species by water or animals; the other is human action by Pre-Columbian native peoples in Brazil nut management which favored its regeneration in the form of stands originating from the planting of the trees. (Shepard and Ramirez 2011; Thomas et al. 2014).

Shepard and Ramirez (2011) considered that the expansion of Brazil nuts occurred across its current broad geographic range rapidly and recently, being influenced to a certain extent by selection and incipient domestication. Genetic studies of chloroplast DNA have revealed low genetic variability within populations suggesting a dissemination of the species from an original narrowly-restricted population.

Another study of the distribution and genetic variability of *Bertholletia excelsa* in the Brazilian Amazon substantiates that the distributional pattern of genetic diversity of nine populations cannot be explained solely by gene flow and local adaptation (Sujii et al. 2015). The authors suggest that Pleistocene glaciation in the southern part of the continent brought about drier and slightly cooler climatic conditions in the Amazon Region which could have led to a contraction of the natural geographic distribution of *B. excelsa* (Thomas et al. 2014; Van der Hammen 1974). In addition, subsequent human dispersal of Brazil nuts could have expanded its distribution and genetic variability, but no research has been done on induced genetic variation.

Practically all recent genetic studies of Brazil nut indicate less genetic diversity (H_e) than that reported for other Amazonian species, with the absence of endogamy, preferential fertilization and genetic evidence confirming self-incompatibility (Baldoni et al. 2017b; Santos et al. 2017; Sujii et al. 2015; Wadt et al. 2015). Baldoni

et al. (2017b) found a pattern of isolation by distance, suggesting cross-pollination among nearby individuals and restricted seed dispersal. Wadt et al. (2015) studied the crossing rate of Brazil nut in different environments (pasture, planted trees, forest). They found that the collection of Brazil nuts for conservation purposes should be made preferentially from native trees because in that situation there was observed the least variation in size of the progeny. On the basis of recommendations of the foregoing authors and the wide occurrence of Brazil nut in the Amazon Basin, whatever germplasm conservation strategy is adopted should include collections from the largest number of populations possible, with at least 30 individuals per population (Silva 2014), in an attempt to maintain intrapopulation variability, as stated by Sujii (2011). These measures are important to avoid loss of fertility in fruit production.

3.3.2 Germplasm Collections

Embrapa Western Amazon in 1997–1999 led the initiative to establish a germplasm bank for Brazil nut in Acre State. However, the germplasm bank was never established despite mother trees having been identified and their seed collected (Wadt and Kaimer 2009).

Embrapa Eastern Amazon had a small collection of Brazil nut accessions, registered with the MMA (Ministry of Environment) under accreditation number 038/2010-SECEX/CGEN. This collection was established in 1969 in an experimental area of the agency in Belém (1°27′21″ S Lat., 48°30′6″ W Long). To this day, the collection is composed of the ten accessions collected in 1966, from an area of natural occurrence in the municipalities of Alenquer and Oriximiná in Pará.

Another germplasm bank was established in the Saracá-Taquera National Forest, Oriximiná, Pará. This collection was part of a project carried out by INPA (National Institute for Amazonian Research), whose objectives, besides the germplasm bank, were to characterize, quantify and conserve the genetic variability of Brazil nut through the accessions constituting the bank. About 500 Brazil nut seedlings were planted, at a spacing of 12 × 12 m, intercalated with seedlings of pioneer forest species with a 3 × 2 m spacing. Collections were made in 35 areas of natural occurrence, extending over 8 regions of the Amazon: Costa Marques RO; Itacoatiara AM; Oriximiná PA; Xapuri AC; Barreirinha AM; Tapauá AM; Novo Aripuanã AM and Laranjal do Jari AP.

Recently, in 2012, Embrapa Roraima initiated a pre-breeding Brazil nut project, as a follow on to the genetic improvement of Brazil nut for fruit production project (Cód SEG 02.13.05.015.00.00). In these projects selections were made of mother trees with high productivity in 7 states of the Brazilian Amazon: Acre, Amazonia, Amapá, Mato Grosso, Pará, Rôndonia and Roraima. Six working collections were established, as either clonal seed orchards or clonal gardens.

3.4 Molecular Breeding

Molecular techniques are viewed as supplementing genetic improvement programs, permitting an acceleration of the process and the creation of genotypes with promising characteristics (Brasileiro and Carneiro 2015). In pre-breeding, when there are envisioned the identification of genes and/or characteristics in populations which have not been previously subjected to any improvement process, molecular techniques have made significant advances. The availability of genetic diversity is fundamental to the genetic improvement of any species, and the knowledge of this diversity in natural populations is the first step to be taken (Nass 2011). For *Bertholletia excelsa*, pre-breeding genetic research provides a solid basis for future programs of improvement and conservation. This is important for the perpetuation of the species, given that in Brazil the tree is currently classified as Vulnerable on the official Ministry of Environment list of species threatened with extinction.

Studies of genetic diversity in natural populations of *Bertholletia excelsa* began in 1988 using isozymes. These revealed the low genetic differences between the populations sampled (Buckley et al. 1988). It is not believed that this represents an example of genetic erosion, but simply the result of the low capacity of the marker used to evaluate genetic diversity; besides, the sampling in this study was narrow.

Genetic studies using molecular markers of DNA, RAPD (random amplified polymorphism DNA) and AFLP (amplified fragment length polymorphism), provided a better understanding of the genetic diversity between and within populations of *Bertholletia excelsa* in the Brazilian Amazon, considering both native forest populations and commercial plantations. Genetic variation within a population was greater than the variation between populations (Coelho 2013; Kanashiro et al. 1997). In addition, it was possible to separate the trees of populations into groups according to their geographic origin (Coelho 2013; Serra et al. 2006). Tropical forest tree species generally exhibit a high proportion of polymorphic loci along with high levels of genetic diversity, the majority of the genetic variation contained within populations and not between them (Hamrick 1994), as has been observed for *B. excelsa* in the molecular studies cited.

Pre-breeding genetic research and conservation have utilized molecular microsatellites, also known as SSRs (simple sequence repeats) and STRs (short tandem repeats). These are repeated sequences that vary in tandem with 1–6 base pairs and are classified according to the size of the repeat units and location in the genome. These markers are highly polymorphic, codominant, and the DNA sequences that flank them normally are preserved between individuals of the same species (Caixeta et al. 2016). For *Bertholletia excelsa*, 19 polymorphic microsatellites were developed (Reis et al. 2009; Sujii et al. 2013), along with a protocol for DNA extraction (Giustina et al. 2018a). These studies have permitted a deeper understanding of the genetic diversity of the species (Cabral et al. 2017; Giustina et al. 2017; Santos et al. 2017; Sujii et al. 2015; Vieira 2014). This research also encompassed other regions such as Amazonian Peru (Reátegui-Zirena et al. 2009). In addition, it was possible to determine that the genetic diversity within progenies of half-siblings was less as

compared with the diversity between progenies from different mother trees (Giustina et al. 2017). Maintenance of genetic diversity within populations is important to assure seed production in quantities sufficient for extractivist exploitation as well as to perpetuate the species in its natural environment (Santos et al. 2017; Sujii et al. 2015; Vieira 2014).

Population fragmentation reduces effective population size and increases the spatial isolation between them, causing increased genetic drift, rates of endogamy and a reduction in genetic flow (Melo 2012; Young and Boyle 2000).

The genetic structure of a species is defined by the distribution of its genetic variability, between and within populations, which is the result of mutation, selection and genetic drift (Wadt 2001). To know the genetic structure of *Bertholletia excelsa* within its natural populations contributes a base for programs of conservation and management. Fragmentation of populations reduces the effective size of the population and increases spatial isolation among them, leading to an increase in genetic drift, rates of endogamy and reduced gene flow (Melo 2012; Young and Boyle 2000).

The distribution of genetic variability in natural populations is defined by the mode of reproduction, breeding system, population size, geographic distribution and gene flow (Hamrick 1983). The breeding system is defined by the way in which individuals, populations or species recombine their genetic variability in each generation to produce progeny, be it autogamous, allogamous or mixed. Knowledge of the particular system is implicit in the furtherance of genetic conservation, genetic improvement and reforestation programs (Goodwillie et al. 2005; Sebbenn 2006).

Bertholletia excelsa is considered to be an allogamous species, which depends upon pollination by bees of the genera *Bombus*, *Xylocopa* and *Centris* for fruit production, and on animals, principally the cutia rodent (*Dasyprocta* spp.) for seed dispersal and reproduction (Cavalcante 2008; Maués 2002; Müller et al. 1995; O'Malley et al. 1988). Anthropomorphic dispersal is also suggested for this species, taking into account its inefficient natural dispersal mechanisms and discontinuous distribution (Shepard and Ramirez 2011).

An early isozyme study of Brazil nut demonstrated the prevalence of cross-pollination along with a low level of endogamy (O'Malley et al. 1988). Microsatellite markers were employed in important studies comparing different Brazil nut populations with different levels of anthropogenic interventions. A study of three Brazil nut tree populations with different levels of anthropomorphic interventions in the Brazilian Amazon – natural forest, isolated trees in pastures and plantations – revealed that the seeds produced by these populations originated from cross-fertilization between different parents. By an estimate of the index of progeny fixation and of adult trees there was observed a selection for heterozygous individuals. The study demonstrated that in efforts of genetic conservation or reforestation with *Bertholletia excelsa*, seed collection should be carried out in natural populations, to guarantee greater genetic variability (Wadt et al. 2015).

Evaluation of other Brazil nut populations in the Brazilian Amazon confirmed the results of the previous paragraph; and the correlated cross-fertilization showed that seeds sampled at the population level were predominantly composed of half-sibs

(66%) and full-sibs (20%); in some cases, cross-fertilization presented inbreeding (Giustina et al. 2018b).

Gene flow can be defined as the movement of genes from one population to another, that is, the evolutionary change in the allele frequency, brought about by the movement of gametes between populations of the same species (Slatkin 1987). An understanding of the evolutionary history and population dynamics of a species is an important step towards its conservation. Genetic variability at low levels associated with genetic structure, the life history of a species and the reproductive system represent the first stage of the extinction of a species, subspecies or population group (Caballero et al. 2010).

Microsatellite molecular markers were also utilized to study gene flow in *Bertholletia excelsa*, considering the importance of this study, inasmuch as fruit production depends upon the natural activities of pollinators. The distances traversed by pollen, by the pollinator, varies from 36 to 2060 m. Both seed and pollen demonstrate a dispersal pattern from isolated locations, indicating a high frequency of crossbreeding between nearby trees and seed dispersal near the mother tree. Fragmentation of Brazil nut tree populations results in a significant reduction in gene flow (Baldoni et al. 2017b). No other information on this subject relative to Brazil nut has been published.

Some initiatives to use SNP (single nucleotide polymorphism) molecular markers, considered to be a new generation of molecular markets, have emerged for the study of *Bertholletia excelsa* (Inglis et al. 2011).

Besides molecular markers, other tools are being utilized to better understand the species. Studies have been done to determine the chromosome numbers of *Bertholletia excelsa.* Mori and Prance (1990b) identified n = 17, while a more recent study by Sodré and Karsburg (2009) determined n = 13. Additional research in this area is required to confirm the correct number of chromosomes in Brazil nut. This is once again an example of the need for further study to fill in the information gaps which exist surrounding this species.

3.5 Conclusions and Prospects

Bertholletia excelsa is widely distributed across *terra firme* forests in the Amazon Basin and most of the Brazil nut production is extracted from natural forests. Its commercialization represents an important source of income to thousands of Amazon people. Therefore its conservation is of utmost importance not only to the local communities that collect and commercialize the nuts, but also to a fair, efficient and productive supply chain from the local collectors to the national and international consumers.

Nowadays, there is a general concern about whether forest tree populations have sufficient genetic diversity to adapt to rapid climate change. Some preliminary studies have also indicated that Brazil nut fruit production is impacted by climatic oscillations, but the question of how the physiological investment in growth versus fruit

production is influenced by climate conditions remains open. It is expected that climate change would affect tree health and fruit production. In the face of a reduction in Brazil nut supply due to climate change, the risk assessment to the local economy is also necessary to develop sound mitigation strategies. One of the strategies to minimize the risk of a future lack of production would be to cultivate the tree, but despite basic studies already carried out, there are no areas of significant cultivation.

This is due, among other factors, to the lack of selected genetic material; cultivated varieties of Brazil nut do not yet exist. In this sense, some research initiatives focused on pre-breeding and genetic improvement of *Bertholletia excelsa* have arisen in order to obtain cultivated trees which are productive, more precocious and produce nuts of high physical and nutritional quality. In addition to genetic improvement, the identification of the most critical areas for Brazil nut tree conservation and the development of practical recommendations for seed sampling, breeding and reforestation strategies are of high relevance. It is hoped that the results of these initiatives are coordinated with the production of propagative materials of high genetic quality leading in the direction of future strategies of tree improvement and conservation of genetic resources, which are so important to the perpetuation of the species.

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Appendix I: Research Institutes Relevant to Brazil Nut

Institution	Specialization and research activities	Website
Embrapa – Brazilian Agricultural Research Corporation	Agricultural research	www.embrapa.br

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Chapter 4
Cashew Nut (*Anacardium occidentale* L.) Breeding Strategies

Suvalaxmi Palei, Rasheswari Dasmohapatra, Sunil Samal, and Gyana Ranjan Rout

Abstract Cashew nut (*Anacardium occidentale* L.) is a tropical, cross-pollinating tree native to South America. Vietnam ranks first in global production of cashew nuts with shells. World average productivity stands at 8136 hg/ha, but in most cashew-growing countries production is below the world average. Low yield is because of lack of soil profile correction, irrigation and pest control and prevalence of orchards of trees grown from seed selections and grafted genotypes. The tree bears male (staminate and bisexual), hermaphrodite and pistillate flowers on the same panicle. Only, 4–6% of flowers are pollinated and reach maturity to bear fruit, the rest are shed away at various stages of development. Generally, the gene pool expands through hybridization with different adaptive values, as long as the hybrids are able to produce segregating progeny in subsequent generations. Evidence of hybrid vigor with an increase of up to 153% in nut yield has been noted as compared to plants derived from out-crossed pollination. Uniform planting materials is a very important input for crop productivity. Cashew improvement programs include development of new commercial varieties having dwarf/semi-dwarf canopy, large nut size with higher shelling percentage, higher kernel grade and tolerance to biotic and abiotic stresses. A hybridization method has been standardized for breeding cashew varieties. Technologies like molecular markers, mutation breeding, molecular breeding, in vitro approach and transgenic breeding and other techniques are expediting the process of analyzing and assessing traits. With the advancements in genetics and molecular biology, genetic engineering has become the primary issue

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in molecular breeding. The present chapter highlights the propagation methods, improvement through conventional breeding, molecular genetic diversity, molecular breeding and conservation strategies.

Keywords Cashew nut · Conservation of genetic resources · Genetic assessment · Molecular breeding · Propagation

4.1 Introduction

Cashew (*Anacardium occidentale* L.) is an important tropical tree of the family Anacardiaceae and is native to the northern parts of South America. Cashew was introduced to Africa and Asia by European explorers during the sixteenth century. In 2015, world production of cashew nuts with shells reached 5.27 million mt. Vietnam is the leading producer. At present, India is the primary country for processing and exporting of cashew nut kernels and also occupies the secondary position in production and consumption. The world average productivity of cashew nuts with shells in 2015 was 8136 hg/ha (FAOSTAT 2015); in most cashew-growing countries, production is lower than the world average. There is also an increasing exploitation of high value byproducts, particularly those made of cashew nut shell liquid. Cashew cultivation and potential production is of major value for marginal smallholder farmers. It is cultivated in the tropical regions of Latin America, Asiatic and African zones (Johnson 1973). Cashew has been widely accepted, becoming a major export commodity crop in many countries. Production of cashew nuts with shells worldwide is equally distributed between Africa and Asia, whereas about 2 million mt of cashew apples are produced in South America and Brazil (FAO 2013a). The cashew industry plays an important role in the economic development of countries like Vietnam, India, Nigeria and Ghana and is considered a key contributor to the agricultural economy. The production of cashew has been steadily increasing over the recent years, showing an increase in cultivated area which exceeded 5.3 million ha in 2012 (FAO 2013b, c). It is estimated that India requires 1.3–1.4 million mt of cashew nuts with shells per annum in order to meet the demand of their existing processing capacity. In 2012, India imported 822 thousand mt of cashew nuts with shells (FAOSTAT 2015). Aliyu (2005) reported that the low productivity of cashew per hectare is attributed to traditional breeding only. There is still to be understood the various methods of vegetative propagation including micropropagation. There are different types of crop management, processing techniques and equipment requirements that have not been fully investigated, thus limiting their potential application. Exploitation of cashew nut shell liquid as green sources for use in the production of biorenewable chemicals, materials and energy has now been outlined (Mgaya et al. 2019). Cashew trees are considered useful for purposes of soil conservation, afforestation and development of waste lands. Therefore, it is necessary to identify the emerging areas of research in cashew.

This chapter highlights the distribution, propagation, breeding strategies, conservation approach and molecular diversity analysis of cashew, an important cash crop.

4.2 Botanical Description

Cashew (*Anacardium occidentale* L.) is an evergreen tree in the Anacardiaceae family. The Anacardiaceae has 60–74 genera and about 400–600 species (Bailey 1961; Brizicky 1962; Khosla et al. 1973; Mitchell and Mori 1987). The genus *Anacardium* has 8 species, but only *A. occidentale* has economic value due to its edible hypocarp (cashew apple) and nutritious kernel. The roots are generally both spreading and deep. The pattern of root distribution depends on soil type, level of crop nutrition, type of planting, age of trees and irrigation. During cashew nut germination, the radical develops quickly and becomes a tap root; further growth produces fibrous, lateral roots. The tap root system gradually becomes a complex structure of massive roots spreading laterally and deeply into the soil. Abdul Salam and Peter (2010) reported that in shallow laterite soils, cashew tree roots extended up to 3 m laterally around the tree and 1 m into the soil. Seedlings having both tap and lateral roots can best withstand transplantation, whereas young seedlings with poorly developed root systems fail to establish themselves during the summer season (Abdul Salam and Peter 2010). The trunk is irregular and short; initial branches grow close to the ground. Cashew leaves are elliptic to obviate, with a notched tip, very green with smooth margins. Leaves are arranged in a spiral manner toward the end of the stem on a small stalk. There are 3–14 leaves on each terminal stem which mature after 20–25 days (Johnson 1973; Ohler 1979; Lim 2012). Flowers are arranged in panicles and constitute both male and hermaphrodite flowers. The panicles have 5 to 11 lateral branches and are 26 cm long. Both male and hermaphrodite flowers bear a single large stamen and 5–9 smaller ones. About 200–1600 flowers per panicle are reported by various researchers (Aliyu and Awopetu 2008; Moncur and Wait 1986; Northwood 1966). Cashew is susceptible to some 10 fungal diseases. Ghini et al. (2011) reported that the two most important diseases are anthracnose foliar blight and fruit rot caused by *Colletotrichum gloeosporioides* Penz. & Sacc. and gummosis of the twigs and trunk caused by *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.); these fungi cause severe damage in all cashew-producing countries.

Martin et al. (1997) reported that flowering occurs at the new periphery of the tree canopy during the vegetative phase, followed by a dry period of 30–60 days. Individual flowers are small, with a small crown of 5 yellowish-green sepals and reddish petals. Rout and Dasmohapatra (unpublished data) reported that the flowers are small with a crown of 5 yellowish-green sepals and reddish petals when grown in the eastern part of India. The cashew tree bears an accessory hypocarpium and the true fruit itself, the seed or nut. The hypocarpium is oval or, alternatively, pear-shaped. Enlargement of the pedicel and the receptacle of the flower develop to form this part of the fruit, which is popularly known as the *cashew apple*. Fully ripe, it is either a yellow or red structure about 5–11 cm long. Later at the base of the apple, a single kidney-shaped drupe develops, 3–5 cm in length. Within *Anacardium occidentale* there are two distinct morphological groups, either the common type or dwarf type. Height of the adult plant reaches 8–14 m in height and a crown that extends to at least 20 m (Johnson 1973; Ohler 1979). Normally, the cashew plants

bear flowers in the third year from transplanting. Generally, the minimum age for stable production is about 8 years, but more commonly 12–14 years. The dwarf-type plants reach no more than 5 m in height, with a homogenous canopy 5–6.5 m wide. Dwarf-type plants have a shorter juvenile phase and also flower within 6–7 months. They reach marketable production in their second or third year from transplanting (Barros 1995; Barros et al. 2002). The performance of the production was obtained in dwarf types and selected in both Brazil and India. Barros et al. (2002) reported that the dwarf type bearing yield averages 1200 kg/ha/year, while the common type produces 379 kg/ha/year, At ripening, the fruits of the different genotypes vary in appearance (Fig. 4.1). Two products such as cashew nut and apple (fruit) are produced from the plant and used for their delicacy and nutritional value. Fruits contain rich in vitamin C and minerals. The fruits were also classified based on color grown in coastal belt of Odisha, India. The weight of the apple ranged between 59.88 g in Kanaka and 102.88 g in Vengurla-7. The length was varying between 4.86 cm (Ullal-2) and 8.73 cm (Priyanka). Juice percentage in fruits and TSS content was maximum in UN-50 (76.00% and 21.06% respectively (Lakshmana et al. 2018).

Fig. 4.1 Different cashew genotypes grown in the southern and eastern parts of India. (Photos by G.R. Rout and R. Dasmohapatra)

4.3 Propagation

4.3.1 Conventional Propagation

There are two primary methods adopted to grow cashew worldwide, propagated either by seeds directly in the field or transplanting nursery-raised plantlets. Azam-Ali and Judge (2001) reported that seedlings have a delicate root system hence transplanting may have a negative impact. They also reported that depending on soil type, seeds are placed at a depth of 5–10 cm. Sowing 2 or 3 seeds together is done to avoid gaps between plants in the field (Ohler 1979). There is limited published research on propagation of cashew through layering. Azam-Ali and Judge (2001) noted that the layering method was gradually being abandoned; as the layer plants are not easily transplanted in all the cases. They also noted that the most successful methods for vegetative propagation in cashew was air layering, providing the opportunity to achieve plantations from clonal material with high yield. For the selection of desirable phenotypes, propagation through grafting was used as an alternative. Different rootstocks of varying age were also tested to reduce the graft mortality; both *Anacardium rhinocarpus* and *A. spruceanum* are useful as rootstock. This method was proven to have the highest influence of grafting success. Wedge grafting performs better than apical side and side grafting (Mahunu et al. 2009). Apart from these traditional cashew grafting techniques, inarching has also been used (Azam-Ali and Judge 2001). In many cases, grafting success is dependent on climate, in particular the mean of the minimum temperature and the number of rainy days per month. The monsoon period in India is most suitable for softwood grafting with a success rate of more than 60% (Swamy et al. 1990).

Generally, there have not been formal breeding programs applied to cashew, so planting materials are obtained from either seeds or vegetative organs from elite trees. In the last five decades, exploration of the genetic diversity of cashew has expanded through germplasm acquisition programs, and also by promoting exchanges among India, South America and Africa (Aliyu and Awopetu 2007). It is reported that there is genetic variation in cashew genetic resources, either by introduction of genetic materials from different geographical origins or materials obtained from possible genetic introgression that coexisted earlier. Mneney et al. (2001) indicated that many researchers have exploited the available genetic pool of cashew around the world to identify economically-valuable traits from elite parental lines and also to exploit heterosis.

4.3.2 Micropropagation

Different biotechnology tools such as embryo culture, meristem culture, cell culture and somatic hybridization through protoplasts have been used in crop improvement programs of many tropical and subtropical plant species. There are very limited

reports on in vitro clonal propagation of cashew. In vitro cultivation of plant cells is a tool for improving their totipotency as reported initially by Haberlandt (1902). The cultivation of isolated cells in synthetic nutrient media is an entirely new approach. At the moment, these new emerging methods are not only used in scientific research but have also gained wide practical application in plant breeding, especially in producing virus-free planting materials. Philip and Unni (1984) reported that cashew is propagated through seeds with high levels of genotypic and phenotypic variability. However, conventional breeding techniques for the improvement of *Anacardium occidentale* have been hampered due to its outcrossing nature. There has been very limited success with vegetative propagation through air layering, mound strolling, grafting or cutting in cashew. Modern techniques like micropropagation and embryo cultures allow for quick multiplication of elite genotypes. Cashew is recalcitrant to in vitro culture techniques and very limited success has been achieved so far (Barghich and Alderson 1983; Mantell et al. 1998). Many researchers have succeeded in developing calli from cashew explants (Falcone and Leva 1987; Leva and Falcone 1990; Sy et al. 1991). Furthermore, the calli developed into small globular protuberances and showed somatic embryo-like structures (Das et al. 1996; Jha 1988; Leva and Falcone 1990; Litz et al. 1984). D'Silva and D'Souza (1992) reported that in vitro rooting and its survivability in soil conditions remain a severe constraint. Some researchers have reported the direct somatic embryogenesis from mature and immature cotyledonary nodes and embryonic axes, but regeneration of complete plantlets has not yet been achieved (Aliyu and Awopetu 2005; Hedge et al. 1990, 1991). Mantell et al. (1998) reported that explant browning is a serious constraint to micropropagation of cashew due to the presence of secondary metabolites which oxidize and cause browning and necrosis of explants (Das et al. 1996; Jha 1988). Meanwhile, different approaches have been tested to solve the browning problem of cashew explants by application of activated charcoal or antioxidants (Bessa and Sardinha 1994a, b; Das et al. 1996; Jha 1988; Lievens et al. 1989; Sardinha et al. 1993; Sy et al. 1991). Das et al. (1996) reported that frequent subculture of cotyledonary nodes into fresh medium with activated charcoal for 7 days increased the viability from 15% to 60%. Either frequent transfer of explants or inclusion of activated charcoal and incubation in the dark for 1 week increased the survival of explants to 90%. Sardinha et al. (1993) reported that the addition of 280 mM of ascorbic acid in the growth medium reduces the browning of the medium and the subsequent necrosis of explants without inhibiting in vitro bud development. The addition of activated charcoal in the culture medium or incubation in the dark for 7 days also had a positive effect on shoot proliferation, but shoot initiation was drastically decreased (Mantell et al. 1998). It was observed that explants collected from young germinated seedlings or juvenile explants were most suitable for clonal propagation of cashew. Mantell et al. (1998) noted that repeated pruning of adult elite trees to be used as source of explants led to better growth in vitro. The age of the explants is the important factor that affects axillary culture of cashew (Mantell et al. 1998). Cashew shoots of mature plants have not been used as explant sources due to the recalcitrance problems. Pierik (1990) reported that the responses of adult tree can be improved through partial rejuvenation of the shoots. It has also been

achieved through micrografting of adult phase meristems onto seedling rootstock. Lievens et al. (1989) and Leva and Falcone (1990) reported promising results obtained with explants collected from seedlings from 6 months to 3 years of age. Lievens et al. (1989) observed that shoot induction and multiplication on liquid Murashige and Skoog (1962) (MS) medium with BAP improved axillary bud development. In some cases, agar impurities have been found to cause problems of browning of explants. MS salt is the most suitable for shoot bud development and plantlet regeneration as reported by Das et al. (1996) and Aliyu and Awopetu (2005). Mantell et al. (1998) reported that MS medium with reduced salts showed optimal response on shoot development in vitro. Earlier, D'Silva and D'Souza (1992) had reported that sucrose concentration has a significant effect on shoot bud development. A higher concentration of sucrose (more than 40 g/l) has a negative impact on shoot bud development according to Mantell et al. (1998). The combination of glucose or maltose appeared to enhance both bud sprouting and number of nodes per culture (D'Silva and D'Souza 1992; Mantell et al. 1998). Different types and concentrations of plant growth regulators used in culture media play critical roles in the induction of in vitro differentiation (D'Silva and D'Souza 1992). Sardinha et al. (1993) and Bessa and Sardinha (1994a) reported that about 65–94% of bud break takes place in culture medium without a growth regulator. A relatively high dose of cytokinins has been used for in vitro bud development of cashew (Das et al. 1996; D'Silva and D'Souza 1992; Leva and Falcone 1990; Lievens et al. 1989). However, only zeatin riboside at a concentration of 5 mg/l stimulated axillary bud development as reported by Leva and Falcone (1990). Kinetin or 2-isopentyladenine in combination with 0.1 mg/l NAA has a positive effect on shoot bud proliferation, but the presence of TDZ and BAP resulted in death of the explants (Lievens et al. 1989).

Mantell et al. (1998) observed that explants cultured continuously in the presence of cytokinins experienced decreased bud sprouting and shoot elongation, which are unsuitable factors to achieve shoot multiplication. Several studies on shoot development from axillary buds of cashew required a two-step procedure. The first step is the addition of high doses of cytokinin to induce bud development, followed by a second low concentration of cytokinin deployed for shoot elongation. The use of gibberellic acid (GA_3) in the medium enhanced bud sprouting and shoot elongation in cashew, but inhibits rooting of the derived plantlets (Leva and Falcone 1990; Lievens et al. 1989; Mantell et al. 1998). Boggetti (1997) reported that rooting abilities were reduced in cashew explants cultured in rooting medium supplemented with GA_3. Root initiation from microshoots derived from nodal explants appeared to be less successful than those from cotyledonary nodal explants (Aliyu and Awopetu 2005), because of their more meristematic/juvenile character and active morphogenic stage. D'Silva and D'Souza (1992) reported that the combination of 2.9 mM IAA and 4.9 mM IBA induces 80.3% rooting in shoots derived from cotyledonary node. Microshoots treated with 5 mg/l NAA induced only 25% rooting (Leva and Falcone 1990) and 2 mg/l IBA reported only 30% rooting (Lievens et al. 1989). Mantell et al. (1998) achieved 42% rooting in microshoots derived from nodal explants of 1-year-old plants treated with 100 mM of IBA for 5 days. Das et al. (1996) reported the induction of rooting from cotyledonary node-derived

microshoots using *Agrobacterium* rhizogenes. Jasik et al. (1996) and Boggetti (1997) achieved induction of rooting from node-derived microshoots of cashew using various wild and disarmed *Agrobacterium* strains.

4.4 Conventional Breeding

4.4.1 Breeding Approaches

Anacardium occidentale L. is an important commercial crop in the tropics and also contributes to social and economic development in various countries (Bezerra et al. 2007; Masawe 2009). It is a diploid with 2n = 42. Cashew nut yields are low because of lack of soil correction, irrigation and pest control and prevalence of orchards of trees grown from seeds instead of selected, grafted and productive genotypes (Aliyu 2012a, b; Oliveira 2007; Rossetti and Montenegro 2012). Many researchers report that inadequate pollination is playing an important role in low productivity of cashew (Freitas 1997; Freitas et al. 2002; Holanda-Neto et al. 2002; Reddi 1987) in addition to other factors.

Increased production is a fundamental goal of plant improvement. But in addition, cashew, being primarily an export-oriented crop, it is also necessary to give high priority to the development of varieties and hybrids with export-grade kernels.

Cashew is a cross-pollinating tree crop (Eradasappa and Mohana 2016; Free and Williams 1976; Palaniswami et al. 1979; Pavithran and Ravindranathan 1974). The tree bears male staminate (Fig. 4.2a), bisexual, hermaphrodite and pistillate flowers (Fig. 4.2b) on the same panicle (Ascenso and Mota 1972; Kumaran et al. 1976a, b;

Fig. 4.2 (**a**) Male cashew flower with an undehisced pink anther, (**b**) Hermaphrodite cashew flower with a long pistil and short stamen. (Source: Eradasappa and Mohana 2016)

Rao and Hassan 1957; Thimmaraju et al. 1980). Abnormal flower types have also been recorded by a number of researchers (Joseph 1979; Mota 1973; Northwood 1966; Kumaran et al. 1976a, b). Cashew plants require at least 4–5 months to complete sequential anthesis in the panicle (Pavithran and Ravindranathan 1974). Male flowers provide pollen grains for the female part of bisexual flowers for pollination and fruit set. Although the total number of flowers in a panicle varies from 200 to 1600, over a flowering period of 70–90 days, less than 10% of those are bisexual flowers. The structure of a hermaphrodite flower includes stamens with short filaments, only half the length of the style, which makes self-pollination difficult and favors cross-pollination. About 85% of the flowers are fertilized under standard conditions, only 4–6% of them reach maturity to bear fruits, with the remaining flowers shed at various stages of development. Earlier, it was reported that pollination takes place by wind (Aiyadurai and Koyamu 1957; Haarer 1954; Rao and Hassan 1957). However, Reddi (1991) and Frietas and Paxton (1996) reported that pollination is by insects. Furthermore, insects like flies (Roubik 1995), moths (Kevan 1975) and bees (Bhattacharya 2004; Paiva et al. 2009; Freitas and Paxton 1996; Heard et al. 1990) have been viewed as the major cashew pollinators, but little information is available about the effective pollinators and use of honeybees for pollination (Freitas 1994). Sundararaju (2000, 2003, 2011) reported that in coastal regions of Karnataka, India, the pollinator activities on cashew were low. However, halictid and honey bees, constantly visit fresh flowers. Among them, the halictid bee, (*Pseudapsis oxybeloides*) was dominant and mainly collects pollen from male flowers as well as nectar from hermaphrodite flowers in visits of 2–5 seconds/flower. Rao and Hassan (1957) reported that peak anthesis is between 0900 and 1100 hours. Hermaphrodite flowers start opening from 0900 to 1000 and continue until 1300 hours. Staminate flowers open from 0900 and continue until about 1500 hours. Damodaran et al. (1965, 1966) reported that staminate flowers open very early in the morning and continue until about 1600 hours. Perfect flowers open mostly between 1000 and 1200 hours. It was reported that over 85% of male flowers opened before 1100 hours, while over 80% of perfect flowers opened between 1000 and 1300 hours (Damodaran et al. 1979). Wunnachit and Sedgley (1992) reported two stages of flower opening per day, one in early morning and another around midday.

Eradasappa et al. (2014) noted a pollen fertility range of 53.2–96.4% in varieties of cultivated species of *Anacardium occidentale*, whereas in the wild species *A. microcarpum*, *A. othonianum* and *A. pumilum*, it was 92.3, 90.5 and 93.8%, respectively. Generally, gene pools expand through hybridization with different adaptive values, providing the hybrids are able to produce segregating progeny in subsequent generations (Stebbins 1974). Evidence of hybrid vigor with an increase of up to 153% in the nut yield, as compared to plants derived from outcrossed pollinations, was reported by Damodaran (1975). Manoj and George (1993) and Cavalcanti et al. (2000) reported the prevalence of heterosis in hybrids of cashew with respect to nut yield, nut weight and kernel weight. Roy et al. (2018) reported that more than 90% heritability was found in characters like canopy area, total numbers of laterals, flowering laterals, duration of flowering, sex ratio and weight of cashew apple. Furthermore, Santos et al. (2010) reported the identification of QTLs (quantitative

trait loci) and MAS (marker-assisted selection) which are of great interest in breeding programs with regard to fruit quality. Physicochemical characteristics including total titrable acidity, phenolics, oligomeric total soluble solids and vitamin-C contents were analyzed in the mapped cashew population. They were detected by QTL intervals and multiple QTL mapping with high phenotypic variation in the segregating F1 generation for all traits, and also identified 18 QTLs linked with fruit quality (Santos et al. 2011). QTLs are promising for marker-assisted selection since they have the greatest phenotypic effects and contribution to phenotypic variation. The first genetic map of cashew was published by Cavalcanti and Wilkinson (2007). QTLs for yield-related traits such as nut weight, male and hermaphrodite flowers have been identified by Cavalcanti et al. (2007). The traits were identified in 71 F1 genotypes of the cross CCP 1001 x CP 96; 3 QTLs were detected for nut weight, 4 for male flowers and 4 for hermaphrodite flowers. They also suggested that QTLs accounted for 3.8–13% of the total phenotypic variance and had phenotypic effects of 31.8–34.2%. The effective potential for marker-assisted selection of the QTL hf-2f and hf-3 m is great and the percentage of phenotypic variation higher than that of the others.

Buso et al. (2011) studied the variability in analytical performance among the tested microsatellite markers. They reported that marker AOBR48 proved to have the highest polymorphic information content. Out of the 100 new markers, 11 showed segregation of alleles from the female parent in the F1 population, while 21 markers showed segregation of alleles from the male parent. Segregation of alleles from both parents in the F1 population was fully informative by only 3 markers. About 29 markers were mapped in this population and 11 markers were positioned in the male and female maps. Aliyu and Awepetu (2007) reported 59 accessions of cashew derived from both local and exotic populations of Nigeria, to assess the extent of variability and pattern of genetic diversity among these populations. They found that 4 distinct morpho-genetically diverse clusters on the basis of multivariate analyses. The groupings formed appeared to be a function of origin, genetic and/or agronomic affinity of the selections and ecogeographical distribution. They also noted that 2 major clusters have been formed. One major cluster having Brazilian populations, the other major cluster having local clones and Indian selections with its unique fruiting and tree-growth habits. The clustering pattern is clearly reflected at the subcluster levels which is the affinity of each genetic population. Fruit characters are the most discriminating parameters for delineating cashew at the varietal level on the basis of PCA (principal component analysis) and potency indices. Mzena et al. (2018) reported that the genetic variability among the genotypes based on plant height, nut size, canopy diameter, number of nuts, number of kernels, kernel weight and yield. The genotypes trait (GT) biplot analysis help to rank the genotypes with respect to agro-morphological attributes. They found there was correlation of yield with number of nuts (r = 0.908), number of kernels (r = 0.918), canopy height (r = 0.372). Moreover, there was insignificant correlation between yield and either nut weight, kernel weight or percentage outturn. This information will help to breeders on developing linkage mapping and implementing selection strategies.

4.4.2 Breeding Objectives and Constraints

Uniform planting material is an essential input for crop productivity to achieve high yield. The main objectives of a cashew improvement program should include development of new commercial varieties having dwarf/semi-dwarf canopy, large nut size with higher shelling percentage, higher kernel grade and tolerance to biotic and abiotic stress. Some researchers suggest that a cashew breeding program should be broken down into five objectives for improvement. First, to develop varieties and hybrids with export grade kernels, large nuts, yield of more than 10 kg/tree/year and a greater fruit setting percentage of up to 18%. Second, to develop plants having dwarf stature with a compact canopy, short flowering phase to reduce the chances of losing crops due to pest infestation and also to minimize the cost of harvesting nuts. Third, to select trees with a high frequency of bisexual flowers and also emphasize the importance of staminate flowers to provide more efficient pollen production so that trees with mixed phase and also high sex ratio are preferred as parents over types which have distinct male and hermaphrodite phases. Fourth, to develop varieties which show field tolerance to the tea mosquito bug (*Helopeltis theivora*). Fifth, to select germplasm having a minimum of 28% shelling percentage in cashew kernels and also to develop varieties with high nutritive value of the kernels.

Paiva et al. (2009) suggested that the breeding of cashew through either clonal improvement (through selecting and evaluating the cloned plants with desired traits) or population movement (selection by evaluating of progenies from selected plants with desired characteristics) or population improvement followed by clonal selection. They also suggested that diversified genetic materials should be considered to the extent possible, not only because clones may not be adaptable to certain ecosystems, but also to avoid the creation of genetic vulnerability. The polycross method is an alternative for improving allogamous species, especially those with small hermaphroditic flowers. This method yields results similar to those obtained through biparental crosses. It was first applied in cashew breeding in 1978 aimed at developing dwarfism, precocity and high nut yield and weight (Barros et al. 1984).

The hybridization method has been standardized for breeding varieties in cashew by various researchers. Cavalcanti et al. (1997) used hybridization and reported the differences among genotypes and their crosses in relation to early nut yield. Subsequently, they detected significant variation with regard to the different traits such as plant height, number of nuts, nut yield, canopy diameter and nut and kernel weight (Cavalcanti et al. 2000). Winter et al. reported highly heterozygous hybrids typically exhibited enhanced vigor. Damodaran (1975) reported that plants obtained from outcrossed pollination have hybrid vigor with increments of up to 153%. Manoj and George (1993) reported the occurrence of heterosis in cashew in relation to nut yield, nut weight and weight of the kernel. Bhat and Kumarn (1998) showed that the paper-roll method of hybridization was better than other methods. The main strategies are to develop of dwarf and semi-compact hybrids for high density planting systems, development of hybrids with high yield and large/medium nut size. Cavalcanti et al. (2003) identified that traits like plant height, canopy diameter, and

number of nuts, nut yield, nut weight and weight of kernel have also been increased. Advances in technology have put many more tools into the hands of breeders. Technologies like molecular markers, mutation breeding, molecular breeding, in vitro approach and transgenic breeding, bioinformatics and other techniques are expediting the process of analyzing and assessing traits (Paiva et al. 2009).

Transgenic breeding is complementary to conventional breeding. With the advancements in genetics and molecular biology, genetic engineering has become one of the essential issues in molecular breeding. Regeneration of transformed plant from tissue/organ is still the key factor which seriously restricts the enhancement of crops through genetic transformation. Sharma et al. (2000) reported that the stable putative regeneration system, target genome and a vector to carry the gene, a candidate gene, and modification of foreign DNA to enhance its expression, identification and characterization of transformed cell with aspirant plants at the molecular levels are prerequisites to genetic transformation. Genetic engineering offers an additional source of diversity through which breeders can develop new resistant varieties and introduce genes which confer resistance. Kiran Nivas et al. (2007) reported a method to introduce insect resistance into cashew trees by transformation, to increase yield and reduce the pesticide load on the environment. They used *Agrobacterium tumefaciens* strain EHA-105 with plasmid pBIN m-gfp5-ER for stable transformation and selection of transformants by using the *nptII* (kanamycin resistance) marker. Successful transformation was also confirmed with PCR amplification of transformed plants developed in vitro using GFP specific primers.

Plant breeding is acknowledged to be a key factor for adaptation of cropping systems to climate change. As a whole, breeding for climate change focuses on genes with large effects on heavy metal, cold, submergence, flooding and drought tolerance, but phenology and stress tolerance are highly polygenic. The most vital elements of a cropping system and its adaptation to climate change are: breeding cycles that provide farmers with a new cultivars developed in and for the current climate; selection of elite germplasm from other agroclimatic regions that currently experience conditions likely to occur in the target region as a result of climate change; evaluation of potential new cultivars under the range of climate conditions they are likely to encounter in commercial cultivation; and seed systems that deliver new varieties to farmers quickly to replace them with changing climate. Climate change severely affects crops in most countries; the systems for delivering these adaptation tools are inadequate. Samal et al. (2003) noted that classical phenotype traits are useful; although the efficiency of selection may be influenced by environmental effects or by developmental stages. The environmental effect can also be minimized by appropriate experimental breeding design. Moreover, cashew breeding is mostly based on selection of useful traits like nut size, nut weight, and sex ratio, length of panicle and yield performance, as reported by Mneney et al. (2001). Several new varieties have been already developed and crop improvement continues to address new challenges of biotic and abiotic stresses. The greatest cashew diversity is reported to exist in the various coastal ecosystems. Due to high heterozygosity coupled with cashew's allogamous nature, plantations have segregated seedling progeny with enormous variability with respect to yield, nut size and growth habit.

As a result, there exist natural recombinants having certain desired high-yielding traits in them, which are identified as *plus* trees for conservation.

4.5 Germplasm Conservation Approaches

Germplasm conservation is one of the emerging areas closely linked to plant improvement programs. Conservation of plant genetic resources can be achieved either in situ or ex situ, or by a combination of the two. Particularly in India, cashew is mainly grown along the West Coast in the states of Maharashtra, Karnataka, Kerala, Goa, Tamil Nadu, Andhra Pradesh and West Bengal; and Odisha along the East Coast. To a very limited extent it is grown in northeastern India, the Andaman and Nicobar Islands and Chattisgarh union territories. Cashew is an important horticultural crop in the country, earning a substantial amount of foreign exchange with the export of over 95,000 mt of cashew kernels, as reported by Bhaskara Rao (2000). As cashew is a cross-pollinated crop and highly heterozygous, segregation has resulted in large variations within plantation populations (Bhaskara Rao and Bhat 1996; Bhaskara Rao and Swamy 1994). Since 1986, cashew germplasm collection and conservation has been carried out at the National Research Centre for Cashew, Puttur, Karnataka. A total of 419 clonal accessions have been collected and conserved in the National Centre in India.

Mantell et al. (1998) reported that MS medium with reduced salts helps in optimal development of shoot production. Earlier, D'Silva and D'Souza (1992) reported that there was significant effect of sucrose concentrations on the number of developing buds in vitro. Minimal concentrations of sucrose, as well as nutrient composition, enhance long-term in vitro culture for conservation. Frequent change in culture and transfer to low concentration of nutrient medium without growth regulators enhanced conservation effectiveness. Castro et al. (2011) characterized 46 accessions of cashew collected in the savanna and maintained under long-term field conservation, all of which exhibited high phenotypic variability. Keller et al. (2011) reported that storage of genetic material at a low temperature is the safest and most cost-effective method to maintain vegetatively-propagated germplasm or to store recalcitrant seeds. Embryo encapsulation, slow-growth dormant bud culture through in vitro and cryopreservation have valuable methods for conservation of valuable genetic resources.

4.6 Molecular Diversity Analysis

During the sixteenth century, cashew was introduced into India in the Goa region and Malabar Coast of Kerala. Wide variation was observed within accessions derived from seed due to the cross-pollinating nature of the crop. Germplasm characterization has been made through morphological as well as molecular levels for

further program improvement. The cashew plant has a high content of phenol and tannin which hinder the extraction of quality genomic DNA from leaf tissues for molecular studies. Rout et al. (2002) established a method of plant DNA extraction from high tannin content leaf tissues of cashew for molecular studies. Archak et al. (2003) reported DNA profiling of 24 selections and 11 hybrids with increased yield and excellent nut characters. They used both randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers to identify genotypes for a breeding program. A total of 94 DNA fragments were generated which differentiated all the varieties. There was no correlation between the relationships based on molecular data and the pedigree of the varieties. Differences in the average similarity coefficients between selections and hybrids was very low, indicating hybridization. Samal et al. (2003, 2004) noted the genetic relationships of 25 varieties of cashew, collected from India on the basis of phenotypic characters and RAPD markers. They found 80 distinct DNA fragments with a range of 0.2–3.0 kb by using 11 selected random decamer primers. Cluster analysis clearly showed 25 varieties of cashew grouped into 2 major clusters based on similarity indices. They reported that Ullal-3 and Dhana (H-1608) showed the highest similarity indices (87%) among the 20 varieties. They also indicated that Vengurla-2 has very close similarity (85%) with variety Vridhachalam-3 (M-26/2). Genotypes Vengurla-2 and Vengurla-3 were not grouped into a single cluster but Vengurla-4 has 82% similarity to Vengurla-3. Aliyu and Awepetu (2007) reported the pattern of diversity among 59 cashew accession breeding populations, assessed using protein-isozyme markers. All the accessions grouped into 6 clusters, indicating moderate diversity among Nigerian cashew collections. The clustering pattern was depended on the ecogeographical origin of the accessions. Closer genetic affinity was observed between Indian and local clonal populations. Dasmohapatra et al. (2014) studied the molecular and agro-morphological assessment of Indian cashew genotypes and found wide genetic variation with regard to nut yield, nut weight, shelling percentage, height and trunk girth of the potentially superior genotypes. Table 4.1 shows the phenotypic variation among 25 genotypes with regard to nut yield, nut weight, shelling percentage and apple weight. A wide variation was noted with regard to fruit quality, fruit color, nut weight, shelling percentage, and nut yield and apple weight. Maximum and minimum plant height was observed in variety Chintamani-1 and Ullal-1, respectively, with a 0.65 CD value. The CD value of nut weight and nut yield was 0.532 and 0.452, respectively (Table 4.1). Nut yield and weight were significantly different between the 25 genotypes. The shelling percentage varied from 26% to 35% in different genotypes. The color of the apple and apple weight also vary in different genotypes of cashew (Fig. 4.3).

Identification of a cashew genotype and its genetic relationships is difficult due to the lack of morphological and floral traits. Significant variation exists among the genotypes due to cross-pollination. DNA profiles of 25 cashew genotypes with 20 random primers were generated with 103 consistent DNA amplified bands with a range of 100–2500 bp. Out of 103 DNA fragments, 19 were monomorphic and the minimum and maximum amplification fragments were generated in primers OPC-18 and OPN-06, respectively. The pattern of RAPD profiles generated by the primers

Table 4.1 Phenotypical characteristics of 25 genotypes of cashew (*Anacardium occidentale* L.)

No.	Variety	Selection / hybrid	Parentage	Plant height (m)	Trunk girth (cm)	Canopy spread (m)		Nut yield (kg/plant)	Nut weight (g)	Shelling %	Apple weight (g)
1	BBSR-1	selection	WBDC-5 (Vengurla 36/3)	2.60	30.76	2.33	3.23	1.58	4.6	30	50
2	Jagannath	Hybrid	Bhubaneswar cluster −2 × VTH 711/4	2.42	31.84	3.49	3.65	3.30	8.6	35	62
3	Balabhadra	Hybrid	Bhubaneswar cluster −1 × H 2/16	2.50	31.13	3.24	3.23	3.69	7.4	33	53
4	BPP- 4	Selection	9/8 Epurupalem	2.23	26.33	1.77	2.00	1.08	5.6	25	30
5	BPP- 6	Selection	T No. 56	2.00	26.33	1.60	1.40	0.72	5.8	26	34
6	BPP- 8	Hybrid	T. No. 1 × T No. 39	2.25	29.37	2.67	2.91	3.93	7.2	29	55
7	Ullal-1	Selection	8/46 Taliparmba	1.87	27.17	2.03	2.13	0.59	6.6	31	35
8	Ullal-3	Selection	5/37 Manchery	2.63	28.50	2.37	2.37	0.74	7.5	30	37
9	Ullal-4	Selection	2/77 TuniAndhra	2.37	27.17	1.60	2.00	0.96	7.2	30	40
10	Chintamani-1	Selection	8/46 Thaliparmba	2.87	31.83	2.87	3.67	1.23	6.4	30	24
11	NRCC-2	Selection	2/9 Dicherla	2.50	27.50	2.73	2.93	1.87	9.0	32	40
12	Vengurla-1	Selection	Ansur-1	2.13	29.50	2.97	3.37	0.98	6.2	30	30
13	Vengurla-4	Hybrid	Midnapore red × Vetore 56	2.03	26.80	1.90	1.80	1.34	8.0	30	42
14	Vengurla −7(H255)	Hybrid	Vengurla −3 × M 10/4 (VRI-1)	2.23	32.00	3.53	3.67	2.28	10.0	31	60
15	Madakkathara −1 (BLA-39-4)	Selection	T No. 39 of Bapatala	2.20	27.83	2.26	2.57	0.66	6.0	27	25
16	Madakkathara −2 (NDR-2-1)	Selection	Neduvellur material	2.13	28.00	2.33	2.17	0.80	7.0	26	30
17	Dhana (H-1608)	Hybrid	ALGD-1 × K-30-1	2.37	29.13	2.48	2.62	2.22	8.0	30	48
18	Kanaka (H-1598)	Hybrid	BLA 139–1 × H-3-13	2.17	28.17	2.03	2.23	1.13	6.0	31	40
19	Priyanka (H-1591)	Hybrid	BLA 139–1 × K-30-1	1.99	28.30	2.75	2.97	1.32	9.8	28	62

(continued)

Table 4.1 (continued)

No.	Variety	Selection / hybrid	Parentage	Plant height (m)	Trunk girth (cm)	Canopy spread (m)		Nut yield (kg/plant)	Nut weight (g)	Shelling %	Apple weight (g)
20	Vengurla −6 (H-68)	Hybrid	Vetore56 × Ansur-1	1.93	29.84	1.86	1.60	1.43	7.4	30	60
21	Amrutha (H-1597)	Hybrid	BLA 139–1 × H-3-13	1.93	26.07	1.30	1.80	0.78	8.0	31	40
22	K-22–1	Selection	Kottarakkara-22 (layer 23)	1.82	27.67	1.72	1.72	1.23	6.0	27	65
23	Bhaskara	Selection	Selection from forest plantation of Gondengram in Goa	2.97	32.84	3.36	3.87	3.36	7.7	27	50
24	Vridhachalam-3	Selection	M 26/2 Edayanchavadi material	1.97	28.17	2.23	2.17	1.98	7.0	29	40
25	Jhargram-1	Selection	T. No. 16 of Bapatala	2.50	29.80	2.90	3.10	1.05	6.0	30	50
	C.D.			0.65	3.88	1.10	0.96	0.45	0.53	2.5	5.42
	SE(m)			0.23	1.36	0.39	0.33	0.16	0.19	0.87	1.90
	C.V.			17.49	8.18	27.8	22.07	17.1	4.51	5.17	7.40

Fig. 4.3 Fruits of 25 cashew genotypes. See Table 4.1 for genotypes description. (Photos by G.R. Rout and R. Dasmohapatra)

OPC 18, OPC 04 and OPA 20 are presented in Fig. 4.4. The polymorphic information content (PIC) showed a variation of 0.32–0.90, with an average PIC value at 0.64 and the marker index (MI) range of 0.54–8.80 with an average of 2.95. Among the 9 DNA fragments amplified by primer OPA 07, 2 unique bands (300 bp and 900 Kb) were observed in varieties BPP-6, Ullal-1 and Ullal-4, which clearly distinguished them from other genotypes. Similarly, another unique band of 800 bp was observed in genotypes Vengurla-4 and Madakkathara with primer OPC 04. They also reported that a total of 116 DNA amplifications with a range of 200–2500 bp were detected among the 25 genotypes using 14 ISSR primers. ISSR primers AM 2 and UBC 872 produced the maximum 13 amplification bands followed by UBC 827. The minimum number of bands was noted in UBC 825 and the average number of bands per primer was 8.21. Out of a total of 116 ISSR fragments, 104 bands (89.6%) were polymorphic. The level of polymorphism generated by ISSRs (89.6%) was more than that of the RAPD primer (81.5%). The pattern of ISSR profiles produced by primers UBC 872, UBC 827 and AM-2 are shown in Fig. 4.5. Combining the RAPD and ISSR markers, a total of 219 bands were detected, out of which 188 bands (85.8%) were polymorphic, with an average of 5.52 polymorphic markers per primer. The maximum polymorphism was observed both in RAPD and ISSR markers with a high level of genetic variation existing within the genotypes because of different genetic sources of origin.

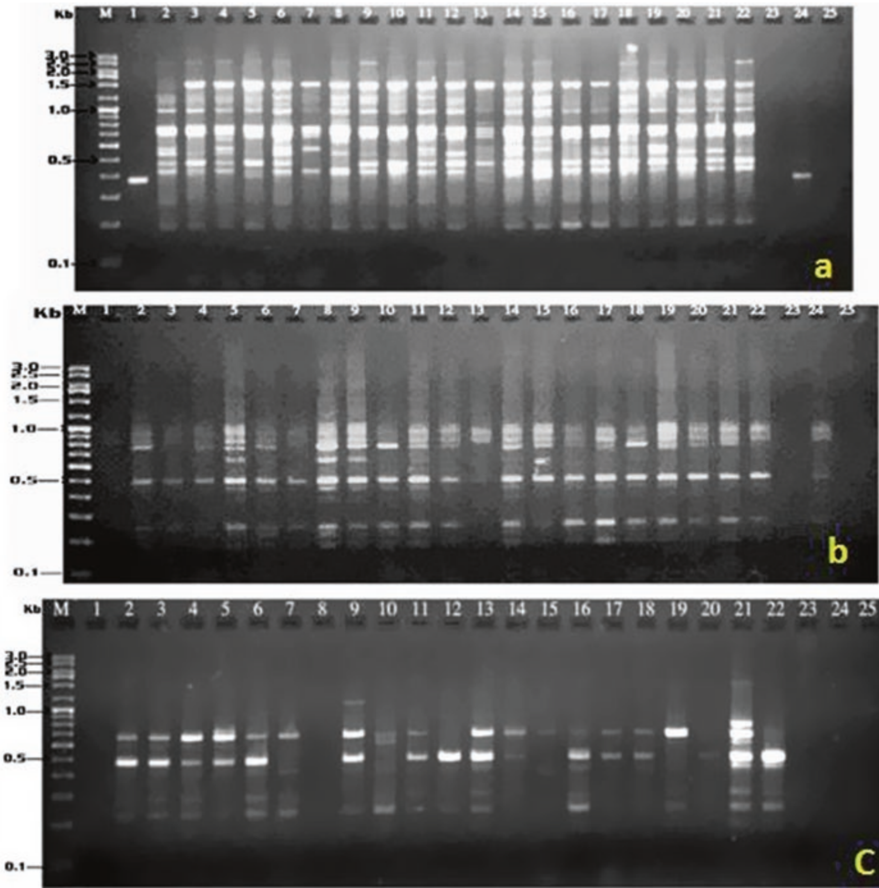

Fig. 4.4 RAPD profile of 25 cashew genotypes generated by using primers OPC 18 (**a**), OPC 04 (**b**) and OPA-20 (**c**). M, kb molecular weight ladder, 1: BBSR-1, 2: Jagannath, 3: Balabhadra, 4: BPP-4, 5: BPP-6, 6: BPP-8 (H-2/16), 7: Ullal-1, 8: Ullal-3, 9: Ullal-4, 10: Chintamani, 11: NRCC-2, 12: Vengurla-1, 13: Vengurla-4, 14: H255, 15: Madakkathara-1 (BLA-39-4), 16: Madakkathara-2, 17: Dhana (H-1608), 18: Kanaka (H-1598), 19: Priyanka (H-1591), 20: H -68, 21: Amrutha, 22: K 22–1, 23: Bhaskara, 24: Vridhachalam-3 (M-26/2), 25: Jhargram. (Source: Dasmohapatra et al. 2014)

The pair-wise comparison of genetic similarity of the 25 genotypes ranged from a maximum of (0.87) between Ullal-1 and Vengurla-1, to a minimum of (0.48) between Bhaskara and Priyanka in the combining of two DNA markers. Genotypes developed through selection showed the maximum genetic variation. A dendrogram plotted using cumulative data of the similarity coefficient derived from RAPD and ISSR profiles is presented in Fig. 4.6 (Dasmohapatra et al. 2014). On the basis of the similarity matrix, there were 2 major clusters with 62% similarity. There were 5 genotypes represented in Cluster-I, whereas 19 genotypes were represented in major cluster-II. Major cluster-II was further divided into 2 minor clusters, III and

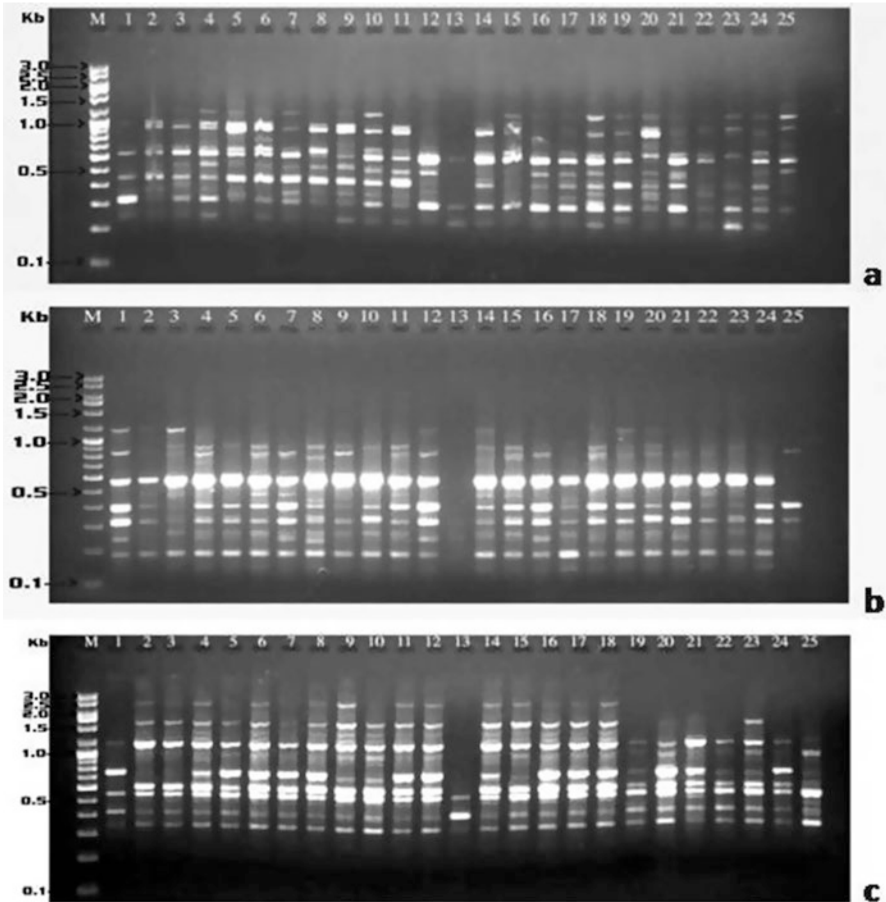

Fig. 4.5 ISSR profile of 25 cashew genotypes generated by using primers UBC 872 (**a**), UBC 827 (**b**), AM- 2 (**c**). M, kb molecular weight ladder, 1: BBSR-1, 2: Jagannath, 3: Balabhadra, 4: BPP-4, 5: BPP-6, 6: BPP-8 (H-2/16), 7: Ullal-1, 8: Ullal-3, 9: Ullal-4, 10: Chintamani, 11: NRCC-2, 12: Vengurla-1, 13: Vengurla-4, 14: H255, 15: Madakkathara-1 (BLA-39-4), 16: Madakkathara-2, 17: Dhana (H-1608), 18: Kanaka (H-1598), 19: Priyanka (H-1591), 20: H -68, 21: Amrutha, 22: K 22–1, 23: Bhaskara, 24: Vridhachalam-3 (M-26/2), 25: Jhargram. (Source: Dasmohapatra et al. 2014)

IV. Minor cluster III contained 1 genotype, Jagannath, with 0.66 similarity and minor cluster IV represented 18 genotypes with 2 sub-minor cluster groups, V and VI. Sub-minor cluster V contained 14 genotypes and VI with 4 genotypes, showed a similarity index of 0.77–0.87. However, sub-minor cluster V further divided into 2 groups, VII and VIII. Cluster VII has 2 genotypes Balabhadra and BPP-4, with 76% similarity among themselves. A total of 12 genotypes were represented in cluster VIII, having 2 groups, IX and X. Cluster IX represented 2 genotypes (Ullal-3 and NRCC-2) with 75% similarity. Cluster X having 10 genotypes, has 2 groups XI and

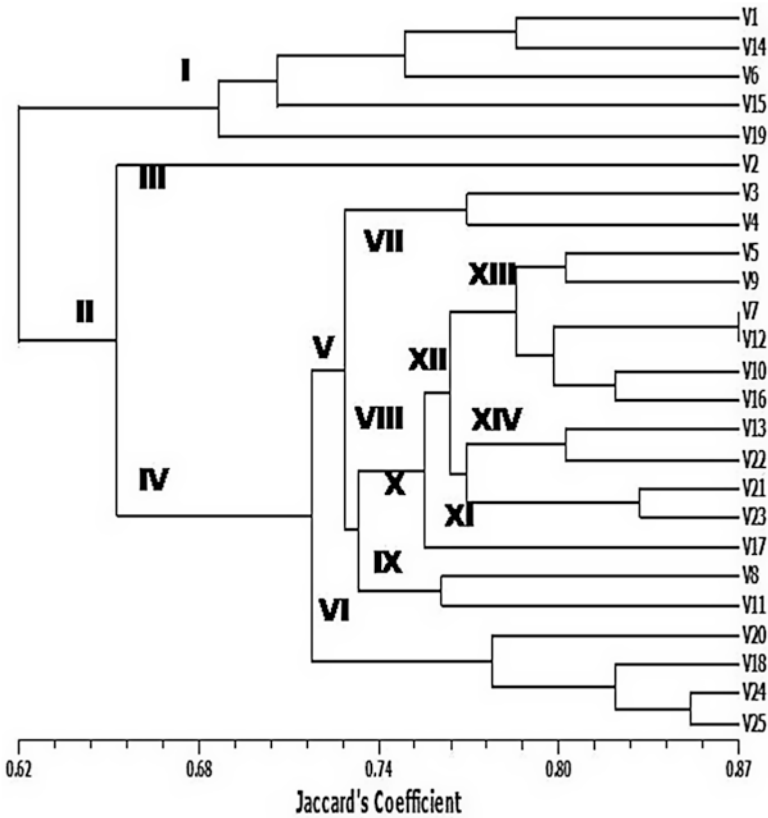

Fig. 4.6 Dendrogram generated from cumulative RAPD and ISSR profile data depicting relationship among the 25 cashew genotypes presented in Table 4.1. (Source: Dasmohapatra et al. 2014)

XII. Cluster XI has 1 genotype (Dhana) and cluster XII has 9 genotypes with 2 clusters, XIII and XIV. Cluster XIII has 6 genotypes with 87% maximum similarity between Ullal-1 and Vengurla-1, as these genotypes were identified through selection. Cluster XIV has 4 genotypes with 83% similarity between Amrutha and Bhaskara.

Genotypes developed from different sources (origin/regions) were found represented in different clusters, indicating a correlation between their morphological attributes and molecular groupings because of the selection of genotypes through crossbreeding. Dhanaraj et al. (2002) and Thimmappaiah et al. (2009) also reported a high percentage of genetic variation among the genotypes. Sika et al. (2015) used SSR markers for genetic characterization of cashew cultivated in Benin. A total of 146 polymorphic bands were produced with low genetic diversity (Shannon index = 0.04) and produced 3 clusters based on their genetic variations. Jena et al. (2017) reported the genetic diversity analysis among 12 varieties of cashew nut on the basis of morphometric, yield performance and DNA markers. They used the

Dice dissimilarity coefficient to discriminate cultivars on the basis of plant growth, flowering, fruit and nut characters. Out of 35 decamer primers, 15 primers generated informative polymorphism (average 79.18%). Both morphometric descriptors and RAPD markers proved their effectiveness in distinguishing the different cultivars in the cashew germplasm of India.

Sethi et al. (2016) used 15 RAPD primers to study the genetic diversity of 20 cashew hybrids and their 8 parents. They found 1742 alleles with an average of 62.2 alleles per genotype, with 87.8% polymorphism. On average 7.66 bands were produced with a range of 4–15 fragments per primer. They observed that hybrids H-6 and D-19 maintained a high level of average genetic dissimilarity with the rest of the tested genotypes. The hybrids comprising the most divergent genetic group showed significantly above-average productivity and these could enrich the cashew gene pool. Thimmappaiah et al. (2016) used different DNA markers (RAPD, ISSR, SSR) to detect the genetic similarity among 40 cashew genotypes. They found 71.8% polymorphism in RAPD, 87.5% in the case of ISSR and 93.3% in terms of SSR markers, which indicated high genetic variation existing among the tested genotypes. PIC (polymorphic information content) and MI (marker index) indicated that SSR and ISSR markers are highly informative. The average genetic similarity was 0.53, 0.63 and 0.76, respectively, revealed predominance of high genetic similarity rather than diversity. The genetic similarity co-efficient with combined markers showed a range of 0.46–0.86, with an average similarity of 0.66, also indicating narrow genetic distances and low diversity occurring between the varieties. The high genetic similarity (0.86) found between V-7 and BPP-5 indicates their close relationship and low genetic divergence. The low similarity (0.46) observed between Ullal-1 and Jhargram-1 indicates high genetic divergence with these varieties (Thimmappaiah et al. 2016). Both RAPD and ISSR markers detected high genetic similarity (0.95) between genotypes Goa11/6 and VRI-3 and also high genetic diversity between Jhargram-1 and Ullal-1, and Jhargram-1 with Chintamani-1. A dendrogram constructed using the data regenerated by 3 markers showed 10–12 similar groupings except for some minor differences. There was little correspondence in the molecular groupings observed between the genotypes originated from the same region or similar morphology. The unique markers may be useful to identify genotypes for breeding programs.

4.7 Conclusions and Prospects

Cashew is a cash crop of major global interest and has been expanding in international markets over recent decades. The crop is mainly cultivated in tropical countries. Among the main producers of cashew nuts, emerging countries like Vietnam, India, Nigeria and Ghana have economies which significantly rely on the cashew industry. Cashew has the potential to enhance societal development in local communities by empowering the most vulnerable groups which are involved in cashew production. By considering the growing population and the increase in smallholder

agriculture, sustainable production of any cropping system, including cashew, needs to address enhanced productivity together with maintaining the stability of other ecosystems. Cashew breeding programs are involved in plant introduction, progeny testing, individual selection and hybrid breeding. The selection of individual hybrid combinations is of great importance for any breeding program. Development of genetic maps for identifying QTLs in cashew and an integrated approach combining conventional and molecular breeding techniques and tools will help advance cashew improvement programs. The collection of core germplasm and its conservation are of utmost importance. Therefore, more research needs to be carried out to understand the governance of the cashew nut production chain for livelihood support to vulnerable groups of people.

Appendices

Appendix I: Research Institutes Concerned with Cashew

Institution	Specialization and research activities	Contact information and website
Odisha University of Agriculture & Technology, Bhubaneswar, India	Agricultural Biotechnology,	http://ouat.ac.in
	Research on Crop improvement, Postharvest Technology/Horticultural research	

Appendix II: Genetic Resources of Cashew

Cultivar	Important traits	Cultivation location
BBSR-1	Shelling percentage	Eastern part of India
Jagannath	High yield	Eastern part of India
Balabhadra	High yield, canopy structure	Eastern part of India
BPP- 4	Nut yield, apple weight	Eastern and southern part of India
BPP- 6	Nut yield, apple weight	Eastern and southern part of India
BPP- 8	Nut yield, apple weight	Eastern and southern part of India
Ullal-1	Trunk girth, nut weight	Northern, eastern and southern parts of India

(continued)

Cultivar	Important traits	Cultivation location
Ullal-3	Nut weight	Northern, eastern and southern parts of India
Ullal-4	Nut weight	Northern, eastern and southern parts of India
Chintamani-1	High yield, canopy structure	Eastern part of India
NRCC-2	Nut weight	Southern part of India
Vengurla-1	Nut weight	Southern part of India
Vengurla-4	Nut weight	Southern part of India
Vengurla – 7(H255)	Nut weight	Southern part of India
Madakkathara −1 (BLA-39-4)	Shelling percentage	Eastern and southern part of India
Madakkathara −2 (NDR-2-1)	High shelling percentage	Eastern and southern part of India
Dhana (H-1608)	High yield	Western and southern part of India
Kanaka (H-1598)	High yield	Western and southern part of India
Priyanka (H-1591)	Nut weight	Western and southern part of India
Vengurla −6 (H-68)	Nut weight	Western and southern part of India
Amrutha (H-1597)	Nut weight	Eastern part of India
K-22–1	Nut weight	Northern, eastern and southern parts of India
Bhaskara	Shelling percentage	Northern, eastern and southern parts of India
Vridhachalam-3	Shelling percentage	Northern, eastern and southern parts of India
Jhargram-1	Nut weight	Northern, eastern and southern parts of India

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Chapter 5
Chestnut (*Castanea* spp. Miller) Breeding

J. Hill Craddock and M. Taylor Perkins

Abstract The chestnuts and chinquapins are a group of about seven species of trees and shrubs in the genus *Castanea.* They are of considerable importance ecologically in all the areas of their natural occurrence, and the chestnuts especially are of great economic value for their lumber and for their nut crop wherever they are cultivated. Two catastrophic diseases, chestnut blight caused by the ascomycete fungus *Cryphonectria parasitica* (Murr.) Barr and *Phytophthora* root rot (ink disease) caused primarily by the soil-borne oomycetes *Phytophthora cinnamomi* Rands and *P. cambivora* (Petri) Buisman, have severely impacted chestnut in Europe and North America. Therefore, much of the breeding work continues to focus on breeding for resistance to these two diseases. The most serious insect pest of *Castanea* is the Asian chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu. Variation in host tolerance to *D. kuriphilus* has led to development of new gall-resistant chestnut cultivars. Interspecific hybridization offers great opportunity to combine the most favorable traits found in the ample genetic diversity of the genus through introgression into locally-adapted populations. Chestnut breeders in eastern Asia have made great strides towards improvement of chestnut fruit quality and crop yields, and researchers in all chestnut growing regions have made gains in disease resistance by using molecular markers and other genomic tools to assist selection. Biotechnologies that include transmissible hypovirulence as a biocontrol for chestnut blight, tissue culture and other micropropagation techniques, and genetic engineering and transformation technologies are complementary to classical plant breeding programs.

Keywords *Castanea* · Chestnut blight · *Cryphonectria parasitica* · Germplasm conservation · Ink disease · *Phytophthora* root rot · *Phytophthora cinnamomi*

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5.1 Introduction

The chestnuts and chinquapins are a group of tree and shrub species in the genus *Castanea* Miller of the Fagaceae. Depending on taxonomic treatment, 7–13 lower taxa are recognized within the genus (Mellano et al. 2012; Nixon 1997; Pereira-Lorenzo et al. 2016; Worthen et al. 2010). Chestnut researchers commonly recognize the following lower taxa: *C. crenata* Siebold & Zucc. (Japanese chestnut), *C. mollissima* Blume (Chinese chestnut), *C. henryi* Rehder & E.H. Wilson (willow leaf or pearl chestnut), and *C. seguinii* Dode from eastern Asia; *C. sativa* Mill. (European or sweet chestnut) from Europe and western Asia; *C. dentata* Borkh. (American chestnut), *C. pumila* (L.) Mill. var. *pumila* (Allegheny chinquapin), and *C. pumila* (L.) Mill. var. *ozarkensis* (Ashe) G.E. Tucker (Ozark chinquapin) from eastern North America (Conedera et al. 2004a; Johnson 1988; Pereira-Lorenzo et al. 2016; Pridnya et al. 1996; Worthen et al. 2010). In English-speaking countries, the common name *chinquapin* is often used in reference to *Castanea* species that typically produce one pistillate flower/nut per cupule, whereas the common name *chestnut* is applied to species that typically produce three pistillate flowers/nuts per cupule. The term *chinquapin* is also applied to other Fagaceae trees in *Quercus*, *Chrysolepis* and *Castanopsis*; *chinquapin* is derived from *chechinquamins*—John Smith's seventeenth century transliteration of the word used by some indigenous peoples of Virginia to refer to *C. pumila* var. *pumila* (Barbour 1986). The native and introduced *Castanea* species found growing throughout the world today are listed in Table 5.1, and their areas of natural occurrence are mapped in Fig. 5.1.

Castanea species are important nut crop and timber producers throughout most of their native and introduced ranges, and have been for many thousands of years (Avanzato and Bounous 2009). The major exception being in eastern North America, where introduced pests and pathogens severely limit cultivation of the native species. Nevertheless, the North American *Castanea* species were ecologically, economically and culturally important in eastern North America prior to the introduction of chestnut blight and *Phytophthora* root rot disease (ink disease) (Anagnostakis 2001; Crandall et al. 1945; Roane et al. 1986). As a result, *C. dentata* and *C. pumila* are the focus of extensive research and restoration breeding programs (Jacobs et al. 2013; Steiner et al. 2017; Thomas et al. 2015; Westbrook et al. 2019; Worthen et al. 2010).

Although there is still much debate about the history of artificial selection and domestication of *Castanea* species around the globe, evidence for selection of chestnut in East Asia and Western Europe dates back many thousands of years (Avanzato and Bounous 2009; Conedera et al. 2004b). In every case, our human ancestors were able to utilize the abundant annual production of fruits from wild chestnut trees, and to alter their environments (using fire, for example) in ways that benefitted both the trees and themselves (Avanzato and Bounous 2009; Krebs et al. 2004). LaBonte et al. (2018) provide genomic evidence for the ancient domestication of *C. mollissima* in China. From Pleistocene refugia in the Caucasus, parts of Italy and Switzerland, and probably also the Iberian Peninsula, *C. sativa* expanded

Table 5.1 *Castanea* germplasm resources; naturally occurring and introduced species and their areas of primary distribution

Species	Distribution
Naturally-occurring species	
C. crenata	Japan, Korea
C. dentata	Eastern North America, Appalachian region
C. henryi	Central and Eastern China
C. mollissima	widely distributed in central and eastern China
C. pumila var. *alabamensis*	Southeastern USA interior uplands
C. pumila var. *ozarkensis*	Ozark Plateau, USA
C. pumila var. *pumila*	Southeastern plateaus and Coastal Plain, USA
(*C. alnifolia, ashei*, *C. floridana*, *C. paucispina*)[a]	Deep South, Gulf Coast and Florida, USA
C. sativa	Europe, Asia Minor, the Caucasus
C. seguinii	Eastern China
Introduced species	
C. crenata	Eastern USA, California, New Zealand, France, Italy, Spain
C. dentata	Midwestern USA, California, Oregon, New Zealand
C. henryi	Rare (only in cultivation)
C. mollissima	Widely distributed in eastern North America
C. pumila sensu lato	Rare (only in cultivation)
C. sativa	California, Oregon, Washington (naturalized), Australia, New Zealand, Chile
C. seguinii	Rare (only in cultivation)

[a]These taxa may not be supported by modern phylogenetic evidence

rapidly to cover an area from the Caspian Sea to the Atlantic Ocean (Conedera et al. 2004; Krebs et al. 2004). Squatriti (2013) reviews evidence that improved cultivars selected from naturally occurring populations of *C. sativa* were being propagated by grafting in tenth century Italy. Pereira-Lorenzo et al. (2018) used microsatellite markers to investigate the role of graft propagation of local ecotypes in the domestication of *C. sativa*. They documented also the great age of some surviving grafts; the two oldest living trees they studied in Spain were grafted in the years 1427 and 1479. In Italy two ancient grafts of cv. Marrone Fiorentino were dated to 1650 and 1700. In contrast, while it is well established that the North American *Castanea* species were widely used by ancient peoples, convincing evidence has not been produced to support a history of artificial selection or domestication for *Castanea* in North America (but see Abrams and Nowacki 2008). Day (1953) describes early archeological evidence of *C. dentata* culture in New England, but no real documentation for selection or improvement prior to the nineteenth century.

Two major diseases—chestnut blight and *Phytophthora* root rot—have impacted chestnut growing globally, and most severely in Europe and North America. The chestnut blight pandemic began in Bronx, New York in 1904, caused by the

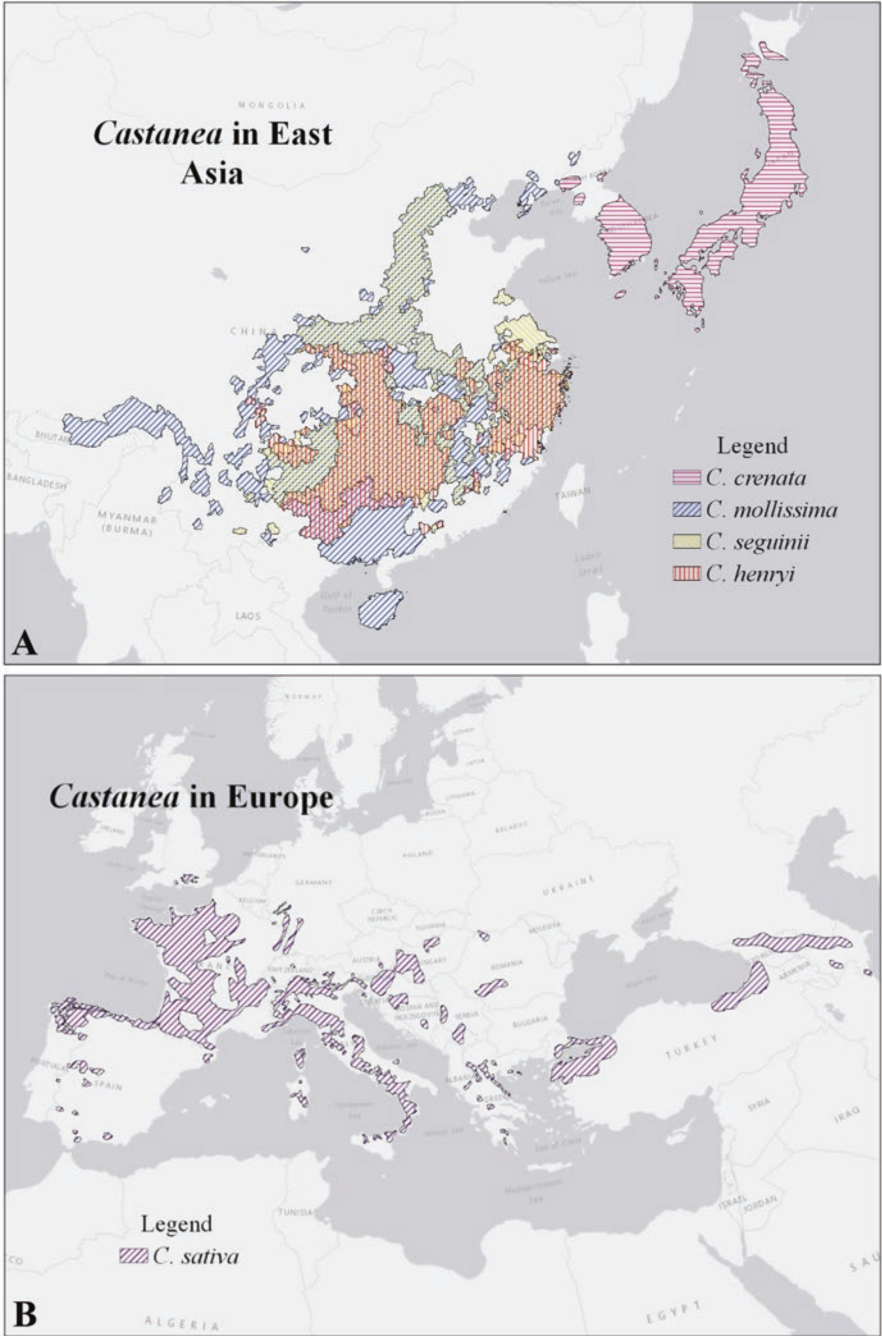

Fig. 5.1 Native and naturalized distributions of *Castanea* species worldwide. (**a**) Distributions of *C. crenata*, *C. mollissima*, *C. henryi* and *C. seguinii* in east Asia. Note: distributions of *C. mollissima,*

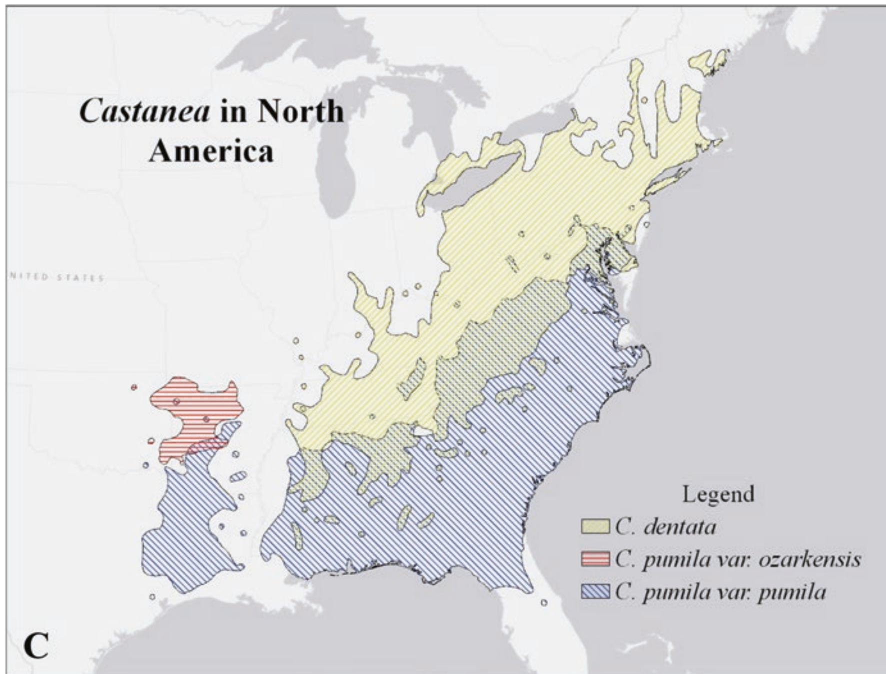

Fig. 5.1 (continued) *C. seguinii* and *C. henryi* are largely sympatric in eastern China. (**b**) Distribution of *C. sativa* in Europe. (**c**) Distributions of *C. dentata*, *C. pumila* var. *pumila* and *C. pumila* var. *ozarkensis* in eastern North America. (Source: Maps created by Erin Taylor using the distribution shapefiles of Fei et al. (2012) for Asian and European species and Little (1977) for North American species. Distribution of *C. crenata* in the Korean peninsula digitized by Erin Taylor using occurrence data from Barstow (2018))

ascomycete fungus *Cryphonectria parasitica* (Murr.) Barr, and within 50 years of its discovery had almost completely destroyed the American chestnut. Chestnut blight subsequently caused grave damage to the European chestnut throughout its native range (Anagnostakis 1987; Robin and Heiniger 2001). *Phytophthora* root rot—called ink disease in Europe—is caused primarily by the soil-borne oomycetes *Phytophthora cinnamomi* Rands and *P. cambivora* (Petri) Buisman, and has been a major limiting factor in chestnut culture in Europe and North America for at least 200 years (Crandall et al. 1945; Vettraino et al. 2005).

The most injurious insect pest of *Castanea* is the Asian chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu. The gall former oviposits in buds during mid-summer, and the larvae deform shoots and leaves at budbreak the following spring, cause drastic crop losses and, in severe cases, death of the tree. The insect is native to China but was accidentally introduced to Korea and Japan, and then into North America and Europe. Variation in host tolerance to *D. kuriphilus* has led to development of new gall-resistant chestnut cultivars.

In this chapter, we review historical developments and recent advances in chestnut breeding for chestnuts as a nut crop, and for restoration of the American chestnut as a functioning member of Appalachian forest communities. We note progress and improvements in nut quality, crop yields and, most importantly, in achieving host tolerance to chestnut blight, *Phytophthora* root rot, and Asian chestnut gall wasp. We also identify important unanswered questions and areas of inquiry that are likely to benefit chestnut breeders in the future.

5.2 Cultivation and Traditional Breeding

Chestnut trees have been cultivated for thousands of years for their sweet, edible nuts, and were valued for their wood and other products by traditional cultures in Europe, Asia and eastern North America (Avanzato and Bounous 2009; Davis 2003). Migrating peoples have carried chestnuts well beyond their areas of natural occurrence, as far as Chile in South America (Grau 2003), southeastern Australia (Casey and Casey 2009), New Zealand (Klinac and Knowles 2009) and the West Coast of North America (Craddock 2009). Chestnuts dried and ground into flour were once a staple food in Mediterranean kitchens, and especially so in the mountainous regions of Turkey, Portugal, Spain, France and Italy, and on the island of Corsica where chestnut culture became a veritable chestnut civilization (Cherubini 1981; Pereira-Lorenzo et al. 2009a, b; Smith 1950; Soylu et al. 2009; Squatriti 2013). Almost every material need was provided for by the chestnut tree, not just food but also timber, poles, wicker, tannin and fuel from the chestnut coppice, bedding for animals and even medicinal astringents from the leaves (Bourgeois 1992; Bruneton-Governatori 1984; Pitte 1986). Along with the grapevine and the olive tree, the European chestnut is one of the great pillars upon which Mediterranean civilization was built (Bellini and Nin 2009; Bignami and Salsotto 1983). The Romans transported chestnut trees to all parts of their empire and chestnut culture later underwent a tremendous expansion during the late classical and early medieval period (Cherubini 1981; Conedera et al. 2004a, b; Jarman et al. 2019, Squatriti 2013). Chinese and Japanese chestnut trees played similar and no less important roles in the civilizations of China, Korea and Japan (Qin and Feng 2009; Saito 2009). A storehouse full of dried chestnuts represented food security – and wealth. Chestnuts were insurance against drought, flood, siege and any of the many other calamities that could befall an agriculture based on annual grain crops (Bruneton-Governatori 1984; Pitte 1986; Smith 1950; Squatriti 2013). Chestnut trees come very close to representing the archetypal tree crop (Smith 1950; Vieitez et al. 1996). They are very long lived; examples of *Castanea sativa* in Europe are known to be more than 1000 years old (Bounous 2002; Vieitez et al. 1996). And even at that great age, they continue to provide annual harvests of delicious, nutritious, easily gathered, easily stored food. Because a mature chestnut orchard resembles a temperate hardwood forest, in both structure and function, it provides significant ecological benefits with consequences far beyond merely producing a fruit (Vieitez et al. 1996).

The tree offers habitat to myriad animal species and acts as the foundation of its community. It anchors soils to steep slopes that would otherwise be almost completely unsuitable for other forms of agriculture (Smith 1950). The chestnut grove moderates the local climate by shading the understory, and transpiring water into the atmosphere. It serves as host for symbiotic relationships with fungi, including the choice edible *Cantharellus cibarius* and *Boletus edulis* mushrooms whose harvest provides a highly valued forest product to the land's stewards (Vieitez et al. 1996). These mutualistic symbionts join the ranks of other chestnut forest fungi involved in decomposition and recycling, mobilizing deep soil nutrients and minerals into the humus layers. The landscape value of the chestnut grove is almost unequalled by that of any other forest type (Bounous and DeGuarda 2002; Vieitez et al. 1996) possibly because the grove is evocative of a simpler, quieter past, when our relationship with nature was closer to the regular cycles of sun and moon and season (Fig. 5.2).

The result of centuries of cultivation and selection by growers is thousands of named varieties of *Castanea crenata*, *C. mollissima*, *C. sativa*, and their hybrids (Anagnostakis 2012; Miller et al. 1996; Pereira-Lorenzo et al. 2012; Rutter et al. 1991; Worthen et al. 2010). Dr. Sandra Anagnostakis, at the Connecticut Agricultural Experiment Station, maintains and updates the registry of chestnut cultivar names on her CAES web page: https://portal.ct.gov/CAES/ABOUT-CAES/Staff-Biographies/Sandra-L-Anagnostakis (Anagnostakis 2019).

Fig. 5.2 The mature chestnut orchard provides many of the same ecosystem services as the temperate hardwood forest. When the trees are widely spaced and pruned up, a rich grassland community can develop in the understory. (Photo by J.H. Craddock)

The history of chestnut breeding was recently reviewed by Pereira-Lorenzo et al. (2016) in their very thorough paper on interspecific hybridization. They discuss germplasm and genetic resources, and genetic diversity and mating systems for the major *Castanea* species, as well as the development of molecular markers and genome-wide approaches to improve selection for desired traits in hybrid progenies. There is a greater focus on cultivar and rootstock development in the review by Pereira-Lorenzo et al. (2012). A history of chestnut breeding in the United States is well illustrated in the review by Anagnostakis (2012) of work begun by Walter Van Fleet in 1894 (Van Fleet 1914). Worthen et al. (2010) provide an excellent historical and theoretical context for breeding and selecting *Castanea dentata* for resistance to *Cryphonectria parasitica* using modern population genetic and genomic tools. Recently, Westbrook et al. (2019) provided genomic and quantitative genetic evidence that phenotypic selection can be used to move alleles conferring disease resistance from Asian *Castanea* species into an American chestnut genetic background (Figs. 5.3, 5.4 and 5.5).

World chestnut production today is increasing. Global chestnut production (nuts and lumber products) declined precipitously in the late nineteenth and early twentieth centuries from a conspiracy of circumstances that included two world wars, two catastrophic diseases (chestnut blight, ink disease), the movement of invasive pests (such as the Asian chestnut gall wasp *Dryocosmus kuriphilus*), changing patterns of settlement and employment (increased urbanization, mechanization of farm labor) and changes in the world's economy and diet (Avanzato and Bounous 2009). But, the situation for chestnut is now improving after reaching a global nadir in the 1970s (Conedera et al. 2004b). Evidence of renewed interest since the 1990s is the number and frequency of international scientific meetings on chestnut worldwide (see the list of Acta on the ISHS web page). The availability of new, improved cultivars means that growers can now choose varieties resistant to the major pests and diseases that have plagued chestnut for much of the past century.

5.3 Germplasm Characterization and Conservation

The difficulty of conservation of *Castanea* genetic resources is compounded by chestnut blight, *Phytophthora* root rot, insect pests and habitat changes. Wild *Castanea* resources in situ are still plentiful but in every case there is special concern for genetic erosion. The Caucasus Biosphere Reserve in the (Republic of) Georgia harbors naturally occurring populations of *C. sativa* in an area thought to be close to the species' center of diversity. Similarly, the Great Smoky Mountains National Park in Tennessee and North Carolina represents a reservoir of diversity close to the area of greatest abundance for *C. dentata*. However, *C. parasitica* and *Phytophthora cinnamomi* and *P. cambivora,* and *Dryocosmus kuriphilus* are perennial threats to that germplasm. In situ conservation depends therefore not only on the political integrity of the Reserve and the Park, but also on the phytopathological conditions present at those locations. There is no such system of parks or reserves

Fig. 5.3 Canker severity on American chestnut hybrids, following inoculation with *Castanea parasitica* strain EP155 (virus-free) and two isogenic hypovirulent strains (CHV1-EP713 and CHV1-Euro7). Host response varies from susceptible to resistant. The susceptible phenotypes are shown on the left. The resistant phenotypes are on the right. The top row are half-sibling B_2F_2s (2nd backcross F_2 hybrids). The bottom row are full-sibling B3s (3rd backcross F_1 hybrids). (Photos by J.H. Craddock)

Fig. 5.4 Roguing susceptible progeny. In backcross breeding for *Castanea parasitica* resistance, American chestnut backcross hybrid progeny are inoculated in their 5th year, and selections are made from years 6 to 10. This selected third backcross tree had adequate blight tolerance, and the tree form and leaf and twig morphology of its *C. dentata* ancestors. (Photo by J.H. Craddock)

in place to conserve germplasm of *C. pumila* var. *pumila* or *C. pumila* var. *ozarkensis*, so conservation is largely the responsibility of engaged citizens and private, non-governmental organizations like The American Chestnut Foundation. Ex situ conservation is also fraught with difficulties. The same diseases and pests conspire to destroy collections of susceptible germplasm, whether it is grown on its own roots or grafted onto *Phytophthora*-resistant rootstocks. The costs and challenges of maintaining collections of grafted plants is exacerbated by the phenomenon of delayed graft failure, which is particularly problematic for some *C. mollissima* genotypes and for interspecific graft combinations. A list of institutions that currently maintain and/or have access to *Castanea* germplasm resources is listed in Appendix I.

5.3.1 Genetic Variation

Germplasm characterization and conservation in the genus *Castanea* has been an active part of chestnut breeding in Europe, Asia and North America (Alexander et al. 2005; Bolvansky and Tarinova 2009). Government and university research programs in each of these regions are currently involved in germplasm conservation (Bounous 2002; Mellano et al. 2018). An important tool in germplasm conservation

Fig. 5.5 Open pollination and controlled pollination can be used to produce seed from selected hybrids. In this illustration, hand pollinations are being used to intercross selected 3rd backcrosses to produce 3rd backcross F2 (B_3F_2) seed for Tennessee seed orchards. (Photo by J.H. Craddock)

work has been the use of molecular methods to decipher genetic relationships among cultivars, and within and among species. And because the wild species are an important source of variation for breeding programs, many research efforts have focused on elucidating genetic variation in natural populations (reviewed by Pereira-Lorenzo et al. 2012, 2016; Worthen et al. 2010).

Values reported for within genus estimates of diversity allow us to rank the species: *Castanea mollissima* is apparently the most highly diverse, and this is maybe not surprising considering its enormous geographic range and the supposed great age of its populations, followed closely by *C. pumila* sensu lato and *C. pumila* var. *ozarkensis*. An intermediate level of diversity is exhibited by populations of *C. crenata* (Nishio et al. 2011b) and *C. sativa* (Mattioni et al. 2013). The structure of this diversity within *C. sativa* varies strongly with geography and is indicative of gene flow from east to west (Mattioni et al. 2013). Within species, diversity for *C. sativa* is generally much higher in the eastern (older) populations in the Caucasus, and generally much lower in the western (younger) populations, particularly in those most strongly influenced by clonal propagation of cultivars such as found in Italy. *Castanea dentata* is apparently lowest in species-level diversity (Huang et al. 1994; Kubisiak and Roberds 2006). However, the values reported by Huang et al. (1994) and Kubisiak and Roberds (2006) may not accurately represent the variation present

in the southernmost regions of its range due to the low number of samples they collected. Chloroplast haplotype diversity in southerly populations of *C. dentata* are remarkably high, and chloroplast haplotype sharing has been documented between *C. dentata* and *C. pumila* (Shaw et al. 2012).

5.3.2 Japan

Castanea crenata is native to Japan (Fig. 5.1) and has been cultivated there for thousands of years. Descriptions of the oldest named cv. Chokoji appear in the literature in the late 1500s (Saito 2009). Cultivar development probably began with selection of wild chestnuts (Kotobuki 1994), but accelerated with urgency in the 1940s after the accidental introduction of gall wasp *Dryocosmus kuriphilus* from China. By the 1950s the gall-wasp resistant cvs. Tanzawa, Tsukuba and Ishizuchi were released. Ginyose, an older variety, was also recognized as resistant. Eventually the gall former adapted to the widely planted new varieties by overcoming their resistance. But, because of effective biocontrol of galls by the parasitoid *Torymus sinensis*, these cultivars are still grown today. Despite their adaptation to the soils and climate of Japan, and their high yields of very large chestnuts (the largest fruit of any chestnut species), these cultivars unfortunately suffer from a trait that was thought to characterize all forms of *C. crenata* – the nuts themselves are very difficult to peel and they have a thick, adherent, astringent and unpalatable pellicle. Hybridization efforts with the easy to peel *C. mollissima* from China did produce some cultivars – Riheiguri is one example – but for the most part the Chinese-Japanese hybrids were either not well adapted to the Japanese growing conditions or the nuts were of inferior quality. New screening techniques for rapid evaluation of pellicle peelability (Shoda et al. 2006) allowed for the discovery and release of the easy peeling cv. Porotan (Saito 2009) and a short list of other cultivars and selections (Takada et al. 2019). The genetics of the easy-peeling trait were worked out by pedigree analysis of the of the original crosses of two difficult-to-peel types, a half-diallel cross of Porotan and two other difficult-to peel cultivars, and a subsequent test cross of Porotan by its difficult-to-peel father and great grandfather Tanzawa (Takada et al. 2012). The authors conclude that the easy-peeling pellicle trait in *C. crenata* is controlled by a major recessive allele at a single locus. Takada et al. (2012) designated the easy-peeling locus *P/p* and offer evidence that the Tsukuba genotype is homozygous dominant (*PP*), the Tanzawa genotype is heterozygous (*Pp*), and the Porotan genotype is homozygous recessive (*pp*). They posit that the underlying mechanism of *P/p* in *C. crenata* may be different than the mechanisms underlying easy peeling in *C. mollissima*. This is based, in part, on the observation that all nuts produced by Porotan are easy to peel regardless of the pollen used to produce the nuts (Takada et al. 2010). This is in contrast with nuts produced by some easy to peel cultivars and hybrids of *C. mollissima* that are pollen-dependent, in other words, they produced difficult to peel nuts when pollinated by *C. crenata* but were easy to peel when pollinated by *C. mollissima*.

5.3.3 Korea

Castanea crenata also occurs naturally in Korea (Fig. 5.1). Although there is some speculation that *Korean native chestnuts* are hybrids between *C. mollissima* and *C. crenata*, and several taxon names have been proposed, most Korean cultivars are morphologically similar to their Japanese counterparts and many named cultivars are known (Kim 2006). Several Japanese cultivars of *C. crenata* were introduced to Korea subsequent to the appearance of the gall wasp *Dryocosmus kuriphilus* and are still widely grown there. Breeding efforts in Korea have focused on gall wasp resistance, chestnut blight resistance, and nut quality. Kim et al. (2005) announced the release of Daehan, a new blight-resistant, gall-wasp resistant cultivar with very high yields of large, good-quality nuts. The cross was made in 1980, preliminary selection was completed by 1990, advanced and regional trials were completed in 2003 and the cultivar was named Daehan in 2004 (Kim et al. 2005).

Variation in other agronomic and ornamental characteristics of *Castanea crenata* are also under selection by breeders (Kim et al. 2014, 2017; Saito 2009). Red leaves and red burs occur in some types and a new red bur cultivar was recently released in Korea (Kim et al. 2014).

5.3.4 China

Castanea mollissima is by far the most important of the Chinese species, as a nut crop, and from a breeding point of view (He et al. 2015). Vast amounts of morphological, physiological and genetic variation exists within the species (Huang 1998; Pereira-Lorenzo et al. 2016). Naturally occurring populations of *C. mollissima* are difficult to study because of the extent of alteration of the Chinese landscape by humans, and because the species exists almost entirely in cultivation (LaBonte et al. 2018). The other wild species in China include *C. henryi* and *C. seguinii*, and to a much lesser extent *C. crenata* (Fig. 5.1). Germplasm characterization of the Chinese species has recently included the complete chloroplast genome of *C. mollissima* cultivars (Zhu et al. 2019b), and complete chloroplast genome sequences for *C. henryi* (Gao et al. 2019) and *C. seguinii* (Zulfiqar et al. 2019). The response of *C. mollissima* to infestation by *Dryocosmus kuriphilus* was investigated by transcriptome analysis (Zhu et al. 2019a).

The ranges of *Castanea henryi* and *C. seguinii* largely overlap with that of *C. mollissima*, but they are found mostly in the wild state, or rarely cultivated locally (Fig. 5.1). Potentially useful genetic variation in tree form and habit, inflorescence type and phenology, and resistance to major diseases and pests exists in these two taxa (Gao et al. 2019; Zulfiqar et al. 2019). *Castanea henryi* is mostly important for its production of timber and other wood product, although the fruits, produced as a single nut per bur, are frequently found in markets throughout its range (Qin and Feng 2009). Some ecotypes of the naturally shrubby *C. seguinii* bloom more or less

continuously throughout the growing season, with bisexual catkins at every node along the branch, and thus may have ripening fruits and fully-blooming flowers present on a single plant at the same time (Rutter et al. 1991). *Castanea seguinii* is regularly coppiced for firewood, but the new shoots are very precocious and will be bearing nuts, which are sold in local markets, within a year or so of cutting (Rutter et al. 1991).

Hundreds of named varieties of *Castanea mollissima* are known and grown in China, the world's largest producer of chestnuts. Great variation exists regionally and many authors divide the cultivars into five separate regional groups: North, Northwest, Southwest, Southeast, and the Yangtze Valley Group. In the far northeast, adjacent to Korea, cultivars of *C. crenata* are grown. Many cultivars are propagated by grafting but seed propagation is also widespread. Cultivars in China are characterized by geographic origin (by regional group, by province or by growing area within province), fruit size and shape, quality, type and content of starches, density of kernels, and a long list of other agronomic characters and processing abilities. Yang et al. (2015) screened chestnut cultivars from ten different ecological regions. They tested a battery of compositional and nutritional factors and found cultivar differences and regional differences. In general, cultivars from the central regions (Hubei province) had higher total carbohydrate content. Cultivars from the deep south (Guandong and Zheijiang provinces) had the highest protein and polyphenol contents. Cultivars originating in the mid-south (Hunan) were highest in flavonoids and simple sugars, but lowest in fat content. A few of the best-known Chinese cultivars of *C. mollissima* are listed in Table 5.2.

5.3.5 Europe

Germplasm resources and characterization of European populations of *Castanea sativa* (Fig. 5.1) have been thoroughly studied and reviewed by Pereira-Lorenzo et al. (2006a, b, 2010, 2011, 2012). Mattioni et al. (2008) used genomic tools to investigate the active role of humans, over the past 9000 years, in determining not only the modern European distribution of *C. sativa*, but also the genetic structure of populations in naturalized forests, coppice stands (intensively managed for wood production), and chestnut orchards (managed for nut production). The species has ample genetic and adaptive variation across its wide geographic and climatic range, reflecting many distinct ecotypes (Casasoli et al. 2004, 2006; Martin et al. 2009; Villani et al. 2009). Boccacci et al. (2004) used SSRs developed in *Quercus* to type *C. sativa* cultivars. Costa et al. (2005) report the results of Portuguese chestnut germplasm characterization using SSRs. Variation exists in many traits of agronomic importance, including disease resistance (Vettraino et al. 2005). Native resistance to *Dryocosmus kuriphilus* in *C. sativa* has been reported recently in populations of grafted trees of the red salernitan ecotype (RSE) growing in the very important chestnut producing region of Campania, Italy (Nugnes et al. 2018). RSE is probably a group of closely related, morphologically similar cultivars including Mercoliana,

Table 5.2 Some examples of important commercially-grown chestnut cultivars, their species, and the areas of their principal cultivation

Cultivar	Type and important traits	Area of cultivation
Amarelal	*C. sativa* Tc	Portugal
Amarelante	*C. sativa* Tc	Spain
Arima	*C. crenata* Tc	Japan, Korea
Belle Epine	*C. sativa* Tc	France
Benton Harbor	*C. mollissima* Ft	Michigan
Bouche de Betizac	Euro-Japanese PRR, ACGW, Cp	Europe, Western USA
Bouche Rouge	*C. sativa* Tc	France
Bournette	Euro-Japanese hybrid PRR, Cp	Europe, Western USA
Colossal	Euro-Japanese hybrid CMS	California, Oregon, Michigan
Comballe	*C. sativa* Tc	France
Dabanhong	*C. mollissima* Tc	Northern China
Daebo	Chinese-Japanese hybrid ACGW	Korea
Eaton	Complex hybrid Cp	Eastern USA
Gideon	*C. mollissima* Cp Prr	Eastern USA
Ginyose	*C. crenata* Tc, ACGW	Japan, Korea
Guihuaxiaoli	*C. mollissima* Tc Cp PRR	Southern China
Hongyou	*C. mollissima* Tc Cp PRR	Northern China
Ishizuchi	*C. crenata* ACGW Cp Prr	Japan, Korea
Injerta	*C. sativa* Tc	Spain
Jinfeng	*C. mollissima* Cp PRR	Northern China
Jiujiazhong	*C. mollissima* Tc	China, widely grown
Judia	*C. sativa* Tc	Portugal
Labor Day	*C. crenata* Ft Cp PRR	Michigan
Longal	*C. sativa* Tc	Portugal, Spain
Marigoule	Euro-Japanese hybrid Cp PRR	Europe, Western USA
Marron d'Olargues	*C. sativa* Tc	France
Marrone di Chiusa Pesio[a]	*C. sativa* M![a] Tc	Italy, California
Martainha	*C. sativa* Tc	Portugal
Nanking	*C. mollissima* Cp PRR	Eastern USA
Negral	*C. sativa* Tc	Spain
Okkwanng	*C. crenata* Cp PRR	Korea
Pallumina[b]	*C. sativa* Tc[b]	Southern Italy
Parede	*C. sativa* Tc	France
Payne	*C. mollissima* Cp PRR	Eastern USA
Porotan	*C. crenata* Ep Cp PRR	Japan
Precoce di Roccamonfina	*C. sativa* Tc	Campania Region, Italy
Precoce Migoule	Euro-Japanese hybrid Cp PRR	Europe, Western USA
Qing	*C. mollissima* Cp PRR	Eastern USA
Riheiguri	Chinese-Japanese hybrid Cp PRR	Japan, Korea
Sandae	*C. crenata* Cp PRR	Korea
Sleeping Giant	Complex hybrid Cp PRR	Eastern USA

(continued)

Table 5.2 (continued)

Cultivar	Type and important traits	Area of cultivation
Tanzawa	*C. crenata* Cp PRR	Japan, Korea
Tsukuba	*C. crenata* Cp PRR	Japan, Korea
Verdeal	*C. sativa* Tc	Portugal

Important Traits: *ACGW* Resistance to Asian chestnut gall wasp, *CMS* cytoplasmic male sterility, *Cp* Resistance to *Cryphonectria parasistica*, *Ep* Easy-Peel, *Ft* freeze tolerance, *M!* Highest quality Marrone cultivar, *PRR Phytophthora* root rot resistance, *Tc* traditional cultivar

[a]The many Italian Marrone cultivars are all very similar, homogeneous genetically, and are differentiated primarily by provenance; famous examples include Marrone di Val di Susa, M. di Luserna, M. di Caprese Michelangelo, M. di Castel de Rio, M. di Marradi, etc

[b]Marketed under the trade name Castagna di Montella IGP (see text for details)

Rossa di San Mango, Verdola and Palummina which differ, curiously, in their response to the gall former. The observation of resistance in RSE was based on lower oviposition rates, higher rates of larval mortality, fewer galls formed and smaller galls with higher rates of predation by *Torymus sinensis*. Prior to this report, there have been only two other indications of potentially resistant *C. sativa* cultivars; Pugnenga from Cuneo, and Savoye, from France (Sartor et al. 2015). The cultivar Palummina is marketed as, and protected by, and is the principal component of the Castagna di Montella IGP protected area, one of the oldest, most recognized and most prestigious denominations of geographic origin in Italy (Regione Campania 2015). Some of the *C. sativa* and hybrid cultivars most widely grown in Europe are listed in Table 5.2.

5.3.6 North America

There are currently recognized two species of *Castanea* naturally occurring in North America: *C. dentata*, the American chestnut, and *C. pumila* sensu lato, the perplexingly variable chinquapins (Craddock 1998; Miller et al. 1996; Perkins 2016; Rutter et al. 1991) (Fig. 5.1). Chinquapins vary widely in leaf shape and vestiture, tree stature, habit and form, across a wide geographic area. Along the Atlantic and Gulf coastal plains, most individuals are multi-stem shrubs maturing at less than 5 m tall. In the fire-prone sand hills of northern Florida, there are populations of *C. pumila* that mature at less than 2 m tall, forming 20-m wide or even larger thickets – each thicket composed apparently of a single, stoloniferous genet whose horizontal stems grow flat upon the ground and root themselves into the ground as they grow (Fig. 5.6). In the Appalachian Mountains, chinquapins are taller and more frequently fewer-stemmed. Some forms in northern Alabama reach heights of 10 m. The tallest chinquapins, in the pre-blight era, were recorded from the Ozark uplands of Arkansas and Missouri, and have been designated as a distinct taxon, *C. pumila* var. *ozarkensis*. The defining characteristic of *C. pumila* sensu lato is that it typically

Fig. 5.6 Trailing chinquapins. At the extreme southernmost edge of its natural distribution, *Castanea pumila* assumes the habit of a stoloniferous shrub about 1 m tall, that forms large thickets up to 20 m (or greater) diameter, each apparently composed of a single genet. This photo was taken in Suwannee County, Florida and shows two adjacent genets that differ in height. Southern ecotypes of *C. pumila* have been assigned to several different taxa since their nineteenth century descriptions, but here we place them in *C. pumila* sensu lato pending further phylogenetic analysis at the population level. (Photo by J.H. Craddock)

produces a single nut per involucre, and that the involucre opens along a single suture into two valves (Fig. 5.7).

Only *Castanea dentata* was ever of any real economic importance, as a valuable source of excellent timber and for its delicious and nutritious nuts (Jaynes 1979; Smith 1950). *Castanea dentata* can grow to be a large tree, while *C. pumila* is a highly variable but usually shrubby, small tree (Paillet 1993). Both species had and continue to have important ecological functions in the forests of the eastern United States (Oak 2002, 2006; Southgate 2006). The chestnut blight pandemic, caused by *Cryphonectria parasitica*, completely removed *C. dentata* from its ecological niche in the forest canopy within 50 years of its first discovery in New York in 1904 (Anagnostakis 1987; Roane et al. 1986; Smith 2000). The rot-resistant remains of fallen giant American chestnut trees may still be found throughout eastern North America. But chestnuts are not extinct; the blight fungus only kills the shoots, not the roots. Although it continues to sprout new shoots from the bases of blight-killed stems, survival of the species varies greatly from site to site (Griffin 2000).

Fig. 5.7 The pistillate inflorescence of *Castanea pumila* will bear up to 9–12 (or more) cupules. Each cupule develops into a prickly involucre containing a single nut. (Photo by J.H. Craddock)

Populations of *Castanea dentata* persist as understory shrubs, suppressed by shade and eventually by blight (Griffin 1992; Parker et al. 1993). Many of the surviving specimens appear as clumps of multiple stems arising from a common root system (clones), although the connections are not always evident. The largest stems are often heavily cankered with blight and usually die before nuts are produced, however, individuals do rarely escape blight long enough to bloom (McWilliams et al. 2006; Stephenson et al. 1991). Surviving American chestnut trees, such as those frequently encountered in the mountains, are valuable for many reasons. Their potential as parents in a backcross breeding restoration program will confer local adaptation to the hybrids and ensure conservation of the native population's genetic diversity (Hebard 2006). Even rarer are large surviving American chestnut trees (LSAs), characterized by swollen cankers with abundant callus. These very rare LSA individuals likely survive due to a combination of favorable circumstances including infection with attenuated (hypovirulent) strains of the blight fungus, ideal site conditions, and possibly a modicum of blight resistance (Fulbright 1999; Griffin 2000; Kolp et al. 2017). Overall, genetic diversity in *C. dentata* appears to be less than that of the other native species *C. pumila* sensu lato, and much lower than that of the Chinese chestnut, *C. mollissima* (Dane et al. 1998; Huang et al. 1994; Worthen et al. 2010).

The apparent distribution of genetic diversity in *Castanea dentata* is such that most of the variation can be found within populations, as is true for metapopulations of many wind-pollinated species that occur over broad ranges (Kubisiak and Roberds 2006), although recent work has shown much greater diversity in the

southernmost populations, indicative of gene flow from southwest to northeast along the main axis of the Appalachian Mountains (Binkley 2008; Gailing and Nelson 2017; Li and Dane 2013; Shaw et al. 2012). The dominance of *C. dentata* in the forest canopy followed by its almost complete disappearance has caused major, long-term changes to the structure and health of the eastern North American forests (Oak 2002, 2006). Understanding the reproductive biology and natural ecology of *C. dentata* will be essential for its successful reintroduction (Dalgleish et al. 2016; Phelps et al. 2006; Wang et al. 2013). From a floristics perspective *C. dentata* is separated from *C. pumila* primarily by the number of pistils per cupule (Fig. 5.8). In the absence of flowering shoots the shrubby, understory post-blight form and habit of the two taxa can be confounding (Shaw et al. 2012).

Many authors have suggested that interspecific hybridization has played a role in forming the highly diverse North American chinquapins (Binkley 2008; Camus 1929; Dode 1908; Elias 1971; Kubisiak and Roberds 2006; Li and Dane 2013; Little 1977; Nixon 1997; Shaw et al. 2012). Chloroplast haplotype sharing between species does occur in areas of sympatry for *Castanea dentata* and *C. pumila* in the southern Appalachian region (Binkley 2008; Li and Dane 2013; Perkins 2016; Shaw et al. 2012), however, the relative importance of interspecific hybridization versus retention of polymorphisms predating speciation has not been investigated.

Fig. 5.8 The pistillate inflorescences of *Castanea* are contained in prickly involucres (the cupules) that typically bear three pistils, and are found at the base of the bisexual catkins. Illustrated here are the pistillate flowers of *C. dentata* at full bloom (peak of pollen receptivity). (Photo by J.H. Craddock)

The commercial chestnut industry in North America is based entirely on introduced germplasm (Craddock 2002, 2009; Craddock and Pellegrino 1992). Nut production is divided into two very separate geographic regions with very different climates, soils and cultures: The East and the West (Nave 1998). The native and introduced *Castanea* species found growing in North America today are listed in Table 5.1. The native species *C. dentata*, and *C. pumila*, which occur naturally only in the East, are not important as nut crops. Commercial cultivars available through the nursery industry are listed in Table 5.2. Distribution of cultivars varies by region: in the West, the most widely-grown cultivars of *C. sativa* and Euro-Japanese hybrids are of recent introduction. Craddock and Pellegrino (1992) list the cultivars they successfully sent through the required 2-year post-entry quarantine. Of those on the list, Marigoule, Bouche de Bétizac and several Italian Marrone cvs. are now grown commercially in Washington, Oregon, California and Michigan. In the East, varieties and hybrids of *C. mollissima* dominate (Craddock et al. 2005). Some varieties, however, are grown only to a very limited extent (one or two growers). California and the Pacific Northwest states of Oregon and Washington produce chestnuts in an area of ideal climate, free of chestnut blight, and free of most of the major insect pests that plague chestnut growers in the East (Table 5.3). The single most important cultivar in the western region is the Euro-Japanese variety Colossal, which is vigorous and very productive. Chestnut flour made from dried Colossal is available commercially. Several western growers are topworking Colossal orchards to the recently introduced Italian Marrone varieties because of the higher nut quality of the Marrone types. Michigan represents a chestnut growing area typical of the American Midwest: very cold winters and hot, humid summers. Although Colossal is grown to a limited extent in the Midwest, it does not have suitable blight resistance for sustained harvests. Most Midwestern production is based on *C. mollissima*. Traditionally, most orchards were planted as seedling trees, which vary greatly in all agronomic characters. Newer orchards include grafted trees of improved cultivars. Problems of limited availability of nursery stock and delayed graft failure of the

Table 5.3 Some injurious insect pests of chestnut

Common name	Order	Family	Species
Chestnut codling moth	Lepidoptera	Tortricidae	*Cydia splendana*
Asian chestnut gall wasp	Hymenoptera	Cynipidae	*Dryocosmus kuriphilus*
Chestnut weevils	Coleoptera	Curculionidae	*Curculio* spp.
Clearwing moth	Lepidoptera	Sesiidae	*Synanthedon vespiformis*
Fire ants[a]	Hymenoptera	Formicidae	*Solenopsis* spp.[a]
Granulate ambrosia beetle	Coleoptera	Scolytidae	*Xylosandrus crassiusculus*
Gypsy moth	Lepidoptera	Eribidae	*Lymantria dispar*
Japanese beetle	Coleoptera	Scarabeaidae	*Popillia japonica*
Polyphemus moth	Lepidoptera	Saturniidae	*Antheraea polyphemus*
Shothole borers	Coleoptera	Scolytidae	*Scolytus* spp.
Yellownecked caterpillar	Lepidoptera	Notodontidae	*Datana ministra*

[a]More likely to attack chestnut growers than chestnut trees

grafted trees have slowed the development of the Midwestern commercial chestnut industry. The University of Missouri Center for Agroforestry has had extensive chestnut cultivar trials underway since 1996. Recent work on cultivar evaluation has uncovered several promising cultivars of *C. mollissima* (Hunt et al. 2004). Shing has large nuts and is exceedingly vigorous and productive. Qing, Payne, Eaton, Homestead and Sleeping Giant all have great commercial potential (Metaxas 2013). In the Appalachian region, where there is still considerable nostalgia for the American chestnut, several cultivars of *C. mollissima* have been developed locally, including Amy, Gideon, Kohr, Mossbarger and Peach. Chinese chestnut is also employed in agroforestry and silvopasture applications in the small farms of Appalachia (Lovell et al. 2018) (Fig. 5.9).

The introduction of Asian chestnut gall wasp into Georgia in the 1970s effectively destroyed the chestnut orchard industry there, but populations of the parasite are apparently now under biocontrol due to the parasitoid wasp *Torymus sinensis*. However, the Southeast has produced several outstanding cultivars of merit, most notably Nanking. Metaxas (2013) presents results of a formal chestnut cultivar evaluation and breeding program at the University of Tennessee at Chattanooga (Fig. 5.10). Qing, Shing and Payne appear outstanding among the *Castanea mollissima* cultivars for precocity and yield. Qing and Payne (Fig. 5.11a) are compact plants and Shing is extremely vigorous. Gideon may be

Fig. 5.9 Sheep are particularly well suited to the chestnut orchard, and in a managed silvopasture system, can contribute significantly to a diversified farm economy. (Photo by J.H. Craddock)

Fig. 5.10 The Euro-Japanese cv. Marigoule was used in crosses with *Castanea mollissima* cv. Gideon in an effort to combine the good tree form and high yields of cv. Marigoule with the better disease resistance and excellent fruit quality of Gideon. (Photo by J.H. Craddock)

Fig. 5.11 *Castanea mollissima* cv. Payne produces an excellent quality nut on a semi-dwarf tree that may be suitable for high density orchard plantings (**a**). *Castanea mollissima* cv. Gideon produces regular crops of an excellent quality, medium sized nut. The cultivar is widely adapted for cultivation across eastern North America (**b**). (Photos by J.H. Craddock)

the best all-around cultivar (Fig. 5.11b) for the eastern United States. Colossal showed great promise for precocity and nut size among the hybrid cultivars but soon proved too susceptible to chestnut blight. Eaton and Sleeping Giant have produced very attractive and good-flavored nuts although Eaton may not have adequate blight tolerance and has suffered from delayed graft failure in Tennessee (Metaxas 2013).

5.4 Cytogenetics

Early investigations of genetic differences between *Castanea* species involved cytogenetic methods. Jaynes (1962) performed chromosome counts in accessions representing each *Castanea* species and nine interspecific hybrids. He found that all *Castanea* species and most of the interspecific hybrids had a somatic chromosome number of 2n = 2× = 24. However, triploidy and aneuploidy were observed in two interspecific hybrids resulting from crosses between *Castanea* species from North America and Eastern Asia (Jaynes 1962). More recently, work by Islam-Faridi et al. (2009, 2011, 2016) has revealed evidence of structural chromosomal differences between *Castanea* species. Islam-Faridi et al. (2011) found that the satellite region distal of the major 18S–28S ribosomal DNA (rDNA) locus in *C. mollissima* is larger than its counterpart in *C. dentata*. By using florescence in situ hybridization (FISH) to investigate a hypothesized reciprocal translocation between chestnut species, Islam-Faridi et al. (2016) found evidence that the *C. mollissima* cv. Vanuxem is heterozygous for a translocation involving linkage groups (LG) H and L.

In addition to the cytogenetic work indicating structural chromosomal differences between *Castanea* species, Sisco et al. (2014) recently shed light on cytoplasmic genome variation among chestnut species. Male sterility in chestnut was observed in the progeny of many intra- and interspecific crosses in the genus (Jaynes 1963, 1964; McKay 1942; Omura and Akihama 1980; Soylu 1992). In North America, male sterility has presented an obstacle to the introgression of disease resistance from *C. mollissima* to *C. dentata* because the male sterile phenotype has been observed in F_1, BC_1 and BC_2 progeny descended from interspecific crosses between *C. dentata* and *C. mollissima* (Shi and Hebard 1997; Sisco et al. 2014) (Fig. 5.12). As a result, chestnut breeders involved in breeding *C. dentata* for blight resistance were limited in crossing designs that would produce male-fertile progeny. By analyzing noncoding chloroplast DNA sequences from the parents and progeny of 17 F_1 and BC_1 crosses involving C. *dentata*, *C. mollissima* and *C. crenata* in various combinations, Sisco et al. (2014) found that male sterility in interspecific hybrids was correlated perfectly with the D chloroplast haplotype of *C. dentata*. Interestingly, *C. dentata* trees containing non-D chloroplast haplotypes (e.g. M and P chloroplast haplotypes) did not produce male-sterile progeny when crossed with *C. mollissima*. Trees with these non-D haplotypes were primarily found in the southern half of the range of *C. dentata*.

Fig. 5.12 The longistaminate catkins in *Castanea dentata* usually reach anthesis before the bisexual catkins do, and before the stigmas of the pistillate flowers are pollen receptive (**a** and **b**). Cytoplasmic male sterility in interspecific hybrids is frequently encountered with certain cytotype/genotype combinations. Many American x Chinese hybrids exhibit completely male-sterile, astaminate catkins, as shown here in a B_2F_2 at full bloom (**c** and **d**). (Photos by J.H. Craddock)

Based on the association of the male-sterile and male-fertile phenotypes (Fig. 5.12) with distinct chloroplast haplotypes of *C. dentata*, the authors posited that the sterility observed in hybrids between *C. dentata* and east Asian *Castanea* spp. is cytoplasmic male sterility (CMS), resulting from an interaction between mitochondrial genes inherited from *C. dentata* and nuclear genes inherited from *C. mollissima*. The findings of Sisco et al. (2014) were of practical value to chestnut breeders because they demonstrated that (1) substantial cytoplasmic diversity of phenotypic significance exists across the range of *C. dentata* and (2) because male sterility in crosses between *C. dentata* and Asian *Castanea* spp. is of the CMS type, male-fertile hybrids can be produced by using D cytotype *C. dentata* as the male parent in crosses with East Asian *Castanea* spp. or by using M or P cytotype *C. dentata* in any combination with East Asian *Castanea* spp. (Sisco et al. 2014).

5.5 Molecular Breeding

5.5.1 Genetic and Genomic Methods in Chestnut Breeding

The recent availability of molecular genetic and genomic tools has allowed investigators to address several recurring questions in the field of chestnut breeding. What is the genetic architecture of resistance to the various pathogens of chestnut? Are genes that confer resistance to one pathogen tightly linked to genes controlling resistance to other pathogens or other desired phenotypes? Can selection strategies informed by molecular genetic and genomic methods produce desired phenotypes more quickly than breeding programs based entirely upon phenotypic selection? Over the past few decades researchers have used quantitative trait locus (QTL) mapping to identify genomic regions controlling several traits of importance for chestnut breeders and growers—namely, resistance to chestnut blight, resistance to *Phytophthora* root rot, nut traits and adaptive traits (Kubisiak et al. 1997, 2013; Nishio et al. 2018b; Santos et al. 2017b; Zhebentyayeva et al. 2014). Comparative genomic and transcriptomic studies have proposed candidate genes for disease resistance (Barakat et al. 2009, 2012; Kubisiak et al. 2013; Serrazina et al. 2015). And, most recently, the availability of genomic resources is beginning to make possible the use of genomic selection in chestnut breeding (Nishio et al. 2018a; Steiner et al. 2017; Westbrook et al. 2019). Nishio et al. (2018b) report genetic maps and QTLs for eight traits based on analysis of single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs) in two breeding populations of *Castanea crenata* cultivars and selections. They showed very strong effects for QTLs associated with harvest date and pericarp splitting, and predict that marker-assisted selection can greatly improve breeding efficiency (Nishio et al. 2018b). Pereira et al. (2012) have reviewed the integration of new biotechnologies into chestnut breeding including molecular markers for selection at the genome level. Historically, isoenzymes, random amplified polymorphic DNAs (RAPDs) and amplification fragment length polymorphisms (AFLPs) were used. More recently SSRs and SNPs have come to the fore. SSR and SNP depend on four primary sources of genomic information: expressed sequence tags (ESTs); genomic DNA libraries enriched for repeating sequences; bacterial artificial chromosomes (BACs) and, increasingly, whole genome sequences (Fang et al. 2013).

5.5.2 Molecular Approaches for Blight Resistance Breeding

In North America, molecular breeding approaches have been used solely within the context of *Castanea dentata* restoration efforts (Burnham et al. 1986; Diskin et al. 2006; Westbrook 2018a, b; Westbrook et al. 2019; Wheeler and Sederoff 2009;

Worthen et al. 2010). In an early paper describing his plan to use the backcross method to introgress blight resistance from *C. mollissima* into *C. dentata*, Burnham (1988) proposed that restriction fragment length polymorphisms (RFLPs) could be used to accelerate the breeding program by selecting backcross progeny that most closely resemble the genotype of the *C. dentata* recurrent parent, then subjecting the marker-selected progeny to phenotypic selection for chestnut blight resistance. Bernatzky and Mulcahy (1992) expanded upon the proposal to use marker-assisted selection (MAS) in *C. dentata* breeding by highlighting the need for genetic linkage maps that can be used to identify DNA markers linked to the genes controlling blight resistance.

Kubisiak et al. (1997) were the first in North America to use genetic mapping to assist chestnut breeding efforts. They used isozyme, RFLP and random amplified polymorphic DNA (RAPD) markers to create a genetic linkage map for an F_2 family derived from two F_1 crosses between *Castanea dentata* and *C. mollissima* and used phenotypic data from inoculation of the F_2 progeny with chestnut blight fungus to identify seven genomic regions that appeared to condition a resistance response in the host. Only three of the seven regions were found to have an intermediate to large effect on resistance. The study of Kubisiak et al. (1997) was encouraging to chestnut breeders because it provided evidence that high levels of blight resistance could be conferred by a limited number of genomic regions, thus indicating that introgression of blight resistance from *C. mollissima* into *C. dentata* may be a realistic objective.

Taking advantage of new genomic resources, Kubisiak et al. (2013) proposed a list of candidate genes for blight resistance that are located within the three QTL intervals identified in the earlier work of Kubisiak et al. (1997). By comparing a genetic map of *Castanea mollissima* with the peach (*Prunus persica* (Rosaceae)) reference genome assembly, Kubisiak et al. (2013) found multiple segmentally homologous regions in the peach genome that span the three blight resistance QTLs in chestnut. The authors found that two of these regions in the annotated peach genome sequence contain genes for resistance to powdery mildew disease. Kubisiak et al. (2013) posited that the orthologous relationships supported by syntenic positions and sequence similarities between peach and chestnut suggest that these genomic regions may contain a set of conserved (prior to the divergence of Fagaceae and Rosaceae) genetic elements whose products respond to fungal invasion.

The development of *Castanea mollissima* genomic resources (Cannon et al. 2017; Sisco et al. 2005) by plant breeders has positioned the Chinese chestnut as a model organism for the study of evolution of woody plants and their relationships to other more closely related herbaceous species. Staton et al. (2015) used the reference genome to focus on disease resistance QTLs of potential importance to chestnut breeders and compare them to sequence data from other species. The unexpected levels of synteny preservation found among unrelated tree species imply that life history may play a more important role than has previously been recognized (Staton et al. 2015).

Recently, genomic selection has been proposed to accelerate recovery of blight resistance in The American Chestnut Foundation's (TACF) backcross breeding

program (Steiner et al. 2017; Westbrook 2017, 2018a, b). In the past, TACF has used progeny testing to identify advanced backcross (BC_3F_2) trees possessing high levels of blight resistance to supply seed for forest plantings. At current rates of progeny testing, it is expected to take 50 years to complete selection on the 5000 mother trees in TACF's seed orchards (Westbrook 2017). Genomic selection, however, can greatly accelerate the process when used with accurate prediction models (Westbrook et al. 2019) (Figs. 5.13 and 5.14). Westbrook et al. (2019) used a genotyping by sequencing (GBS) approach to develop and apply a genomic prediction model to populations of segregating B_3F_2 American chestnut hybrids. They used a training population of more than 7000 B_3F_3 progeny of more than 500 open-pollinated, selected B_3F_2s. The B_3F_2s were descended from two *Castanea mollissima* sources of resistance (cvs. Clapper and Graves) and were planted in a seed orchard experimental design which had been screened by inoculation with two strains of *C. parasitica* (SG2-3, Ep155) and culled (Hebard 1994, 2006). The genomic predictions of progeny canker severity were as accurate as phenotypic estimates for Clapper B_3F_2s, but heritability was too low for the descendants of cv. Graves (Westbrook et al. 2019). Westbrook et al. (2019) conclude that the forthcoming chromosome-scale reference genome for *C. dentata*, combined with the genotypes for thousands of backcross individuals currently in the TACF state chapter programs, will greatly facilitate the elucidation of the *C. mollissima* contributions to blight resistance.

Fig. 5.13 American chestnut hybrid seed orchard. A minimum of 20 genetic families (non-first cousin lines) of B_3F_2 hybrid seeds are planted at very high density (0.6 m × 1.2 m) in 150-tree plots, in anticipation of high selection pressure. Genomic prediction of *Castanea parasitica* tolerance, which is currently under development, will allow us to choose the very best 2–5 trees per 150-tree plot. The fully selected seed orchard should contain 40–100 blight tolerant trees with *C. dentata* morphology. (Photo by J.H. Craddock)

Fig. 5.14 Three stages of selection in an American chestnut seed orchard. The B_3F_2 trees in the foreground are at the end of their first growing season after planting at very high density (0.3 m × 2 m). In the middle ground are 3-year-olds that have been inoculated with virus-free isolates of *Castanea parasitica*. The taller trees in the background are 6-year-olds that were selected for *C. parasitica* tolerance, tree form, and *C. dentata* leaf and twig morphological characters. (Photo by J.H. Craddock)

5.5.3 Molecular Approaches for *Phytophthora* Root Rot Resistance Breeding

Amid the growing realization that *Phytophthora* root rot (PRR) is an important obstacle to growth of chestnut in the southern United States, multiple groups began investigation of the genetic architecture of PRR resistance in the first decade of the twenty-first century. Kubisiak (2010) presented a major effect QTL for PRR resistance on chestnut linkage group (LG) E in BC_1 progeny derived from crossing a PRR-susceptible *Castanea dentata* individual with a *C. dentata-mollissima* F_1 descended from the *C. mollissima* cv. Nanking source of PRR resistance. In a study of multiple BC_1 and BC_4 mapping populations descended from PRR resistance sources *C. mollissima* cv. Nanking and *C. mollissima* cv. Mahogany, Zhebentyayeva et al. (2014) identified a major effect QTL for PRR resistance on LG_E, providing further support of the hypothesis of a limited number of genomic regions controlling PRR resistance.

Because PRR is also a major impediment to cultivation of *Castanea sativa* in Europe, genetic and genomic tools have been used to understand and employ PRR-resistance in breeding of this species (Robin et al. 2006). Santos et al. (2015b, 2017b) developed and used SSR and SNP markers to map QTLs for PRR resistance in two F_1 families derived from interspecific crosses between the PRR-susceptible species *C. sativa* and the PRR-resistant species *C. crenata*. Santos et al. (2017b) identified QTLs for PRR resistance on LG_E and LG_K, which was consistent with preliminary results obtained by Zhebentyayeva (pers comm to Santos (2017b)) from descendants of crosses between *C. dentata* and *C. mollissima*. Santos et al. (2017b) hypothesized that the finding of common resistance QTLs within *C. crenata* and *C. mollissima* may indicate shared resistance mechanisms to fungal or fungal-like pathogens across the *Castanea* genus.

In Europe, comparison of the root transcriptomes of PRR-susceptible *Castanea sativa* and PRR-resistant *C. crenata* after inoculation with *Phytophthora cinnamomi* resulted in the identification of 283 genes that are differentially expressed in response to the pathogen. Santos et al. (2017a) filtered this list of 283 differentially expressed genes to identify 8 candidate genes for resistance to *P. cinnamomi*. Using digital polymerase chain reaction (dPCR), Santos et al. (2017a) identified one gene, *Cast_Gnk2-like*, that best discriminates between susceptible and resistant genotypes. Santos et al. (2017a) also found that pre-formed defenses are crucial for the resistance of *C. crenata* to *P. cinnamomi* infection.

Perkins et al. (2019) report on the discovery of new sources of host resistance to *Phytophthora cinnamomi* in the TACF backcross breeding program (Fig. 5.15). They screened ten interspecific hybrid families in a nursery setting, and although the primary scope of their effort was introgression of disease resistance into *Castanea dentata* for ecological restoration purposes, the first backcross progeny used in the trial all descend from *C. mollissima* cultivars of merit: Amy, Byron, Gideon, Lindstrom-99, Payne and Petersburg and so on may be of interest in the development of new nut cultivars with the flavor profile of *C. dentata* (Perkins et al. 2019).

5.5.4 Genetic Resources for Understanding Insect Resistance

Alma (2002) lists more than 40 kinds of insects and mites known to cause damage to *Castanea* species. They attack the roots, bark, twigs, leaves and fruits. Some of the most injurious pests are listed in Table 5.3. On a global scale, the chestnut gall wasp, *Dryocosmus kuriphilus* is surely the one insect that causes the most damage to chestnut production; when introduced from China to Japan in the 1940s, it caused drastic reductions in the harvest of *C. crenata* (Saito 2009), and soon after its discovery in the state of Georgia, USA, commercial production based on *C. mollissima* was almost completely wiped out (Payne et al. 1983). Its accidental introduction to Italy was quickly followed by a similar, catastrophic drop in yield – some areas in Cuneo Province lost the entire crop within a few years of infestation (Brussino et al.

Fig. 5.15 Resistance to *Phytophthora cinnamomi* can be determined by screening trees in containers during their first year of growth in the nursery. Resistant progeny are then transplanted into PRR symptomatic orchards for screening against *Cryphonectria parasitica.* Phenotypic selection at the F_1 and BC_1 generations was sufficient to improve the average PRR resistance for these lines (Perkins et al. 2019). These first backcross American hybrids descend from a 2004 cross of a wild type Tennessee *C. dentata* x *C. mollissima* cv. Gideon. The selected F_1s were backcrossed to other wild type Tennessee *C. dentata* in 2014. (Photo by J.H. Craddock)

2002; Sartor et al. 2015). A biocontrol, based on the parasitoid wasp *Torymus sinensis*, native to China, is currently deployed in Japan, Korea, Italy and USA, but the gall wasp remains problematic; episodic infestations continue to impact harvests worldwide, and the pest continues to spread in Europe (Ferracini et al. 2019; Gehring et al. 2018; Quacchia et al. 2008; Sartor et al. 2015). The best long term control may be genetic host resistance to infestation. Variation in gall wasp tolerance was first explored in the 1940s in Japan (Oho and Shimura 1970), and several gall-wasp resistant selections of *C. crenata* were released as cultivars in the 1950s (Saito 2009; Shimura 1972). Despite the continued ravages of the insect and its apparent ability to overcome resistance, Tsukuba, Tanzawa, Ishizuchi and Ginyose are still the most widely grown cultivars in Japan today (Pereira-Lorenzo et al. 2012; Saito 2009). The better, more resistant cvs. Kunimi and Shiho, were released in the 1980s but they are not so widely grown because about the time of their first availability the gall wasp parasitoid *T. sinensis* was released and began effecting biocontrol of *D. kuriphilus*. Since the 1990s, the focus of the Japanese chestnut breeders has returned to improvements in nut quality, particularly ease of pellicle removal in cultivars of *C. crenata* (Nishio et al. 2013; Saito 2009).

Dini et al. (2012) report on the discovery of a possibly novel mechanism for gall wasp resistance in the Euro-Japanese hybrid Bouche de Bétizac. They describe what they interpret to be a hypersensitive response in swelling buds to infestation by *Dryocosmus kuriphilus*. They used 3,3′-diaminobenzidine (DAB) in an assay for in vivo production of H_2O_2, itself an indicator of the stress-response glycoproteins germin and germin-like proteins (GLPs). GLPs are markers for stress response gene products and have oxalate oxidase (OxO) activity related to programmed cell death and the hypersensitive response (HR). They observed a positive DAB response in all buds of the resistant cv. Bouche de Bétizac but in none of the buds of the susceptible cv. Madonna, regardless of infestation state. They detected production of putative GLP using Real Time PCR. The observed hypersensitive response in Bouche de Bétizac has allowed the cultivar to remain free of galls after more than 10 years of exposure (Dini et al. 2012).

The molecular basis of *Castanea mollissima* responses to *Dryocosmus kuriphilus* infestations was very thoroughly investigated by Zhu et al. (2019a) through transcriptomic analysis of differentially-expressed genes during the different gall-formation stages in the life cycle of the wasp. In response to attack by the gall former, plant responses included plant hormone signaling, changes in transcription products, stimulation of Ca^{2+}-mediated signal transduction pathways, and activation of secondary metabolic and other stress induced changes. The authors conclude that the genes involved represent *C. mollissima* gall defense genes (Zhu et al. 2019a).

5.5.5 Deciphering the Genetics of Agronomic and Nut Traits

The genetic architecture of important agronomic and nut traits in *Castanea crenata* and *C. mollissima* has been investigated by researchers in Japan, China and the USA. Researchers at the NARO Institute of Fruit Tree and Tea Science, Japan, used genetic linkage mapping and pedigree analysis to understand genetic control of nut traits (Nishio et al. 2011a, 2014b). The easy-peeling pellicle trait in the *C. crenata* cv. Porotan, the only commercial cultivar of *C. crenata* that possesses this trait, was investigated by Nishio et al. (2013) and Takada et al. (2019). Nishio et al. (2013) found that the easy-peeling pellicle was conferred in their *C. crenata* breeding populations by a recessive allele at a single gene. They also identified multiple SSR markers tightly linked to this locus. The difficulty of pellicle removal in *C. crenata* is a major disadvantage for commercial use of the species; thus, the SSR markers developed by Nishio et al. (2013) and the germplasm discovered by Takada et al. (2019) are currently being used to breed new *C. crenata* cultivars with easy-peel nuts (Nishio et al. 2018a, b; Takada et al. 2019).

With MAS for easy-peeling pellicles currently in use at the Institute of Fruit Tree and Tea Science, an additional objective of the breeding program is to release cultivars with easy-peeling pellicles and different nut harvesting dates from *Castanea crenata* cv. Porotan (Nishio et al. 2018b). For this purpose, Nishio et al. (2018b) used QTL mapping to study the genetic control of several important agro-

nomic and nut traits in *C. crenata*: nut harvest date, nut weight, pericarp splitting, peach moth (*Conogethes punctiferalis*) infestation, number of burs per tree, number of nuts per tree, trunk diameter and yield per tree. One significant QTL each was identified for nut weight, peach moth infestation, number of burs per tree, number of nuts per tree, trunk diameter and yield per tree, while multiple significant QTLs were identified for both harvest date and pericarp splitting. The percentage of total phenotypic variance explained by the QTLs for harvest date and pericarp splitting was high (47.5–60.8% and 33.4–41.7%, respectively) and the SSR and SNP markers used in the study are expected to have immediate use for MAS in *C. crenata* breeding (Nishio et al. 2018b). Next, Nishio et al. (2018a) revisited study of many of these traits using a genome-wide association study on 99 phenotypically diverse *C. crenata* cultivars. Of the 12 total QTLs identified, 4 were associated with harvest date, 4 with nut weight, 3 with peach moth infestation and 1 with pericarp splitting. Accuracies of genomic selection were high for nut harvest (0.841) and moderate for peach moth infestation (0.604), indicating that genomic selection may reduce the cost of phenotypic evaluation of these traits by allowing selection at the seedling stage.

Microsatellite-based paternity analyses, using SSRs, allowed Nishio et al. (2014a, 2019) to estimate the effective pollen dispersal distance for cross-pollination in orchards of *Castanea crenata* cultivars. Because many Japanese chestnut cultivars are difficult to peel, and because it is difficult to separate the easy-peel nuts of cv. Porotan from the difficult-to-peel types after they have been harvested, the recommendation is to plant cv. Porotan alone in solid rows or blocks so that the easy-peel nuts can be harvested separately. The authors note that the effective pollen distance is not very far and thus suggest that the pollinizer varieties should be in adjacent rows or staggered in the row of the main cultivar (Nishio et al. 2014a, 2019).

QTL mapping has been used to facilitate marker-assisted selection in *Castanea mollissima* breeding programs in China where the desired traits mostly concern nut quality and harvest date. Ji et al. (2018) used the genotyping-by-sequencing method to construct a high density linkage map and identify QTLs for five nut traits. Three of the QTLs were associated with single nut weight, two were associated with nut thickness, five with nut width, one for nut height, and six QTLs associated with ripening period (Ji et al. 2018).

5.5.6 Future Prospects in Molecular Breeding of Chestnut

Despite the recent advances listed above, several important questions remain unanswered. Do the same genes control blight resistance in different chestnut species? Do the same genes control blight resistance in different cultivars within the same species? Are there different alleles encoding blight resistance within the

Fig. 5.16 The prickles on the bur of the American chestnut may be the longest, proportionately, of any species (**a**). The fruits of *Castanea dentata* are typically covered in silky hairs (**b**). (Photo by J.H. Craddock)

same loci? Are there different loci for *Phytophthora* root rot resistance across different species and cultivars? What about viruses? Although the focus of many molecular studies in chestnut have been on breeding for disease resistance, several other important traits that vary in interesting ways may be amenable to molecular breeding approaches. These include nut crop yield, bud-bust phenology, tree architecture, and drought tolerance, nut flavors, nut higher lipid contents, nut morphology (Fig. 5.16). Moreover, untapped genetic potential exists in North American species. The excellent taste of American chestnuts and Ozark chinquapins, related to their lipid contents (Senter et al. 1994), and adaptation to fire disturbance in coastal chinquapins are traits of potential agronomic/culinary utility. Genetic study of the stoloniferous shrub (<1 m) plant architecture of the *Castanea pumila* coastal ecotype might facilitate production of a high-quality nut crop. Hybridization of trailing chinquapin with disease-resistant East Asian species that produce large chestnuts could produce dwarf cultivars suitable for intensive, high-density cultivation.

5.6 Biotechnology

Biotechnologies, including biological controls, tissue culture techniques, genetic engineering and genomic marker systems, have great potential to advance restoration of *Castanea dentata* in eastern North America and to improve chestnut culture worldwide. The contributions, prospects and limitations of novel biotechnologies to forest tree breeding have been reviewed by Häggman et al. (2016) and by Tuskan et al. (2018).

5.6.1 Induced Mutation Breeding

Chestnut breeding using induced mutations was attempted in the USA after the chestnut blight pandemic decimated *Castanea dentata* populations in the twentieth century. Starting in the 1950s, multiple breeding programs irradiated thousands of *C. dentata* nuts and scions with the goal of generating blight-resistant mutants (Diller and Clapper 1965; Samman and Thor 1975; Singleton and Dietz 1974). Large plantings containing irradiated seedlings and their progeny were established in five U.S. states. However, no blight-resistant mutants were identified (Burnham 1988). Dietz (1978) attempted induced mutation breeding using ionizing radiation to achieve resistance to *C. parasitica*. Some of his irradiated trees, and some of their intercrossed progeny still exist in orchard plantings in Maryland, and are maintained by The American Chestnut Foundation (Burnworth 2002). Recent examination of surviving trees by Craddock (pers obs) indicated canker severity well within the range of *C. dentata* wild type trees infected with hypovirulent strains of *C. parasitica*, with no real evidence of increased resistance in irradiated trees or their progeny. Interest in American chestnut irradiation programs began to dwindle in the 1980s (Curry 2014).

5.6.2 Hypovirulence

Biological control of chestnut blight is based on transmissible hypovirulence, a phenomenon marked by the reduced virulence of the pathogenic fungus, rendering the fungus less damaging to the host plant (Grente 1965). Effective biocontrol was first observed on *Castanea sativa* in Italy (Biraghi 1950a, b) although the nature of the causal agent was not determined for more than 20 years. A dsRNA virus transmits the hypovirulent phenotype and the viral RNA can transform normal lethal cankers into slower-growing superficial bark cankers that do not kill the tree (Ćurković-Perica et al. 2017; Grente and Berthelet-Sauret 1978; Peever et al. 1997; Roane et al. 1986). In the 1970s hypoviruses were found in Michigan associated with recovering stands of *C. dentata* (Roane et al. 1986), although some of the Michigan hypovirulent strains were associated with mutations in the *C. parasitica* mitochondrial DNA (Fulbright 1999). By slowing the growth of the fungus, the virus allows the tree to live and bear fruit (Griffin 2000).

The biology of hypovirulence has been studied and reviewed extensively (Anagnostakis et al. 1998; Baidyaroy et al. 2000; Chen and Nuss 1999; Dierauf et al. 1997; Fulbright 1999; Griffin et al. 2006; MacDonald and Double 2006; Monteiro-Vitorello et al. 2000). Hebard et al. (1984) showed that host resistance in *Castanea* is a key factor allowing expression of hypovirulence (superficial cankers with reduced canker expansion). Barriers to virus transmission, one of the chief limitations to effective biocontrol, can be overcome using biotechnologies (Choi et al. 2012; Double et al. 2017; Zhang and Nuss 2016).

5.6.3 Genetic Engineering

The marriage of in vitro culture and molecular biology of trees, including chestnut, was reviewed by Chang et al. (2018). Beginning around the same time as the American Chestnut Foundation's backcross breeding program, scientists at the State University of New York's College of Environmental Science and Forestry (SUNY-ESF) and collaborators have pursued a genetic engineering approach to increase blight resistance in *Castanea dentata* (Chang et al. 2018; Steiner et al. 2017). An important breakthrough in this area occurred with development of a method to establish *C. dentata* somatic embryo cultures (Caraway et al. 1994; Merkle et al. 1991). Newhouse et al. (2014) used in vitro methods to study heterologous expression of oxalate oxidase in transformed *C. dentata* that show high levels of tolerance to *C. parasitica* infection. In vitro homologous expression of a CsCh3 chitinase in transformed somatic embryos of *C. sativa* with potential resistance to *C. parasitica* was reported by Corredoira et al. (2012, 2016). The use of somatic embryos facilitated the design of an effective transformation system for *C. dentata* (Andrade et al. 2009, Polin et al. 2006; Xing et al. 1999). The main blight tolerance gene chosen by researchers in this area is a gene encoding oxalate oxidase, which catalyzes the degradation of oxalate into H_2O_2 and CO_2 and is produced in a variety of unrelated plants, such as strawberry, beet, peanut, apricot and most cereal grains (Chang et al. 2018; Steiner et al. 2017). Oxalate production by some plant pathogenic fungi, including the chestnut blight fungus, induces a programmed cell death response in plant tissue that is required for disease development (Hebard and Shain 1988; Kim et al. 2008). Based on observations that virulent strains of *C. parasitica* produce greater quantities of oxalate than hypovirulent strains (Chen et al. 2010; Havir and Anagnostakis 1983), the oxalate oxidase gene was chosen as a lead candidate gene for transformation (Chang et al. 2018). Tests of transgenic *C. dentata* plants expressing the oxalate oxidase transgene have shown that these plants exhibit enhanced resistance to *C. parasitica* and transmit blight resistance to a portion of their progeny (Newhouse et al. 2014; Zhang et al. 2013). The first field trials of transgenic *C. dentata* were planted in 2006 under a permit from the United States Department of Agriculture, Animal Plant Health Inspection Service (USDA-APHIS) (Steiner et al. 2017). There is some concern in the scientific community about public resistance to a transgenic approach (Barnhill-Dilling and Delborne 2019). Researchers at SUNY-ESF are currently in the process of seeking deregulation of the best-performing transgenic lines through USDA-APHIS (Chang et al. 2018).

Researchers in the United States have proposed that genetic engineering can be merged with backcross breeding to create *Castanea dentata* populations that combine most, if not all, of the following attributes: (1) blight resistance from the OxO transgene, (2) *Phytophthora* root rot resistance introgressed from the East Asian *Castanea* species, (3) blight resistance introgressed from the East Asian *Castanea* species, (4) adaptation to local environmental conditions from a diverse set of *C.*

dentata recurrent parents and (5) an effective population size sufficiently large as to be at minimal long-term risk of extinction due to inbreeding and genetic drift (Steiner et al. 2017; Westbrook 2018a, b). The long-term breeding goal for *C. dentata* in North America is unique in that resistance to two diseases must be introgressed into wild populations to facilitate restoration of the species (Westbrook et al. 2019; Worthen et al. 2010).

Biotechnology research on chestnut in Europe and Asia is fundamentally different from that in North America because the main emphasis is on cultivar development and preservation, rather than species restoration. As a result, more effort has been spent on micropropagation and preservation of superior genotypes than on transformation. One very effective use of micropropagated material in plant breeding is the ability to clone hybrid progeny before screening. Santos et al. (2015a, 2017a) used plantlets generated by in vitro propagation to study the genetics of resistance to ink disease. The ability to clone progenies, before screening, is a powerful tool for selection for disease resistance (Santos et al. 2015a). Researchers in Europe are also in the process of using genetic engineering to increase resistance to both blight and ink disease in *C. sativa*. Corredoira et al. (2012) engineered *C. sativa* to overexpress a native thaumatin-like protein gene (*CsTL1*), which encodes a protein that was earlier shown to possess in vitro antifungal activity against *Trichoderma viride* and *Fusarium oxysporum*. A gene from *C. sativa* was used to transform hybrid poplars by Moreno-Cortez et al. (2012). They used the *C. sativa RAV1* gene which is homologous to an *Arabidopsis* TEM gene. The transgenic poplars overexpressed *CsRAV1* and showed early formation of current-growing-season branching, a possibly adaptive trait the authors consider important to biomass production (Moreno-Cortez et al. 2012).

5.7 Conclusions and Prospects

Chestnut researchers and growers now have at their disposal new tools, new techniques, new information and better access to genetic resources that should, in the near future, permit great gains in the movement towards several important breeding objectives: increased resistance to the most limiting chestnut diseases, tolerance to the most troublesome pests and improvements in fruit quality and yields. Cutting-edge gene editing technologies such as those based on the CRISPR/Cas9 systems may have tremendous applications for chestnut (Häggman et al. 2016; Tuskan et al. 2018). The American Chestnut Foundation

Fig. 5.17 These third backcross American chestnut hybrids were bred from some of the southernmost populations of *Castanea dentata.* They exhibit good tree form, adequate blight resistance, and represent a potentially very important germplasm resource for adaptive variation as the species copes with climate change. (Photo by J.H. Craddock)

is currently funding range-wide, and genome-wide sampling in an effort to document adaptive variation in *Castanea dentata* that may be useful to the species as it confronts rapid climate change (Westbrook 2018a, b; Westbrook et al. 2019), and conversations have begun within the scientific community about possible assisted migration as a conservation strategy (Fig. 5.17). Habitat modelling to predict future geographic ranges can be based on current and historic occurrence data for the trees and for their pathogens and pests (Phillips et al. 2006; Wang et al. 2010, 2013). Application of the knowledge presented here offers great opportunities to improve forest stands of chestnut for the sustainable production of high-quality lumber and other forest products in East Asia, North America and in Europe.

Appendices

Appendix I: Research Institutes Relevant to Chestnut

Institute	Specialization and research activities	Contact information, including website
The American Chestnut Foundation, Asheville, NC, USA	American chestnut restoration	Dr. Jared Westbrook email: chestnut@acf.org website: https://www.acf.org/
American Chestnut Research and Restoration Project, SUNY College of Environmental Science and Forestry, Forest Biotechnology Working Group Syracuse, NY, USA	Basic and applied research that will lead to the development of a blight-resistant American chestnut tree. Perform cutting edge research that will enhance our understanding of forest tree biology and lead to improved productivity and biodiversity of our forested ecosystems	Dr. William A. Powell email: wapowell@esf.edu website: https://www.esf.edu/chestnut/
Beijing Advanced Innovation Center for Tree Breeding by Molecular Design Beijing University of Agriculture Beijing, China	Genetics and molecular breeding of tree crops	Dr. Ling Qin email: qinlingbac@126.com
Centre for Research and Technology of Agro-Environment and Biological Sciences University of Trás-os-Montes e Alto Douro Vila Real, Portugal	Chestnut physiology, abiotic and biotic stresses, and breeding	Dr. José Gomes-Laranjo email: jlaranjo@utad.pt web: https://www.citab.utad.pt/
Chattanooga Chestnut Project, University of Tennessee at Chattanooga, TN, USA	Cultivar development, American chestnut restoration, genetic diversity and evolution of North American *Castanea* species	Dr. J. Hill Craddock email: hill-craddock@utc.edu website: https://www.utc.edu/biology-geology-environmental-science/profiles/faculty/wmn758.php

Institute	Specialization and research activities	Contact information, including website
Chestnut Research and Development Centre Chiusa Pesio, Italy	Research on several topics related to chestnut cultivation, including chestnut germplasm, advanced propagation techniques, optimization of cultural practices, pests and diseases management, nursery and extension services	Prof. Gabriele Beccaro email: centro.castanicoltura@unito.it web: http://www.centrocastanicoltura.unito.it/
Dept. of Biological Sciences Mississippi State University Starkville, MS, USA	Molecular interactions of plant pathogenic fungi and their hosts, particularly between chestnut blight fungus and the American chestnut	Dr. Angus Dawe email: dawe@biology.msstate.edu website: https://www.biology.msstate.edu/people/staff.php?id=ald662
Dept. of Biological Sciences University of Notre Dame Notre Dame, IN, USA	Population dynamics, spatial genetic structure, gene flow, adaptive genetic variation and spontaneous interspecific hybridization	Dr. Jeanne Romero-Severson email: jromeros@nd.edu website: https://biology.nd.edu/people/jeanne-romero-severson/
Department of Crop Production and Engineering Projects High Polytechnic School Lugo Campus University of Santiago de CompostelaLugo Spain	Research in chestnut with morphological and molecular markers, genetic diversity evaluation, identification and catalogue of local cultivars,	Dr. Santiago Pereira-Lorenzo email: santiago.pereira.lorenzo@usc.es website: http://www.usc.es/en/departamentos/prodvexg/profesores/pereiralorenzosantiagopublicacion
Dept. of Plant and Environmental Sciences Clemson University Clemson, SC, USA	Diseases caused by *Phytophthora* spp. and rust diseases, with current work on root rot of chestnut	Dr. Steven Jeffers email: sjffrs@clemson.edu website: https://www.clemson.edu/cafls/faculty_staff/profiles/sjffrs
Dept. of Plant Biology Michigan State University East Lansing, MI, USA	Interactions between American chestnut, its blight pathogen, and intracellular hyperparasites that alter pathogen virulence	Dr. Andrew M. Jarosz email: amjarosz@msu.edu website: https://plantbiology.natsci.msu.edu/directory/andrew-m-jarosz/
Division of Plant and Soil Sciences West Virginia University Morgantown, WV, USA	Biological control of chestnut blight using transmissible hypovirulence	Dr. William L. MacDonald email: macd@wvu.edu website: https://www.davis.wvu.edu/faculty-staff/directory/william-macdonald
Faculty of Agriculture Dept. of Horticulture Ondokuz Mayis University Samsun, Turkey	Evaluation of chestnut hybrids for cultivar and rootstock characteristics, postharvest treatment of chestnuts	Prof. Dr. Ümit Serdar email: userdar@omu.edu.tr website: https://personel.omu.edu.tr/tr/userdar

Institute	Specialization and research activities	Contact information, including website
Forest Health Research and Education Center University of Kentucky Lexington, KY, USA	Conservation and restoration of forested ecosystems using genetics-based biological research, social science research, and educational outreach on factors affecting tree and forest health	Dr. C. Dana Nelson email: dananelson@fs.fed.us website: http://www2.ca.uky.edu/Forestry/fhrec/index.html
Institute of Botany, Jiangsu Province and Chinese Academy of Sciences Nanjing, China	Genetics and breeding of *C. mollissima*	Dr. Guomin Geng, email: 13951970164@163.com website: http://old.cnbg.net/English/Yjx/formindex.aspx
ISHS Chestnut Working Group	Coordinates chestnut research worldwide	Chairman: Dr. José Gomes Laranjo email: jlaranjo@utad.pt web: https://www.ishs.org/chestnuts
NARO Institute of Fruit Tree and Tea Science Tsukuba, Japan	Cultivar development, production systems, integrated pest management, elucidation of fruit functionality, regulatory mechanisms governing fruit quality	Dr. Toshihiro Saito email: www.naro.affrc.go.jp website: http://www.naro.affrc.go.jp/english/fruit/
Research Institute on Terrestrial Ecosystems Italian National Research Council Porano, Italy	Population genetics and genomics of *C. sativa*	Dr. Fiorella Villani email: fiorella.villani@ibaf.cnr.it website: https://www.cnr.it/en/institute/125/research-institute-on-terrestrial-ecosystems-iret
The Schatz Center for Tree Molecular Genetics Pennsylvania State University University Park, PA, USA	Research and training in forest genetics related projects in the areas of genomics, conservation genetics, and biotechnology	Dr. John Carlson email: jec16@psu.edu website: https://ecosystems.psu.edu/research/centers/schatz
Scientific-Research Centre of Agriculture Tbilisi, Georgia	In situ and ex situ management of *C. sativa* genetic resources in Georgia	Dr. Zviad Bobokashvili email: bobokashvili@hotmail.com website: http://srca.gov.ge/en
Shandong Provincial Key Laboratory of Fruit Tree Biotechnology Breeding, Shandong Institute of Pomology Tai'an, China	Genetic diversity and breeding of *C. mollissima*, home of the national chestnut germplasm repository of China	Dr. Qingzhong Liu email: qzliu@sdip.cn website: http://www.saas.ac.cn/saas/?content-6497.html

Institute	Specialization and research activities	Contact information, including website
Sistemas Agrários e Florestais e Sanidade Vegetal Instituto Nacional de Investigação Agrária e Veterinária Lisbon, Portugal	Forest genetics, molecular biology and genomics of woody species including *C. sativa*, and understanding resistance of *Castanea* spp. to *Phytophthora*	Dr. Rita Lourenço Costa email: rita.lcosta@iniav.pt website: http://www.iniav.pt/gca/index.php?id=1410
South China Botanical Garden Chinese Academy of Sciences Guangzhou, China	Plant *ex situ* conservation and inventory, plant conservation genetics	Dr. Hongwen Huang email: huanghw@mail.scbg.ac.cn website: http://english.scbg.cas.cn/
Southern Institute of Forest Genetics, US Forest Service Saucier, MS, USA	Genetics/genomics of disease resistance, genetic diversity of American chestnut	Dr. C. Dana Nelson email: dananelson@fs.fed.us website: https://srs.fs.usda.gov/sifg/FERMCharterFINAL.html
Swiss Federal Institute for Forest, Snow and Landscape Research WSL Cadenazzo, Switzerland	WSL explores the dynamics of the terrestrial environment, and the use and protection of natural habitats and cultural landscapes.	Dr. Marco Conederaemail: marco.conedera@wsl.ch web: https://www.wsl.ch/en.html
Tree Genetics and Breeding Lab, Aristotle University of Thessaloniki Thessaloniki, Greece	Genetics and epigenetics of *C. sativa*, breeding and micropropagation of *C. sativa*,	Prof. F.A. Aravanopoulos, email: aravanop@for.auth.gr https://users.auth.gr/~aravanop/index_en.html
Unité Mixte de Recherche Biodiversité, des Gènes aux Communautés Institut National de la Recherche Agronomique Bordeaux, France	Genetic determinism of diseases of chestnut; interactions between gall wasp, *C. parasitica* and endophytic fungi; evolution of *C. parasitica* and its hypovirus	Dr. Cécile Robin email: robin@bordeaux.inra.fr https://www6.bordeaux-aquitaine.inra.fr/biogeco_eng/Staff/Staff-directory/Q-Z/Robin-Cecile
USDA Forest Service Hardwood Tree Improvement and Regeneration Center, Department of Forestry and Natural Resources Purdue University West Lafayette, IN, USA	Genetics of forest trees including development and use of DNA markers and sequence data to study genetic variability, evolution and spatial distribution of variability, breeding trees for plantations and species restoration	Dr. Keith Woeste, email: woeste@purdue.edu website: https://htirc.org/

Appendix II: Chestnut Genetic Resources

Information is presented in Table 5.2.

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Chapter 6
Hazelnut (*Corylus* spp.) Breeding

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Abstract Hazelnut is an economically important tree nut whose production is mostly destined to the confectionery industry with a demand that currently exceeds supply. Its cultivation remains substantially based on named selections from local, wild vegetation. Public breeding programs were not initiated until the 1960s and only two, both in the USA, are in operation today that are relatively large. Oregon State University has produced new cultivars with Gasaway resistance to the fungus *Anisogramma anomala*, causal agent of eastern filbert blight (EFB), a major disease in North America; these cultivars are being widely planted. In China, cold-hardy hybrid cultivars from *Corylus heterophylla* and *C. avellana* were recently released and are planted in northeastern China. In the past 25 years, molecular markers have facilitated a much better understanding of genetic diversity in the genus *Corylus*, aided the construction of linkage maps and allowed for marker-assisted selection for disease resistance. The genome of *C. avellana* was sequenced and assembled, and DNA markers identified from the transcriptome, providing the basis for the isolation of important genes, including those related to nut quality and adaptive and phenological traits. Many new genotypes expressing eastern filbert blight (EFB) resistance have been identified in the germplasm, and subsequent linked DNA markers developed, allowing new approaches to breeding for durable resistance. Micropropagation is routinely used in the USA, Chile and Italy for multiplication,

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but work with other in vitro techniques is less advanced. Genetic engineering has not been developed in hazelnut due to regeneration difficulties from somatic tissues but recent advances have established a protocol for organogenesis. More research is being carried out to assemble a high-quality hazelnut genome and achieve somatic embryogenesis. The results from this research will provide knowledge and tools enabling the isolation of genes and molecular markers, and the application of genome editing techniques to hazelnut.

Keywords Breeding · Climatic adaptation · Disease resistance · Genetic diversity · Linkage map · Marker-assisted selection · Nut quality

6.1 Introduction

Hazelnuts have been used by humans as a food since ancient times. Specialized cultivation has developed more recently across the Mediterranean Basin, based on the species *Corylus avellana*, and later in other areas of the world, including China where *C. heterophylla* and its hybrid selections with *C. avellana* are grown. Currently, hazelnut is an important tree nut whose success is due to the strong demand for nuts from the confectionary industry.

In spite of this situation, the cultivation is still based on ancient cultivars selected from the wild and most of the world production is from two countries: Turkey and Italy. The breeding programs initiated in the 1960s were aimed at improving yield and nut quality, climatic adaptation and resistance to diseases.

The objective of this chapter is to present the state of the art concerning the hazelnut breeding carried out in the world with particular emphasis on advancements based on use of modern technologies. The chapter will present information on genetic resources available within the genus *Corylus*, major breeding goals for hazelnut, results obtained by current and past breeding programs as well as breeding and molecular techniques currently available.

6.1.1 Botanical Classification and Distribution

Hazelnuts (other common names are cobnut and filbert) belong to the order Fagales, family Betulaceae, subfamily Coryloideae, genus *Corylus* L. and are native to the temperate zones of the northern hemisphere (Europe, Asia Minor, Asia, North America). Other families in the order Fagales include Juglandaceae [e.g., *Juglans* (walnut), *Carya* (pecan and hickory)] and Fagaceae [e.g., *Fagus* (beech), *Castanea* (chestnut) and *Quercus* (oak)]. The number of species within the genus *Corylus* varies between 9 and 25, depending on the taxonomic authority, with current revisions based on morphological, molecular and hybridization studies suggesting 13 major species assigned to 4 subsections (Molnar 2011). Two species occur in

Europe and Asia Minor (*C. avellana* L., *C. colurna* L.), three in North America (*C. americana* Marshall, *C. cornuta* Marshall, *C. californica* Marshall), and one in the Himalayas (*C. jacquemontii* Decaisne). The remaining species are endemic to eastern Asia and include *C. chinensis* Franchet, *C. fargesii* (Franch.) C.K. Schneid., *C. ferox* Wallich, *C. heterophylla* Fisher, *C. kweichowensis* Hu, *C. yunnanensis* A. Camus and *C. sieboldiana* Blume (Whitcher and Wen 2001). Of these, 8 are shrubby species due to the continuous production of shoots (suckers) from the crown of the plant, and 5 are tree species (Table 6.1). Subsection *Corylus* includes 5 species whose major similarity is the leafy, overlapping involucre (husk) covering the nut (*C. avellana, C. americana, C. heterophylla, C. kweichowensis,* and *C. yunnanensis*); subsection *Colurnaea* includes 4 species that grow as single-stem trees (*C. colurna, C. chinensis, C. fargesii* and *C. jacquemontii*); subsection *Siphonochlamys* includes 3 species with tubular, bristle-covered involucres (*C. cornuta, C. californica* and *C. sieboldiana*); subsection *Acanthochlamys* solely includes *C. ferox*, which is unique in the genus having a chestnut (*Castanea* sp.)-like involucre covered in spines (Erdogan and Mehlenbacher 2000a, b; Whitcher and Wen 2001). *Corylus fargesii* (likely syn. of *C. papyraceae* Hickel) has yet to be officially placed in a subsection, likely to be *Colurnaea* (Molnar 2011).

Table 6.1 Hazelnut species and their geographic distribution

Species	Common name	Distribution
Shrubs with leafy husks		
C. avellana	European hazel	Europe, Caucasus, western Asia
C. americana	American hazel	Eastern North America
C. heterophylla	Siberian hazel	Northeast China, Japan, Korea, Russian far east
C. kweichowensis	Guizhou hazel	Middle-southern China (Sichuan, Hunan, Hubei, Anhui, Guizhou, Shaanxi, Henan and Zhejiang provinces)
C. yunnanensis	Yunnan hazel	China (Sichuan and Yunnan provinces)
Shrubs with bristly husks		
C. cornuta	Beaked hazel	Appalachian Mountains (USA) to Gaspè peninsula (Quebec) to central British Columbia (Canada)
C. californica	California hazel	Southern British Columbia (Canada) to central California (USA)
C. sieboldiana	–	Japan, Korea, northeast China, Russian far east
Trees with single stems		
C. colurna	Turkish tree hazel	Balkans, Turkey and Republic of Georgia
C. jacquemontii	Indian tree hazel	Northern India and Pakistan
C. chinensis	Chinese tree hazel	Middle-southern China (Sichuan, Hunan, Hubei, Anhui, Guizhou, Shaanxi, Henan and Zhejiang provinces)
C. fargesii	Paperbark tree hazel	Middle-southern China (Sichuan, Hunan, Hubei, Anhui, Guizhou, Shaanxi, Henan and Zhejiang provinces)
Tree with chestnut-like bur		
C. ferox	Tibetan hazel	South-central China to Tibet, Bhutan and Nepal

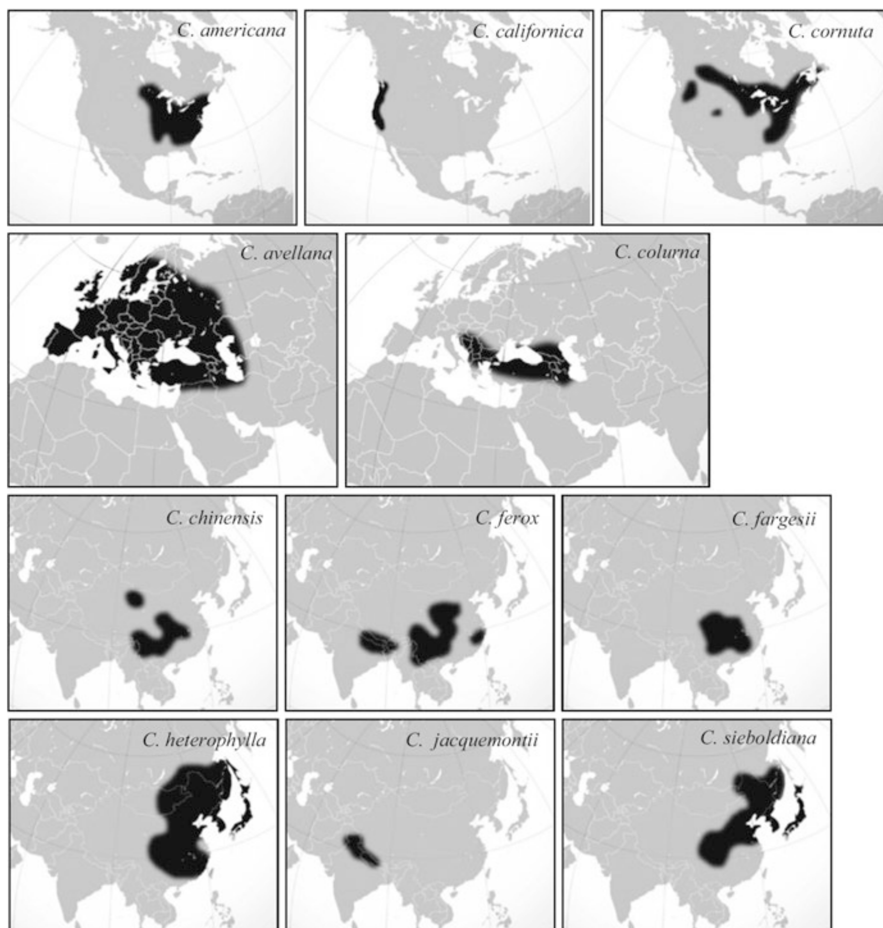

Fig. 6.1 Geografic distribution of *Corylus* species. *C. heterophylla* includes *C. kweichowensis* and *C. yunnanensis* which are considered by some taxonomists to be botanical varieties of *C. heterophylla*. (Source: Bassil et al. 2013)

All *Corylus* species (Fig. 6.1) bear edible nuts but their size and seed (kernel) traits vary considerably. *Corylus avellana* is the major species cultivated for the commercial production of the kernel.

Corylus avellana, also known as the European hazelnut, has a natural geographical distribution that extends in Europe and western Asia from Portugal, Ireland, Orkney Islands, Norway, Sweden, Russia, Kazakhstan, Iran, Iraq, Syria, Lebanon, Greece, Italy and Spain. It is naturally occurring from sea level up to 1500 m elevation. It is common in mixed hardwood forests, and along paths and rural roads. It was more recently introduced for cultivation in North America and China, and in the Southern Hemisphere, primarily in Chile. *Corylus avellana* is a very polymorphic species based on its morphology. It has been divided into subgroups that were con-

sidered as separate species by some authors: C. *maxima* Mill., *C. pontica* Koch. and *C. colchica* (Kasapligil 1972). However, experts now agree that these species should be placed within *C. avellana* since they show continuous variation in morphology, hybridize readily and overlap in geographic distribution; further, DNA fingerprint data cluster *C. maxima* and *C. avellana* together supporting a common species origin (Erdogan and Mehlenbacher 2000a, 2002; Mehlenbacher 1991a; Rovira 1997; Thompson et al. 1996).

The wide distribution of *Corylus avellana* underlines its large genetic and morphological variability and climatic adaptation. However, its profitable area of cultivation is limited to mild climates in temperate regions, often characterized by their proximity to the sea or other large bodies of water. As distance increases from favorable areas, yield and kernel quality typically decreases. In addition, some cultivars may show variation in adaptation to local ecological conditions. For example, a recent study showed the Spanish cv. Negret and multiple Turkish cultivars were more sensitive to adverse conditions than other cultivars from Europe and the USA, including cvs. Willamette, Pauetet and Tonda di Giffoni (Erdogan and Ozdemir 2018). Further, hazelnut has a chilling requirement that must be satisfied for catkin elongation, female flower development and leaf budburst (Mehlenbacher 1991b), presenting challenges to proper development and/or consistent production in some regions.

Corylus avellana plants are 3–7 m tall, have broad foliage (simple, alternative leaves), and a shrub-like growth habit (bush) that is constituted by a variable number of stems that are generated by the continuous emission of suckers (basal shoots), developed from adventitious buds in the area of the root collar. The youngest suckers are on the outside of the clump.

Hazelnuts are a wind-pollinated, monoecious species that exhibit a sporophytic self-incompatibility system. The male (staminate) flowers are arranged in cylindrical inflorescences called catkins (Figs. 6.2 and 6.3). Each catkin produces more than 5 million wind-dispersed (anemophilous) pollen grains. The female flowers are gathered in inflorescences (7–12 flowers) inside the mixed vegetative buds. Staminate and pistillate flowering times may not overlap, and cultivars can be homogamous, protogynous or protandrous depending on the cultivar and the region it is grown. Flowering takes place during the winter months (December to March in the Northern Hemisphere), and dates of peak bloom can fluctuate by several weeks each year depending on localized weather patterns. At time of bloom, the pistillate flower consists of a rudimentary ovary with two short styles ending with a red-colored stigma emerging from the bud scales of a mixed floral and vegetative bud. After pollination, the stigma dries while the formation of the ovary begins at the time of leaf budburst (March) and is completed at the end of May/early June when fertilization of the pollen nucleus and egg cell occurs.

Recognized species have been described by several authors (Aiello and Dillard 2007; eFloras 2009; Farris 2000; Kasapligil 1972; Mehlenbacher 1991a; Molnar 2011; Thompson et al. 1996).

Corylus americana, the wild American hazelnut, is native to a wide area spanning much of eastern North America. Several *C. americana* cultivars have been

Fig. 6.2 Elongated catkins and female inflorescences at bloom. (Photo by R. Botta)

Fig. 6.3 Clasping husk (involucre) representative of Turkish cultivars. Nuts do not drop freely at maturity and are hand harvested. (Photo by R. Botta)

selected from the wild that produce relatively large-size nuts with acceptable quality; however, plants of the species produce tiny nuts with thick shells reducing their appeal as a commercial crop. Wild American hazelnuts were harvested in the past for local consumption and sale. The species is the natural host of *Anisogramma anomala* (Pack) E. Müller, the fungus that causes the disease EFB. *Corylus americana* is generally highly tolerant of EFB, with plants developing only small cankers or none at all (Capik and Molnar 2012). Hybrids of *C. americana* and *C. avellana* show promise for expanding hazelnut production into colder regions of North America (Molnar 2011).

Corylus colurna, the Turkish tree hazel, is native to Turkey, the Balkan Peninsula and the Caucasus. It is also grown across parts of Europe and North America as an ornamental shade tree where it naturally forms an attractive pyramidal crown and displays interesting scaly, corky bark and heavily textured leaves. Its small, thick-shelled nuts are collected from the wild and consumed and sold locally, including for use in making candy. The trees are also valued for their high-quality timber. They can also be used as non-suckering rootstocks of the cultivated hazelnut *C. avellana*.

Corylus heterophylla, the Siberian hazel, is native to a wide area that spans parts of China, Japan, Korea and eastern Russian. This species is similar to *C. americana* in many traits, including their small nuts with thick shells, while also holding promise for breeding widely adapted interspecific hybrid cultivars. Selections exist that are extremely cold-hardy and tolerant of drought, especially those from northeast China where snowless winters and −30 °C temperatures are common. Further, selections exist that drop the nuts from the involucres at maturity, a commercially valuable trait, which is unlike most *C. americana*.

Corylus kweichowensis and *C. yunnanensis* are very similar to *C. heterophylla* but are adapted to different areas and climates. *Corylus kweichowensis* lacks the stolons of *C. heterophylla* and grows in clumps of stems; *C. yunnanensis* is adapted to subtropical conditions and has very pubescent leaves.

Corylus cornuta, the beaked hazel, grows wild across much of the northern USA and southern Canada into regions where winter temperatures drop to −50 °C. This species has very early maturing nuts and a very stoloniferous, spreading-growth habit. It is known to be one of the first to recover after wildfires. It can naturally develop into dense thickets, a trait useful for soil reclamation and for wildlife habitat but not beneficial to most commercial orchards.

Corylus californica is found growing in the coastal mountains of the Pacific Northwest of North America. It has only been utilized as a food source to a minor extent due to its small, thick-shelled nuts, which are very early maturing. Its early maturity, plus its lack of stolons and shorter husks, may make it more useful for breeding than *C. cornuta*, if cold hardiness is not a primary objective.

Corylus sieboldiana (*C. mandshurica*), a shrubby Asian hazel, grows across much of eastern and northern Asia, including the Russian Far East, Japan, Korea, and central and northeastern China. The species is considered to be very cold-hardy, with many traits similar to *C. cornuta* and *C. californica*; however, its nut maturity date is later (Erdogan 1999). This species is less abundant than *C. heterophylla* in

the overlapping areas of distribution because it requires fertile soils, rich in organic matter and moisture. Nuts are harvested and consumed locally but the species is less interesting for breeding than *C. heterophylla* due to the long clasping husk which have needlelike hairs and tiny nuts.

Corylus chinensis, the Chinese tree hazel, is distributed across southern China where it is found as scattered trees on moist, forested mountain slopes. It grows as large, single-trunk trees, 20–40 m tall with trunk diameters up to 2 m. Its bark is considerably thinner and smoother than *C. colurna*. Its nuts are tiny and very thick shelled and covered in thick fleshy involucres. Its timber has been used in China for furniture and paneling. Seedling trees were found to be very fast growing and resistant to EFB in trials at Rutgers University, thus may hold value as non-suckering rootstock (Molnar unpublished).

Corylus jacquemontii, the Indian tree hazel. The species is native to parts of northeast Afghanistan, northern India, northern Pakistan and western Nepal where it can be found at 1900–3000 m elevation. The species grows as single trunk trees, 12–15 m tall. Its bark is thinner and less corky than *C. colurna*. Its small (up to 1.5 cm), thick-shelled nuts have been reported to be easier to remove from the involucre than *C. colurna*. Nuts are harvested from the wild and sold in local markets.

Corylus fargesii, the paperbark tree hazel, is a single-trunk tree up to 25 m tall. The unique peeling bark of older stems and trunk are similar to that of river birch (*Betula nigra* L.). The species is distributed throughout mountain valleys of the Henan, Sichuan, Hubei, Shangxi, Gangsu and Guizhou provinces of China. Morphological examination suggests *C. fargesii* be placed in subsection *Colurnaea*, although further investigation is needed to clarify its taxonomic position. *Corylus fargesii* is likely a synonym for *C. papyraceae* Hickel.

Corylus ferox, the Tibetan hazel, is native to the eastern Himalaya from Bhutan, northeast India, northern Myanmar and Nepal to parts of the Yunnan, Sichang and Xizang provinces of China. It can be found at 1700–3800 m elevation. It grows as a small to medium size tree (6–9 m, occasionally to 16 m tall and 30 cm in diameter) with smooth bark. Its nuts develop in clusters of 3–6 in spiny, cup-shaped involucres very similar to spiny chestnut (*Castanea* L.) burrs. *Corylus ferox* var. *thibetica* (Batal.) Franch. has less pubescence on the base of the involucre than *C. ferox* and lacks pubescence on buds and immature stems. It is native to the Gansu, Guizhou, Hubei, Ningxia, Shaanxi, Sichuan, Xizang and Yunnan provinces of China, growing at 1500–3600 m elevation.

6.1.2 Economic Importance and Producing Countries

The hazelnut is currently rated fifth in the world for surface area harvested (about 672,000 ha in 2017; FAO 2019) among the cultivated tree nut species, after cashew, walnut, almond and pistachio. The average annual production (data 2012–2017; FAO 2019) of in-shell hazelnuts is about 865,000 mt and is concentrated mainly in two countries: Turkey (570,000 mt) and Italy (104,000 mt). Other significant

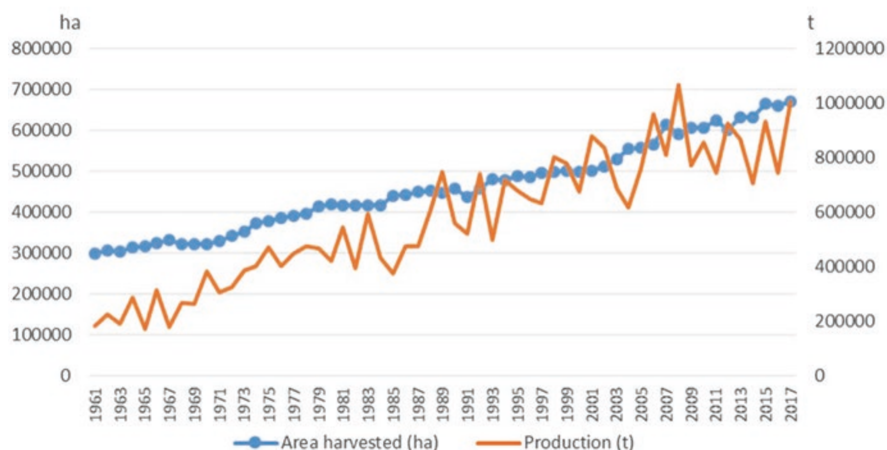

Fig. 6.4 World area harvested (ha) and production (t) of in shell hazelnuts in the period 1961–2017. (FAO (2019))

producing countries are the USA (34,000 mt), Azerbaijan and Republic of Georgia (33,000 and 31,000 mt, respectively), and China (25,000 mt). Hazelnuts are also cultivated in Chile, Spain, France, Romania, Serbia and Russia. On average, the commercial value of production exceeds USD 2 billion per year, but world market prices vary between years due to fluctuations in volume of production in Turkey, which depends on the climatic trends and possible impacts from spring frosts in the Black Sea Region. Currently, the kernel market represents about 93% of the world market for hazelnut with the remaining sold as in-shell nuts.

Hazelnuts are mostly destined to the confectionary industry where kernels are roasted and used to produce a large number of foods such as creams (nut butters), candies, baked-goods and other snacks. Due to the strong demand of kernels for the confectionary and baked-goods industry, the surface area planted and tonnage harvested have shown a trend of continuous growth from 1961 to 2017 (FAO 2019), and there are new plantings of significant size in multiple countries, including Italy, the USA, Chile and several in eastern Europe (Fig. 6.4).

6.1.3 *Domestication, Selection and Early Improvements*

Hazelnut use by humans spans the geographic range of *Corylus* and dates back at least 8400 years BC in western and central Europe, North America and China (Holstein et al. 2018). At the end of the last ice age, hazelnut was one of the first shrubby plants to move from southern to northern Europe. Between 7500 and 5500 years BC, they were abundant in the forests of the boreal regions, up to the British Isles and part of Scandinavia. At that time, hazelnut was considered an important food in the human diet, as testified by findings from Mesolithic and Neolithic sites in Sweden, Norway and Denmark.

The genus *Corylus* is believed to be named for the involucre that surrounds the nut, which resembles a helmet that in classical Greek language is called *córys*. The species name *avellana* is thought to derive either from the name of the location Abellina in the Middle East, a center of early production, or from Abella (currently Avella), which is a town in Campania (region of southern Italy) where hazelnuts were also historically grown. Cato (II century BC), in Chapter VIII of the *De re rustica*, used the word *avellanas* to designate hazelnuts, while Pliny the Elder (first century AD) in book XV of the *Naturalis Historia* (77–79 AD) uses the term *abellinas*. According to Pliny, the cultivated hazelnut comes from Abellina in the Middle East, corresponding to today's Damascus Valley. The plants were then brought to the Black Sea region (Ponto) and later to Greece. From Greece they were introduced to Campania and orchards were established at Abella, a town located near Mt. Vesuvius.

The first historical evidence of commercial trade of hazelnuts was reported in Turkey and dates back to the Middle Bronze age (c. 1950–1830 BC; Fairbairn et al. 2014). In the western Mediterranean Basin, Campania, current Italy, is considered the oldest center of hazelnut cultivation (Trotter 1921). Archaeological findings show that the cultivation was present at least from the fifth to the fourth centuries BC. However, the earliest evidence of specialized hazelnut cultivation in Italy can be found in documents (contracts between growers and landowners) of the high Middle Ages (800–900 AD; Botta and Valentini 2018).

In the traditional area of cultivation across the Mediterranean Basin, in particular in Turkey, Italy and Spain, production still relies on traditional cultivars that were selected from the local, wild vegetation. Major cultivars are Tombul, Foşa and Palaz in Turkey; Negret in Spain and Tonda Gentile delle Langhe (TGdL, syn. Tonda Gentile, Tonda Gentile Trilobata), Tonda di Giffoni and Tonda Romana in Italy. The origin of these cultivars is poorly documented, but their success was established in the twentieth century when the confectionary industry defined the quality standards required for processing.

In Northeastern China, *Corylus heterophylla* is naturally found widespread in forests, and its nuts have been traditionally harvested from the wild and used as a food since ancient times. Evidence of its cultivation dates back to 1100–600 years BC (*The Book of Songs*) but the development of management methods to increase yield and harvest efficiency of the wild populations is recent. In the twenty-first century specialized cultivation (currently over 50,000 ha) is based on hybrids between *C. heterophylla* and *C. avellana* (named Ping'ou hybrids), which express increased yield and nut quality over the native species parent and improved cold tolerance over the European (Wang et al. 2018).

6.1.4 Modern Breeding Activities

Major modern breeding programs started in Italy, France and the USA in the 1960s, and in China and in Turkey in the early 1980s (Table 6.2). In Europe, the aims were to improve productivity and adaptability, and maintain or increase kernel quality for

Table 6.2 Public hazelnut breeding programs since 1960 and current status

Country	Location	Project Leader	Current status	Releases and selections
China	Economic Forestry Research Institute. Dalian, Liaoning	Weijian Liang Xie Ming	Continuing	Bokehong, Dawei, Jinling, Pingdinghuang, Yuzhui and 14 numbered selections
China	China Research Institute of Forestry, Chinese Academy of Forestry, Beijing	Guixi Wang	Continuing	4 cvs. (Liaozhen 1, Liaozhen 2, Liaozhen 4, Liaozhen 9); more than 10 selections in trials (including cultivars and pollinizers)
France	INRA Bordeaux	Eric Germain	Terminated	Fercoril-Corabel®, Feriale, Ferwiller
Italy	University of Torino	Piero Romisondo Giovanni Me Nadia Valentini Roberto Botta	Continuing (small)	Daria, UNITO 101, UNITO 119, UNITO 3L, UNITO G1, UNITO L35; 4 Tonda Gentile delle Langhe clones; suckerless rootstocks under evaluation
Italy	University of Tuscia, Viterbo	Valerio Cristofori	Continuing (small)	Madonnella, Romanella (under evaluation)
Italy	University of Perugia	Agostino Tombesi Daniela Farinelli	Terminated	Tonda Francescana®, Tonda Etrusca, Volumnia (I, II, III, IV); 12 Tonda Romana clones
Romania	University of Craiova	Mihai Botu	Continuing (small)	Arutela, Cozia, Natval, Primaval, Romavel, Roverd, Uriase de Vâlcea, Vâlcea 22, Valverd
Spain	IRTA Mas Bové, Reus	Mena Mas	Terminated	none
Turkey	Hazelnut Research Institute, Giresun	Ahmet Okay	Terminated	Allah Verdi, Giresun Melezi, Okay-28; 14 Tombul clones under evaluation
USA	Oregon State University	Shawn Mehlenbacher	Continuing	11 cultivars, 12 pollinizers, 3 ornamentals (Table 6.3)
USA	Rutgers University. New Jersey	Thomas Molnar	Continuing	6 selections in trials

industrial use. There were also efforts to improve the quality and productivity of cultivars for table use (in-shell market).

Italy The University of Torino released four selections from the crossing Cosford × Tonda Gentile delle Langhe (Daria, UNITO 101, UNITO 119, UNITO 3L) and one from Payrone × Tonda Romana (UNITO G1), which are proposed as new cultivars and pollinators of cv. Tonda Gentile delle Langhe, while bearing nuts of comparable quality (Valentini and Me 1999); one additional selection is proposed for the in-shell market (UNITO L35, Tonda Gentile delle Langhe × Lansing, Valentini et al. 2001a). Four clones of Tonda Gentile delle Langhe were also released as tested true-to-type improved material for new orchards (UNITO-AD17,

UNITO-MT4, UNITO-MT5, and UNITO-PD6). Current work is focused on non-suckering rootstocks obtained from *Corylus colurna* and *C. avellana*. At the University of Tuscia in Viterbo, two selections (Madonnella and Romanella) with kernels suitable for confectionary uses are under evaluation (Tombesi et al. 2017). The University of Perugia has released two cultivars derived from the crossing Tonda Romana × Tonda di Giffoni (Tonda Francescana® and Tonda Etrusca), four selections derived from o.p. of Tonda Romana or Tonda di Giffoni named (Volumnia I, II, III, IV; Tombesi et al. 2017) and 12 clones of Tonda Romana.

France INRA (Institut National de la Recherche Agronomique) Bordeaux breeding program started in the 1960s and terminated in the early 1990s. Three releases from the program include Fercoril-Corabel® (Fertile de Coutard o.p.), a cultivar for the in-shell market (Germain 1989), Ferwiller, a pollinizer (Hall's Giant × Tonda Romana) and Feriale [Imperiale de Trebizonde (=Kargalak) × Butler] for the kernel market.

Romania The breeding program started at SCDP (Staţiunea De Cercetare Dezvoltare PT. Pomicultură) Vâlcea in the early 1980s (today affiliated with the University of Craiova) and continues. Three cultivars for in-shell market were released named Vâlcea 22 (selected clone of Kargalak), Cozia (Hall's Giant o.p.) and Uriaşe de Vâlcea (Ennis × Purple Filbert); five cultivars for the kernel market named Romavel (Hall's Giant o.p.), Arutela (Hall's Giant × TGdL), Valverd (mutation of cv. Red Lambert), Primval (Hall's Giant o.p.) and Natval (Vâlcea 22 × Ennis) and one ornamental cultivar (Roverd, Red Lambert × Daviana) (Botu et al. 2009; Vicol et al. 2013).

Turkey The Hazelnut Research Institute in Giresun initiated a breeding program by crossing the cvs. Tombul and Kargalak in 1981, and from these efforts released cvs. Giresun Melezi and Okay 28. Another cultivar named Allah Verdi, originating from a chance seedling or local selection, was also released. Currently, 14 Tombul clones are being evaluated in replicated trials (Balik et al. 2018).

USA Hazelnut genetic improvement efforts started in the early 1900s in the eastern USA. In 1919, J.F. Jones, of Lancaster, PA, made the first reported *Corylus americana* × *C. avellana* crosses using the wild selection Rush as the *C. americana* parent and a number of European cultivars. Although no cultivars were released from his efforts, Rush continued to be used as a parent plant by C.A. Reed of the Bureau of Plant Industry, USDA Beltsville, MD and G.H. Slate of the New York State Agricultural Experiment Station in Geneva, NY. From 1928 to 1930, Reed and Slate each developed additional hybrids using Rush × *C. avellana* cultivars and oversaw their subsequent evaluation (Slate 1969). While further breeding was not continued, from these efforts the New York F1 hybrids (NY 616, NY 398, NY 110, etc.) were selected, of which a number are still available from nurseries and are held

in the germplasm collection of the USDA, Corvallis, OR. Additionally, throughout the 1930s and 1940s, C. Weschcke of River Falls, WI, used *C. americana* Winkler from Iowa to develop many additional interspecific hybrids (Weschcke 1953, 1963). While no cultivars were released, improvements were made in the populations of hybrid trees, especially in adaptation to the cold climate of Wisconsin and resistance to EFB. Seeds from surviving trees in Weschcke's planting were later used to continue hybrid breeding efforts by P. Rutter of Canton, MN (Rutter 1987), which continue to this day. From this work, seedlings have been disseminated widely throughout the Upper Midwest, USA (Demchik et al. 2011). Notably, the Arbor Day Foundation (Nebraska City, NE) established 5000 seedlings from Rutter in 1996, and from this planting several consistently high yielding, EFB-resistant selections have been identified (Capik and Molnar 2012). More recently, additional clonal selections of Weschcke-Rutter origin are under replicated evaluation across Wisconsin and Minnesota by the Upper Midwest Hazelnut Development Initiative (Braun et al. 2018). Further efforts to develop hybrid hazelnuts are also in progress by the Hybrid Hazelnut Consortium that includes Oregon State University, Rutgers University, the University of Nebraska, Lincoln/Nebraska Forest Service and the Arbor Day Foundation. This group pools plant genetic resources, land and other infrastructure to collaboratively develop improved EFB-resistant hybrid hazelnuts for adaptation to colder regions around the USA (Molnar and Capik 2012a).

USA At Oregon State University (OSU), a major effort for the genetic improvement of hazelnut was initiated in 1969, with an emphasis on suitability to the blanched kernel market and resistance to *Anisogramma anomala*, the cause of EFB. The program has 27 releases to date, including 12 cultivars (Fig. 6.5), 12 pollinizers and 3 ornamentals. The most interesting are listed (Table 6.3). At Rutgers University, New Jersey (eastern USA) hazelnut evaluation and breeding efforts began in 1996 with a primary objective to identify and developed improved germplasm and cultivars resistant to EFB and adapted to the cold climate of the northeastern USA (Molnar et al. 2005, 2018a). Four *Corylus avellana* breeding selections are expected to be released in 2020.

China Interspecific breeding activities, started in 1980 in northeastern China (Dalian, Liaoning Province), were aimed at obtaining selections with the cold hardiness of *Corylus heterophylla* and the improved yields and nut quality of *C. avellana,* with the interspecific hybrids subsequently named Ping'ou hybrids. In 1999, five cultivars were released that included Pingdinghuan, Bokehong, Dawei, Jinling and Yuzui (Xie et al. 2005). Since 2000, 14 selections were chosen for widespread planting in China. Ping'ou hybrids are reported to have improved cold resistance and high yields (Wang et al. 2018). Further work is aimed at obtaining cold-hardy pollenizers and breeding for southern areas based on hybridizing *C. kweichowensis* and *C. avellana*.

Fig. 6.5 Nuts of *Corylus avellana* cv. McDonald recently released by OSU. (Photo by R. McCluskey)

6.2 Cultivation and Breeding Goals

The steady increase in the planted area and the strong demand for hazelnuts of superior quality for the confectionary industry have influenced both cultural practices and cultivar choice. This is particularly true for new orchards. However, in regions of ancient cultivation, such as those on the Black Sea coast, there are still topographic, social, economic and environmental constraints that slow down innovation and adoption of new practices. In addition, climatic change and the expansion of the crop into new areas of the world, such as in the southern hemisphere, are influencing breeding objectives and cultural needs.

6.2.1 Current Cultivation Practices

Hazelnut cultivars are highly heterozygous and clonally propagated. Most cultivars have a monoclonal origin but some older cultivars, such as Tombul in Turkey, are comprised of collections of different clones with similar phenotypes. Clonal propagation of hazelnut has historically been carried out by utilizing the rooted suckers that are naturally produced around the crown of the plant. In commercial nurseries, it has mainly been propagated by mound layerage. Propagation by the rooting of stem cuttings is also possible but not very common, since the practice can be very challenging with the percentage of well-rooted cuttings that develop into

Table 6.3 Hazelnut cultivars from Oregon State University and year of release

Cultivar	Year of release	References
Nut producers		
PollyO (USPP)[a]	2018	–
McDonald (USPP)	2014	Mehlenbacher et al. (2016)
Wepster (USPP)	2013	Mehlenbacher et al. (2014)
Dorris (USPP)	2012	Mehlenbacher et al. (2013)
Tonda Pacifica (USPP)	2010	Mehlenbacher et al. (2011a)
Jefferson	2009	Mehlenbacher et al. (2011b)
Yamhill	2008	Mehlenbacher et al. (2009)
Sacajawea	2006	Mehlenbacher et al. (2008)
Clark	1999	Mehlenbacher et al. (2001)
Lewis	1997	Mehlenbacher et al. (2000)
Pollinizers		
Felix (USPP)	2012	Mehlenbacher et al. (2018a)
York (USPP)	2012	Mehlenbacher et al. (2018a)
Theta	2009	Mehlenbacher et al. (2012)
Gamma	2002	Mehlenbacher and Smith (2004)
Epsilon	2002	Mehlenbacher and Smith (2004)
Ornamentals		
Burgundy Lace (USPP)	2016	Mehlenbacher et al. (2018b)
Red Dragon (USPP)	2008	Mehlenbacher and Smith (2009)

[a]USPP denotes cultivars protected by a United States Plant Patent

plants suitable for planting in an orchard found to be variable, depending on genotype, phenological stage of the cutting and environmental factors (Contessa et al. 2011; Cristofori et al. 2010). Today, micropropagation is routinely used in Oregon, USA, and is increasing in use elsewhere, but also remains challenging due to requiring expensive facilities and special expertise, especially as hazelnut has proven to be more difficult than many other species to propagate in vitro.

Grafting can also be used to propagate hazelnuts. Grafting onto seedlings of *Corylus colurna* (Fig. 6.6) generally results in a suckerless tree (removal of suckers is a constant requirement and expense for hazelnut orchards). The interspecific *C. colurna* x *C. avellana* clonal rootstocks Dundee and Newberg produce few suckers and vigorous trees. Approximately 5% of hazelnut orchards were planted with trees grafted on *C. colurna* rootstock in Oregon. They are older than 30 years in Oregon at present and considered as healthy and productive as plants on their own roots.

Cultivar choice takes into account multiple factors, but the major ones are environmental adaptation, local traditions and market destination, which today is primarily the confectionary industry. Today, only a small number of cultivars with optimal traits for the industry (production of high-quality, round-shaped kernels), are available and planted worldwide. They include the Italian cvs. Tonda Gentile delle Langhe, Tonda di Giffoni and Tonda Romana, of which the first is less productive than the others and the latter has less pellicle removal after kernel

Fig. 6.6 Hazelnut cv. Tonda Gentile delle Langhe grafted onto *Corylus colurna* seedling rootstocks in Italy. (Photo by R. Botta)

roasting. Another cultivar that is still planted outside Europe for its adaptability, large nut size, and high yield is Barcelona (syn. Castanyera, Fertile de Coutard), but which is hindered by the lower quality of the kernel and thick shell of the nut. Recent releases from Oregon State University combine the high quality kernels of Tonda Gentile delle Langhe with improved yields and EFB resistance. In Turkey, late spring frosts often cause serious crop losses, especially in the eastern Black Sea region. Although there is almost no land available for new plantings, some growers push the growing limits up to 1000 m elevation with the late leafing cv. Çakıldak to minimize frost damage. This cultivar is also preferred at middle and higher elevations around Ordu, although it is low in flavor. Some existing orchards are renewed in a small extent (<1%) with the high quality Tombul in Giresun and west of Trabzon, and with Foşa and Giresun Yabanisi, a local grower selection, at low and higher elevations, respectively, in other parts of Trabzon. Recently, a grower selection called Yomra is also being planted in renewals in the plains of Samsun. No scientific data have been published to date, but late leafing and vigorous growth are its highlights. However, the name Yomra may cause some confusion since there is already an existing cultivar with the same name, which is a synonym of Foşa. Orchards in the Western Black Sea region (Bartın, Zonguldak, Düzce, Sakarya

Kocaeli, Bolu) consist of mixed cvs. such as Karafındık, Mincane, Çakıldak and Foşa but mostly Tombul has been planted in recent renewals.

Due to their self-incompatibility, the main production cultivars are grown together with genetically-compatible pollinizers chosen to shed pollen at the time of the female flowers of the main cultivars are receptive. The percentage of pollinizers required to have a proper pollination is at least 10% of the orchard trees, generally with two pollinizer cultivars planted per orchard, each with different bloom periods, to ensure the dissemination of cross-compatible pollen.

Hazelnut orchards are planted with trees at regular spacing. Spacing depends on plant vigor, which can be cultivar specific, and the training system. In Turkey, hazelnuts are traditionally grown as a clump called an *ocak* consisting of about 6 trees established in a circle of about 1.2 m in diameter (Fig. 6.7). Ocaks are spaced in the field on a grid of 4 × 4 m or 4 × 5 m on sloping land with shallow soils and 6 × 6 m on level areas with deep soils. In Western Europe (Italy, Spain, France) single trees are planted at distances of 4–6 m within the row (2.5–3 m in case of double-density) and 5–6 m between rows. In Oregon, standard spacing is 6 × 6 m, while double-density is 3 × 6 m. When the double-density field layout is chosen, the intention is to remove every other tree when the tips of the branches of neighboring trees begin to touch, 8–12 years after planting.

Fig. 6.7 *Ocak* planting system in Turkey, which consists of about six trees planted in a circle (Photo by V. Erdogan)

Fig. 6.8 Free-vase training system used in the Langhe district, Italy. (Photo by R. Botta)

The traditional training scheme favors the natural development of the plant into a bush, but limits the number of main trunks to 4–5. This form is achieved by desuckering the plant 3–5 times a year mechanically or more efficiently using an herbicide to kill the young shoots (15–20 cm). In modern orchards, plants with a single stem are often preferred for easier management (Fig. 6.8). In this case, a single shoot is trained to develop strong scaffold branches at about 1–1.5 m from the crown, beginning from the first year of planting and with all suckers removed during the season.

The tendency to produce suckers varies among cultivars; however, since most can produce 100–150 suckers per year, desuckering is an expensive operation that accounts for 15–25% of total cultural costs, depending on the technique used. The use of low/non-suckering rootstocks, selected from *Corylus colurna* and its hybrids, can permanently reduce the need for desuckering. Other pruning practices are carried out manually or mechanically and start when the plant crowns are closing and there is a risk of reduced penetration of light in the canopy. In the Black Sea region, a rejuvenation pruning is typically done to the ocak when stems get old, weak and fail to bear, which can be between 25–40 years of age. The orchard floor is managed either by maintaining the soil surface clean from weeds or leaving a grass strip between rows that is cut short before harvesting.

A well established and well managed orchard initiated with disease-free, healthy plant material, in a suitable environment, may not require treatments against pathogens. However, orchards are not always free of pathogen concerns. These can include: virus (apple mosaic virus, AMV) and bacteria, such as *Pseudomonas avellanae* (Psallidas) Janse et al. (agent of bacterial twig dieback) and *Xanthomonas arboricola* pv. *corylina* (Miller et al.) Vauterin et al. (bacterial blight), whose

infection can be prevented using copper salts; fungi such as *Piggottia coryli* (Desm.) B. Sutton (anthracnose), *Cytospora corylicola* Sacc. (cytospora canker) and *Erysiphe corylacearum* U. Braun & S. Takam., the agent of powdery mildew similar to *Phyllactinia guttata* (Wallr. ex Fr.) Lév., but more aggressive, recently spread in The Republic of Georgia and Turkey. A special mention concerns the fungus *Anisogramma anomala*, native to eastern North America but now present in the Pacific Northwest, which causes EFB, a serious threat to plant survival. While management that includes scouting for infected stems, heavy pruning and fungicide applications can help reduce the impacts of EFB, these efforts are expensive and not always fully effective. Thus, breeding for resistance to the pathogen is considered the most sustainable approach for the management of the disease. Considerable efforts are underway at Oregon State University and Rutgers University to develop new hazelnut cultivars expressing durable resistance (Mehlenbacher 2018; Molnar et al. 2018a).

Arthropod pests also require special attention and may necessitate interventions one or more times during the year to avoid yield loss and a reduction in kernel quality. The main pests in general are big bud mite (*Phytoptus avellanae* Nal.), nut weevil (*Curculio nucum* L.) and the true bugs. The true bug group includes indigenous species such as *Gonocerus acuteangulatus* Goeze, *Coreus marginatus* L., *Palomena prasina* L. and *Nezara viridula* L. and the introduced species of Asian origin *Halyomorpha halys* Stål (brown marmorated stink bug). This insect is now present in the USA, as well as parts of Europe, the Caucasus and Turkey, and has become a major pest due to its polyphagy, gregarious behavior, ability of adults to attack hazelnuts throughout the season (Bosco et al. 2018; Hedstrom et al. 2014) and the lack of specific parasitoids. Further, the filbertworm (*Melissopus latigerranus* Wals.), present only in North America, causes damage to nuts similar to that of the nut weevil, but to date it is easily controlled by pesticides.

Nut maturity is reached in August to October depending on the cultivar, area of cultivation and elevation. At harvest time, the nuts fall to the ground, either free or in the husk. However, nuts are still collected by hand in areas when the topography or other factors do not allow machine harvesting, such as in Turkey where they are harvested by hand from the plant while still in the husk. In this case, the husk is removed by hand or machine (*patoz*) after first drying for 3–5 days. In all cases, nuts require drying to a moisture content of about 10%, which correspond to <6% moisture in the kernel, to be suitable for storage and/or sale. Drying has been traditionally carried out in the farmyard with nuts spread out in a thin layer under the sun for 4–5 days. However, rains and high humidity can hinder the drying process, causing delays and deterioration of the kernels. Today, electrical or heated forced-air dryers are used to provide more consistent results and are considered vital in the proper postharvest management of hazelnuts.

Dry nuts can be stored at room temperature for over 8 months in the shell without significant quality loss (Ghirardello et al. 2013); kernels can be preserved up to 1 year in a storage room at 2–4 °C. In Turkey, in-shell hazelnuts may be stored for as long as 24 months in climate-controlled warehouses, allowing storage of the crop for an extended period when it exceeds domestic and/or international demands.

6.2.2 Current Agricultural Problems and Challenges

Hazelnut is currently in a state of significant expansion of the surface area planted and harvested worldwide, including land outside of traditional production regions. This increase in production in new regions, compounded by the expected impacts of climate change, prioritizes the need to identify and/or develop new cultivars suitable for different growing environments, including those more tolerant to heat stress and drought as well as wide temperature fluctuations. In addition, the attention of authorities and public opinion towards the sustainability and impacts of agricultural systems indicates that cultivation techniques must be directed to address desires to reduce chemical use, pollution due to fertilizers, water and fossil fuel consumption and CO2 emissions. The logistical and economic challenges associated with assembling and managing sufficient hand labor in the agricultural sectors of Western countries is another major problem that is changing cultivation techniques, driving an increase in mechanization (also expected to become relevant in the future in all hazelnut-producing countries). Further, in many existing situations, for example in old production centers such as the Black Sea region, orchard age (>100 years) and small farm size (1–2 ha) and limited access to various inputs, make further mechanization unlikely, and have led to reduced yield and lower farm income.

Hazelnut is considered a relatively low impact crop with low input requirements; in many countries it has been grown prevalently in areas where major crops, such as cereals or other high-value fruit species, cannot be grown with profit, due to a less than ideal environmental (soil type, no possibility of irrigation) and/or orographic (hills) conditions. Yet, further steps can be taken to expand upon this valuable aspect of hazelnut production through the adoption of innovative and more sustainable methods of cultivation, based also on cultivars deemed more tolerant to environmental constrains and biotic factors, including pest and pathogens of severe impact. To reach these goals without losing quality and yield, the use of biotechnological tools and a greater understanding of hazelnut genetics in support of traditional techniques should prove highly advantageous.

6.2.3 Breeding Goals

Breeding goals may change depending on environmental conditions and the presence of various pathogens or pests in the different hazelnut growing regions around the world. The major goals are summarized in the following list.

Vegetative traits
Common goals

- Reduced sucker emission
- Upright trees with moderate vigor
- Late vegetative budbreak to avoid spring frosts

Specific goals

- Cold hardiness (especially of the catkins) for expanding the crop to northern areas with severe winters
- Low chilling requirement for expanding the crop to subtropical regions
- Tolerance to adverse soil conditions, such as high/low pH and heavy soil
- Improved tolerance to drought*

Reproductive traits
Common goals

- Early bearing (precocity)
- Abundant male (pollenizer) and female flowering
- High flower fertility
- Self-compatibility
- High yield
- Early nut maturity
- Short period of nut fall (concentrated drop at maturity)
- Husk not clasping to allow nut to drop free at maturity (ease of mechanical harvest)
- Well-sealed nut shell (lack of split sutures)
- High percent kernel (thin shells)
- Low percentage of nuts with defects (blanks, twin kernels, shrivels, poorly-filled nuts, moldy kernels, black tips, brown-stained nuts)
- Kernels uniform in size and round shape
- Long storage life (oil less prone to rancidity)*
- High nutritional and nutraceutical value*

Kernel market

- Round nut and kernel shape
- Small to medium size kernel, 13–15 mm in diameter with low percent of broken kernels after shelling
- Ease of pellicle removal after kernel roasting
- Good flavor after kernel roasting

In-shell market (table consumption)

- Large nut size
- Attractive and intact (no splits) nut shell; shell lacks pubescence (clean and shiny not dull)
- Kernel with good flavor
- Little or no fiber on the pellicle of the kernel

Pest and pathogens
Resistance/tolerance to major pests

- Big bud mite (*Phytoptus avellanae*)
- Nut weevil (*Curculio nucum*)

Resistance/tolerance to major diseases

- Eastern filbert blight (*Anisogramma anomala*)
- Anthracnose (*Piggottia coryli*)∗
- Cytospora canker (*Cytospora corylicola*)∗
- Powdery mildew (*Erysiphe corylacearum*)∗
- Bacterial blight (*Xanthomonas arboricola* pv. *corylin*a)
- Bacterial twig dieback (*Pseudomonas syringae* pv. *coryli*)∗

Ornamental attributes for garden use

- Contorted and weeping stems
- Red/purple or yellow leaves
- Pink/red fall color
- Dissected, lobed or truncated leaves
- Frilly involucres
- Attractive peeling bark

∗Traits still to be tested for genetic variability within genus *Corylus* and that are currently not pursued breeding goals.

Most traits of interest are polygenic and some of them have medium to high heritability (Table 6.4). Self-incompatibility, red leaf color and EFB resistance from cv. Gasaway and several other sources are traits inherited in a simple dominant Mendelian manner. Other traits known to be simply inherited are: contorted growth habit, the cutleaf trait, cream-colored pollen and yellow style color, but are recessive (Mehlenbacher 2018).

6.3 Germplasm Biodiversity and Conservation

Hazelnut biodiversity is very high due to its natural range of distribution, spanning different climate and soil types. Several authors have studied the genetic structure of wild and cultivated germplasm.

6.3.1 Cultivar Characterization, Germplasm Diversity and Phylogeny

Identification and correct naming of accessions and analysis of genetic diversity in wild populations and germplasm collections (ex situ, in situ) are important in the management and exploitation of plant genetic resources (Boccacci et al. 2013). Morphological and phenological descriptors are influenced by environmental factors and are often unreliable indicators to discriminate cultivars; moreover, when

Table 6.4 Narrow-sense heritability of traits in hazelnut (Adapted from Yao and Mehlenbacher 2000)

Trait	Yao and Mehlenbacher (2000)[a] ∗Valentini et al. (2004)
Big bud mite susceptibility	0.78∗
Time of leaf budbreak	0.72 + 0.05
Time of catkin elongation	0.68 + 0.05
Time of stigma exertion	0.58 + 0.05
Husk length	0.82 + 0.07
Relative husk length	0.91 + 0.06
Nuts per cluster	0.67 + 0.11
Time of nut maturity	0.86 + 0.11
Nut length	0.68 + 0.05
Nut width	0.78 + 0.05
Nut depth	0.89 + 0.05
Shape index	0.65 + 0.10
Nut weight	0.63 + 0.04
Kernel weight	0.67 + 0.05
Percent kernel	0.87 + 0.07
Fiber amount	0.56 + 0.09
Blanching ability	0.64 + 0.07
Kernel compression index	0.88 + 0.08

[a]Point estimate + standard error

plants are not yet bearing, cultivar identification based on plant traits can be very difficult (Boccacci et al. 2005).

DNA markers, due to their high discriminating power at a relatively low cost, have proven to be a convenient method to accurately identify hazelnut cultivars, clarify cases of homonymy and synonymy, study the origin of the cultivated varieties, determine parentage and assess genetic relationships among cultivars. RAPDs (random amplified polymorphic DNA), AFLPs (amplified fragment length polymorphism) and especially SSRs (simple sequence repeats) have been extensively used for germplasm characterization.

RAPD markers were used to construct maps (Table 6.5) and to select markers linked to the incompatibility S-locus (Pomper et al. 1998) and to the trait of resistance to EFB found in Gasaway (Mehlenbacher et al. 2004). Studies using AFLP and ISSR markers have been limited and include work by Chen et al. (2005), Ferrari et al. (2005), Kafkas et al. (2009) and Martins et al. (2014). Microsatellite or SSR molecular markers were developed at OSU and at the University of Torino (Bassil et al. 2005a, b; Boccacci et al. 2005; Gürcan and Mehlenbacher 2010a, b; Gürcan et al. 2010a) and have been routinely used for germplasm characterization, since they have significant advantages over other types of markers. Some 720 SSR markers have been developed to date, from enriched libraries, ISSR fragments, transcriptome sequences and genome sequences (Bhattarai and Mehlenbacher 2018). Moreover, SSR markers identified in *Corylus avellana* are transferable

Table 6.5 Studies carried out on the genetic diversity of *Corylus* using nuclear simple sequence repeat (SSR) markers

Reference	No. nSSR loci analyzed	Germplasm evaluated	Main results
Boccacci et al. (2006)	16	78 cvs. from various germplasm repositories	The study confirmed several synonyms described in the literature, and new cases of synonymy were identified. The parentage of some North American cvs. (Butler, Ennis, Royal) and the French cv. Fercoril-Corabel was investigated. An evident differentiation of the northern European cvs. from the southern European ones and from the Turkish cv. was highlighted
Boccacci et al. (2008)	16	33 Spanish cvs. including local germplasm and 18 minor cvs. from northeastern Spain	A high level of genetic diversity was observed in 33 genotypes, although a high number of them showed a close genetic relationship. Negret-type cvs. were found to be distinct from Negret, and some cvs. within this germplasm appeared to be seedlings of Negret. The 18 minor cvs. showed unique profiles and no new case of synonymy was detected
Gökirmak et al. (2009)	21	270 accessions from USDA Collection in Corvallis (OR) USA	Of the 270 accessions analyzed, 198 had unique fingerprints while 72 were duplicates. The genetic dendrogram revealed four major geographical groups: Central European, Black Sea, English and Spanish-Italian
Bassil et al. (2009)	12	29 cvs. from USDA collection in Parlier (CA) USA	SSR markers were used to fingerprint trees in the Corvallis (OR) backup collection, established in Parlier (CA) and the results were compared to the fingerprints of the same accessions in the Corvallis collection. This work allowed for the elimination of misidentified accessions and for further refinement of the fingerprinting set
Boccacci and Botta (2010)	16	75 accessions from Spain, Italy, Turkey and Iran	A set of SSR loci was selected and proposed as molecular descriptors for hazelnut. Genetic differentiation among cvs. from different regions was significant; statistical analysis indicated that hazelnuts were domesticated independently in the four areas, suggesting that present cvs. have multiple origins

(continued)

Table 6.5 (continued)

Reference	No. nSSR loci analyzed	Germplasm evaluated	Main results
Gürcan et al. (2010b)	12	117 cvs. from Turkey, Republic of Georgia, Azerbaijan	Genetic diversity was investigated in accessions from three countries and compared with cvs. from Spain and Italy. A high level of genetic diversity was observed in the Black Sea accessions. A moderate shift in allelic frequencies was seen between accessions from the Black Sea and the Spanish-Italian accessions. SSR analysis identified the putative parents of two Turkish cvs.
Campa et al. (2011)	13	40 wild hazelnuts collected in northern Spain, and 76 cultivated hazelnuts (62 local selections and 14 reference cvs.)	Microsatellite analysis revealed considerable diversity and a high level of differentiation between the locally cultivated forms and the remaining materials. The results indicate that hazelnuts accessions from northern Spain can be divided in three groups: accessions clearly differentiated within the Spanish-Italian gene pool, accessions derived from hybridization, and accessions probably derived through exchange with other geographical areas
Erfatpour et al. (2011)	15	90 samples from Northern Iran	A population of 90 hazelnut plants from the Talesh Mountains (Guilan province, northern Iran) was studied. A dendrogram was generated by UPGMA clustering method that placed the 90 samples into 7 main groups. A high genetic diversity was found in the studied population
Sathuvalli and Mehlenbacher (2012)	21	154 accessions (87 *C. americana* and 67 *C. americana* x *C. avellana* hybrids)	A high level of genetic diversity was observed in the American accessions. The plants used were grown in field collections at the USDA-ARS-NCGR, the hazelnut breeding program at Oregon State University and the Arbor Day Farm, USA. Seven major groups resulted from a genetic dendrogram: European cvs. and cv. Rush hybrids, two groups of Arbor Day Farm hybrids, three groups of *C. americana* accessions, and a mixed group of hybrid and American accessions. The genetic diversity among the American hazelnut accessions is useful in breeding hybrids that will allow expansion of hazelnut production into eastern North America

(continued)

Table 6.5 (continued)

Reference	No. nSSR loci analyzed	Germplasm evaluated	Main results
Bassil et al. (2013)	7	114 *Corylus* accessions including 11 species and 44 interspecific hybrids	Among 23 SSRs tested, seven were highly polymorphic and used to perform neighbor-joining clustering and structure analysis. The results supported inclusion of *C. maxima* within the large polymorphic species, *C. avellana*. Moreover, the results indicated that *C. californica* is a distinct species rather than a botanical variety of *C. cornuta*
Boccacci et al. (2013)	10	118 accession including 42 southern Europe unique landraces, 57 reference cvs. and 19 wild accessions	Analysis of genetic relationships and population structure indicated the existence of three primary centers of diversity in the Mediterranean basin: northwestern Spain (Tarragona) and southern Italy (Campania) in the West and Black Sea (Turkey) in the East. Moreover, the data suggest the existence of secondary gene pools in the Iberian (Asturias) and Italian (Liguria, Latium) peninsulas
Muehlbauer et al. (2014)	17	323 unique accessions including EFB-resistant and tolerant germplasm of uncertain origins	Microsatellite markers were used to investigate the genetic diversity and population structure of 323 unique accessions, in comparison with reference accessions representing a wide diversity of *Corylus* cvs. breeding selections and interspecific hybrids. Results indicated that EFB resistance is relatively widespread across the genus *Corylus* and showed 11 consensus groups with EFB-resistant or tolerant accessions in all
Martins et al. (2015)	16	58 accessions including 19 landraces, 13 wild accessions from Portugal and 26 reference cvs.	High level of polymorphism was found. A neighbor-joining dendrogram showed a clear separation of the wild genotypes from the landraces and reference cvs. This study provided new insights on the origin of Portuguese landraces. The high genetic diversity found among wild genotypes may indicate that Portugal was a refuge during the last ice age and reinforce the idea that Portugal may also have been a natural reservoir for the wild genotypes

(continued)

Table 6.5 (continued)

Reference	No. nSSR loci analyzed	Germplasm evaluated	Main results
Zong et al. (2015)	10	348 *C. mandshurica* individuals among 12 populations in China	SSRs were applied to evaluate the genetic diversity and population structure. SSR markers expressed a relatively high level of genetic diversity. The results of genetic dendrogram and structure analysis suggested that *C. mandshurica* populations fell into two main clusters: group I accessions were located in Northeast China, and group II accessions were in North China
Brown et al. (2016)	7	850 wild hazels from 29 sites in Ireland (25 sites across Northern Ireland along with 4 sites in the Republic of Ireland)	High-resolution microsatellite markers were used to analyze levels and patterns of genetic diversity in the species. The results indicate that hazel populations exhibit high levels of genetic diversity along with low levels of population differentiation, suggesting extensive gene flow
Öztürk et al. (2017)	49	102 accessions of Slovenian germplasm, including 54 wild accessions and 48 cvs.	This study examined molecular genetic diversity and population structure. In general, cvs. and wild accessions clustered separately. An association mapping panel composed of cvs. and wild accessions had considerable variation for the nut and kernel quality traits. 49 SSR markers were significantly associated with nut and kernel traits
Demchik et al. 2018	10	1140 individuals sampled from 25 populations across Wisconsin, Iowa, Minnesota and North Dakota, USA	This study used molecular markers to investigate genetic diversity in 25 populations across central-north USA states. Results showed a very high genetic diversity within populations and some tendency toward population differentiation. Genetic distance among the populations was not correlated with geographic distance. Selection of individuals for use in breeding should be based primarily on phenotype, including individuals of genetically differentiated populations

across the genus as well as in related genera in the Betulaceae family (Gürcan and Mehlenbacher 2010b). SSR markers were used to fingerprint cultivars, identify synonyms, determine parentage and assess genetic relationships among cultivars; they were also used to investigate the genetic diversity and structure in collections and wild populations, or compare local cultivars and wild hazelnuts (Table 6.5).

According to several studies (Boccacci and Botta 2009, 2010; Gökirmak et al. 2009; Gürcan et al. 2010a), *Corylus avellana* was domesticated independently in

six different areas: the British Islands, central Europe, Spain, Italy, the Black Sea region and Iran. The results reported by Boccacci et al. (2013) indicate the existence of three main germplasm groups in the Mediterranean Basin, corresponding to three putative domestication areas: northwestern Spain (Tarragona), southern Italy (Campania) and the Black Sea region (Turkey). Secondary gene pools were observed in the Iberian (Asturias) and Italian (Latium, Liguria) peninsulas, where local cultivars were domesticated from wild forms and/or from crossings with introduced ancient domesticated varieties.

Studies of hazelnut genetic diversity show that there is a huge amount of genetic variability in *Corylus avellana*; to date about 500 cultivars have been described in the literature (Mehlenbacher 2018). Mislabeling and the existence of synonyms and homonyms are important challenges for germplasm conservation that can in most cases be solved using a set of SSR markers; however, SSRs have a limitation of not being able to identify closely-related cultivars derived from clonal variation (Valentini et al. 2014), and other marker types are required. Phenotypic variability existing among clones of major cultivars, in response to clonal propagation for many decades if not centuries, underlines the necessity to carry out clonal selection work and genetic certification. At the University of Torino, Italy, studies on clonal variation within cv. Tonda Gentile delle Langhe are being carried out. Preliminary results show the presence of some 130,000 polymorphisms (mostly SNPs) between clones (unpublished data).

The chloroplast genome has a lower evolutionary rate than the nuclear genome; therefore, chloroplast SSRs (cpSSRs) can provided insight into origin and diffusion of the species. CpSSRs analysis was used for investigating the post-glacial migration of wild *Corylus avellana* populations in Europe (Palmé and Vendramin 2002), studying the origin and diffusion of hazelnut cultivars in the Mediterranean Basin (Boccacci and Botta 2009; Martins et al. 2013) and the phylogeographical relationships in *Corylus* species (Bassil et al. 2013) (Table 6.6).

A single, most common chlorotype was found in very high frequency across northern and central Europe suggesting probable migration from the Pyrenees following the most recent ice age. In contrast, a higher diversity of chlorotypes were identified in refugia in southern Italy, the Balkans and northern Portugal. This work and others support the understanding that *Corylus avellana* appears to have had three domestication areas: Mediterranean, Turkish and Iranian. Further, among the *Corylus* genus, the greatest species diversity is found in Asia, particularly in China, suggesting this region is its center of origin (Mehlenbacher 2018).

Finally, EST-SSRs are powerful tools for linkage map construction, comparative mapping, marker-assisted selection and evolutionary studies. In silico identification and characterization of unique SSRs derived from Betulaceae ESTs was carried out to develop conserved orthologous markers for genetic analysis of different Betulaceae species. Ten EST-SSR from *Betula pendula* Roth (Gürcan and Mehlenbacher 2010b) and 24 EST-SSR from *Alnus glutinosa* L., *Betula pendula* Roth, *Betula platyphylla* Suckaczev (Boccacci et al. 2015) were characterized in *Corylus* spp. The EST-SSRs developed in these studies increase the number of SSR markers currently available for *C. avellana*, representing not only suitable marker

Table 6.6 Studies of chloroplast diversity in *Corylus* based on simple sequence repeat markers

Reference	No. cpSSR loci analyzed	Plant material	No. chlorotypes	Main results
Palmé and Vendramin (2002)	3	248 *C. avellana* wild individuals from 26 locations across Europe	7	A most common chlorotype (A) was found in 76% of the individuals and in most geographical areas except in the southeastern Europe. One chlorotype (B), present in only 4% of the individuals, was found in Spain, Germany, France and Corsica. Additional chlorotypes (C, D, E and F) were found in southern and central Italy, Corsica and the Balkans. The pattern of chloroplast haplotypes divide Europe into two main areas: northern and western Europe, and Italy and the Balkan area
Boccacci and Botta (2009)	4	75 *C. avellana* cvs. from Spain, Italy, Turkey and Iran	4	A single most common chlorotype was present in all groups. Results suggest hazelnut was domesticated independently in three areas: the Mediterranean, Turkey and Iran with limited germplasm exchange with the western to eastern Mediterranean Basin. Moreover, the results suggest considerable exchange of germplasm between Italy and Spain, which probably occurred during Roman times
Martins et al. (2013)	4	35 *C. avellana* accessions, including 3 cvs., 19 Portuguese landraces and 13 wild plants	11	A dominant chlorotype was frequently found in landraces and cultivars. Four chlorotypes were found only in wild hazelnuts. The results suggest that northern Portugal was a refugium for hazel during the last ice age
Bassil et al. (2013)	6	114 *Corylus* accessions representing 11 species and 44 interspecific hybrids	21	Six cpSSRs were polymorphic in *Corylus* species and generated 21 distinct chlorotypes. The phylogeographical relationships in the 11 *Corylus* species support Asia as a refugium where several hazelnut lineages survived during glaciation and from which *Corylus* species continued to evolve through the Mediterranean to Europe, and across the Atlantic and/or the Bering land bridge to North America

for genotyping and population genetic analyses, but representing ideal markers for genetic and comparative mapping and marker-assisted selection in hazelnut.

6.3.2 Genetic Resources Conservation Approaches

Conservation of hazelnut germplasm is carried out prevalently ex situ, in collections where hazelnut cultivars and landraces are planted in replicates (see Appendix 1). Tissue cryopreservation studies were carried out using seed embryo axes (Reed et al. 1994) but no strategies of conservation in vitro are being currently used.

6.3.3 Cytogenetics

The chromosome number of the genus is 2n = 22, although with small karyotypic differences (Guo et al. 2009; Thompson et al. 1996). The genome size of *Corylus avellana* was estimated 378 Mbp (empirically determined by flow cytometry at the USDA-ARS-NCGR in Oregon in 2012; Rowley et al. 2018).

Observations carried out during meiosis indicate that the decreased pollen fertility (40–70%) observed in some cultivars, including Barcelona, Negret, Tonda di Giffoni, Segorbe and Tonda Gentile delle Langhe, is related to the presence of reciprocal translocations (Salesses 1973; Salesses and Bonnet 1988). While only half of the pollen is expected to be viable due to the unbalanced gametes that result from the irregular meiosis, the productivity of these cultivars is not reduced in a noticeable way. However, abnormalities like this should be considered carefully as they can have significant effects in a breeding program. In addition, reciprocal translocations complicate the construction of genetic maps (Lehmensiek et al. 2005); in fact, linkage relationships in a translocation heterozygote are altered, as recombination between loci may be significantly reduced, particularly close to the translocation breakpoints.

During the development of maps for a progeny Tonda Gentile delle Langhe × Hall's Giant, Torello Marinoni et al. (2018) were able to disentangle *pseudo-linkage* between markers applying a bioinformatic approach (Farré et al. 2011). In this work, the markers around the translocation breakpoints were identified and the two chromosomes arms of each linkage group involved in the translocation in Tonda Gentile delle Langhe were aligned with the corresponding LGs of Hall's Giant.

These findings indicate that to bolster future breeding strategies and to better understand the impact of the reciprocal translocation on yield, it is worthwhile to expand the study on the occurrence of these genetic modifications to a broader range of cultivars, observing the effect on pollen and embryo sac fertility.

6.4 Traditional Breeding and Role of Biotechnologies

Despite its sporophytic self-incompatibility system, *Corylus* species are rather well amenable to breeding, especially in comparison to other temperate nut crops of world importance. Generation times are relatively short (3–6 years to first flowering in *C. avellana*, maybe longer in other species of the genus); plants are smaller in size; female inflorescences stay receptive to pollen for multiple weeks and are easy to isolate from foreign pollen; staminate flowers are simple to emasculate and pollen is abundant, easily collected and can be stored for up to 1 year; and hand crosses can produce significant numbers of seeds (Thompson et al. 1996). Considering its commercial nut attributes and high level of phenotypic and genotypic variability, it is expected that most genetic improvement efforts will continue to be centered on the use of cultivated and wild germplasm of *C. avellana*, with other species used as donor parents to contribute desired traits as needed using a backcrossing strategy.

6.4.1 Traditional Breeding Methodologies

Hazelnut breeding methodologies include setting breeding objectives, the collection and evaluation of germplasm, selection of eligible parents, crossing between selected parents, collection of seeds, and then the growing of seedlings in the greenhouse and later in the field for evaluation. At this stage, although still in its infancy for hazelnut, marker-assisted selection can be used to help identify genotypes carrying a specific trait. Typically, in fall of the first year, plants are transplanted to the open field to begin initial screening and selection. They begin to flower after 4–5 years, and the first nuts can be evaluated and individuals with non-ideal traits subsequently eliminated; however, a complete selection/evaluation cycle takes about 8 years from germination, as multiple years are needed to determine single-plant performance across seasons. Selected, superior genotypes at this point could enter replicated clonal yield trials to assess their performance in the longer term and across locations, and/or could be selected for use in continued breeding efforts.

In an ideal scenario, the release of a new cultivar requires 17–18 years from the year the cross was performed. However, despite this long timeline, since hazelnut shows a high level of heterozygosity, the selections of first generation progeny may not provide all the desired traits in one individual and continued breeding is necessary. This is particularly true when the introgression of a needed character or trait, such as resistance to EFB, requires starting from a parent with poor quality nuts and/or other attributes detrimental to commercial production. This timeline can become even greater when considering traits under multigenic control. Thus, the use of wild materials in breeding should be considered very carefully, with attention on also using the best available, diverse recurrent parents. Fortunately, the high genetic diversity within cultivated *Corylus avellana* and the only very recent

breeding of the crop in general presents great opportunities for systematic improvement across many traits.

6.4.1.1 Flower Biology and Controlled Pollination

Hazelnuts are monoecious and wind-pollinated. They flower during the winter and are dichogamous. The dates of bloom range from early December to March depending on the genotype, geographic location and year. Reproduction is limited by a sporophytic self-incompatibility system, which in *Corylus avellana*, the species most studied in this regard, is controlled by a single locus designated as the S-locus with 33 alleles (Mehlenbacher 2014). The stigmatic surface is the site of the incompatibility reaction. Dominance or codominance of the alleles is expressed in the pollen, whereas all known S-alleles are codominant in the pistil (Mehlenbacher 1997; Mehlenbacher and Thompson 1988). Interestingly, female flowers can stay receptive to fertilization for up to 3 months. Self-pollination of most cultivars results in null or very low nut set. Partial self-compatibility was reported in some cultivars, such as Montebello (syn. Nocchione), Tombul, Palaz, Çakıldak, Kalınkara, Sivri and a few offspring of Montebello (Cakır and Genc 1971, Mehlenbacher 2014; Mehlenbacher and Smith 1991). Self-incompatibility also exists in other *Corylus* species (Erdogan and Mehlenbacher 2001).

A useful first step in planning the crosses is to determine the S alleles of the potential parents to be sure that the desired combination is compatible. In autumn, before any pollen is released and pistillate flowers have not yet emerged, the plant selected as a female parent must be isolated to exclude foreign wind-borne pollen. The entire tree can be covered in a pollen-proof structure (Fig. 6.9), or when smaller amounts of seed are desired, pollen-proof bags can be placed over individual limbs. Before covering, the plant must be emasculated (clipping off the catkins). Although most will set few or no nuts with their own pollen, emasculations help avoid potential uncontrolled pollination and simplifies the procedure by reducing large clouds of self-pollen that would be present in the isolation chamber or bags. Pollen is collected from a selected male parent plant by removing catkins from the tree just after they have begun to elongate and while turgid, bright yellow anthers remain visible, preferably in the morning prior to pollen release. Once collected, they are placed indoors in a still room on clean white paper at 15–20 °C to promote anther dehiscence, typically overnight. The next morning, pollen is gently shaken from the catkins onto the paper then poured into glass or plastic vials and stored in a freezer at −18/−20 °C until its use for pollination. The pollen can be manually applied on a windless day and preferably when humidity is very high, using a paintbrush or a fingertip. The pollen-proof chamber can be removed at the end of the female flowering period when the stigmas are dry and dark brown colored.

Fig. 6.9 The setting up of a pollen-proof structure around the female parent tree destined for use in a controlled cross. (Photo by S.A. Mehlenbacher)

6.4.1.2 Seed Management and Germination

Nuts are collected from the crossed plant before they fall to the ground, typically when the nuts can be easily separated from the husk, which is about the time the shells turn at least partially brown. They are stored in mesh bags and put into a cooler at 4 °C for about 3 months. In November, nuts are soaked for 2–3 days in a bucket of water, changing the water after 24 h. After soaking, the nuts are provided a moist chilling (stratification) treatment, for example using an equal volume of moistened vermiculite in a plastic box, covered with a polyethylene bag, and stored in a cooler at 4 °C for 3–5 months. In March, the box is left at room temperature (18–20 °C) for a week to promote germination. Note that germination can be aided by soaking nuts with carefully cracked shells in gibberellic acid (50–100 ppm) for 12–24 h. Seeds that show root tips are planted into containers with a substrate composed of peat and perlite (3:1 ratio) and placed in a greenhouse until autumn. By late summer, seedlings reach about 1 m tall, are hardened outdoors for a few weeks and then are transplanted to the field.

6.4.1.3 Field Planting

In the fall, young plants are transplanted to the open field for initial screening and selection. Most consider the best time for planting seedlings to be mid-October to early-November; plants grow vigorously and have high survival rates during the second year due to the considerable root growth that can occur during the first autumn and early spring prior to the heat of summer. The spacing for seedlings trees is at least 1 m in the row and 3 m between rows. With closer spacing, it becomes difficult to separate nuts from adjacent trees, light exposure can be insufficient and it becomes challenging to move equipment through the field.

6.4.1.4 Selection

Seedlings are regularly observed for the most important traits following evaluation systems developed with international cooperation (Bioversity International 2008; Thompson et al. 1978; UPOV 1979). Evaluation of traits follows the timeline described in Table 6.7, with a hierarchy of minimum standards set for each trait by the breeder that is adjusted based on expectations of each progeny and breeding line, with rigorous culling practiced to eliminate poor performers as they become evident. Superior individual seedlings expressing desirable traits, identified in the progeny row trials, are propagated by mound layering and subsequently evaluated in primary replicated clonal yield trials for important agronomic and nut traits using a single-tree plot design with 5–10 replications of each selection and well-known control cultivars.

Table 6.7 Main objectives of hazelnut breeding and age of tree at evaluation

Objective	Age at evaluation
Bud mite resistance	4
EFB resistance	1[a]
Precocity	4–5
Early maturity	5–8
Free-falling nuts at harvest	5–8
Round nut shape	4–5
Few nut and kernel defects	4–16
High percent kernel	4–5
Easy kernel blanching	5–8
High yield	4–16

Source: adapted from Molnar (2011)

[a]Using marker assisted selection if segregating for the Gasaway R-gene

6.4.2 Hybridization

Despite that the interspecific hybridization potential within *Corylus* is high (Erdogan and Mehlenbacher 2000b; Thompson et al. 1996), strategies used for improving hazelnut have been mostly based on intraspecific crossings of *C. avellana*. This focus is due to its far superior nut quality, yield and other commercial production characteristics when compared to the non-cultivated species in the genus. In general, successful hybrids can be easily obtained between members of the same subsection, while crosses between the different subsections are more restricted, but still possible in many cases (Table 6.8; Erdogan and Mehlenbacher 2000b; Molnar 2011). While some species intercross freely, some can be crossed with difficulty, some can be crossed only in one direction (unilateral incompatibility) and the others cannot be crossed in either direction. Unsuccessful interspecific crosses may result in low or zero seed set, a high frequency of blanks, abnormal embryos, reduced germination, and/or stunted seedling vigor, chlorosis or death. In general, crosses involving *C. avellana* were more successful when it was the pollen parent (Erdogan and Mehlenbacher 2000b). Compatibility between *C. jacquemontii*, *C. fargesii* and *C. ferox* and several other hazelnut species have yet to be evaluated.

Desirable and economically useful traits lacking or difficult to find in *Corylus avellana* that could be of interest to improve cultivars include (Table 6.9): non-suckering growth habit, tolerance of alkaline soils, extreme precocity, early maturing nuts, extreme cold hardiness, drought and heat tolerance, ornamental attributes, unique incompatibility alleles and novel sources of resistance to EFB. Several attractive ornamental attributes are present in the genus including contorted/twisted stems, pendulous/weeping branches, red/purple and yellow/golden leaves, highly dissected leaves (cut-leaf habit), lobed and truncated leaf habits (*C. heterophylla*), bright pink and red fall color (*C. americana*), frilly involucres (*C. americana*) and attractive peeling bark (*C. fargesii*).

Corylus jacquemontii, *C. chinensis*, *C. fargesii*, and *C. ferox* (not reported in the table) are interesting for their non-suckering tree form and for ornamental attributes; however, they have small, thick-shelled nuts with husks that retain the nuts at maturity, and a limited germplasm base currently available.

Within the *Corylus* genus, three species have been used most prevalently in breeding with *C. avellana*: *C. colurna*, *C. americana* and *C. heterophylla.*

Corylus colurna is considered interesting for its stress tolerance, vigor, and single stem growth habit. In particular, the use of non-suckering trees, either directly or as rootstocks, would reduce the need for herbicides and/or hand labor for desuckering plants in commercial orchards. Seedlings of *C. colurna* have been successfully used as rootstocks for *C. avellana* (Cerovic et al. 2009; Ninic-Todorovic et al. 2012), showing good graft compatibility and subsequent growth, although its use is complicated by poor germination rates of the seed and slow growth of the plants in the nursery, as well as reported transplanting problems (Lagerstedt 1976). Today, hundreds of plants grafted on unselected seedlings of *C. colurna* are produced in Serbia,

Table 6.8 Hybridization potential between representative *Corylus* species (adapted from Molnar 2011)

	Species	*C. avellana*	*C. americana*	*C. heterophylla*	*C. cornuta*	*C. californica*	*C. sieboldiana*	*C. colurna*	*C. chinensis*
Compatibility (as female parent)	*C. avellana*	••	••	••			•	•	
	C. americana	••	••	•		•			
	C. heterophylla	•		••					
	C. cornuta	⊙		••	••	••	••		
	C. californica	••	••	••	••	••	••	••	•
	C. sieboldiana	⊙	••		••	••	••		
	C. colurna	••		•		•		••	••
	C. chinensis	••	••					••	••

•• High level of compatibility • Cross is compatible ⊙ Compatibility reported but not regular

Table 6.9 Positive and negative attributes found in major hazelnut species (adapted from Molnar 2011)

		C. avellana	*C. americana*	*C. heterophylla*	*C. cornuta*	*C. californica*	*C. sieboldiana*	*C. colurna*
Positive attributes	Diversity of cultivated forms	••						
	Large nut and kernel size	••						
	Early maturing nuts	•			••	••		
	High yield potential	••	•	•				
	Cold hardiness	•	••	••	••		••	••
	Drought tolerance		•	•	•		•	•
	Heat tolerance	•	•	•				
	Small growth habit (high density planting)		•	•	•			
	Non-suckering tree form							•
	Releases nuts from involucres	••		•				
	Precocious (produce nuts at young age)		•	••				
	Ornamental attributes	•	•	•				•
	Resistance to EFB	•	••	⊙	•	•	⊙	•

(continued)

Table 6.9 (continued)

		C. avellana	*C. americana*	*C. heterophylla*	*C. cornuta*	*C. californica*	*C. sieboldiana*	*C. colurna*
Possible limitations	Small, thick-shelled nuts		•	•	••	•	••	•
	Late maturing nuts		•				•	
	Cold sensitive	•				•		
	Husk retains nut at maturity		••	•	•	•	•	•
	Involucres covered with irritating hairs				••	•	••	
	Suckering growth habit	•	••	••	••	••	••	
	Susceptible to EFB	••						⊙
	Not precocious							•
	Limited germplasm available			•			•	•

• Characteristic observed •• Characteristic very prominent ⊙ Characteristic observed on rare occasion but more evaluation needed

and propagation studies are performed in Turkey; however, data on the performance of the grafted trees in the medium and long term are not yet available. Significant opportunities likely exist to identify specific seed sources as well as select clonal *C. colurna* for improved non-suckering rootstocks of *C. avellana*.

Corylus colurna can also be used in interspecific hybridization programs, especially in terms of developing vigorously growing, stress-tolerant, non-suckering clonal rootstocks (Lagerstedt 1975, 1990). *Corylus colurna* and its hybrids have also shown resistance to EFB and big bud mites (Chen et al. 2007; Coyne et al. 1998; Farris 1978, 2000; Lunde et al. 2000), demonstrating their potential value for breeding. However, it is rather difficult to hybridize *C. colurna* and *C. avellana*; trials by Erdogan and Mehlenbacher (2000b) indicate that when attempting to make the cross, *C. colurna* should be used as the female parent and breeders should perform large numbers of pollinations but expect a lot of blank shells and only a small number of hybrid seedlings.

Released selections were obtained mostly by growing large numbers of open-pollinated seed either of *Corylus colurna* or *C. avellana*. For example, in 1990, the clonal rootstocks Newberg (USOR 1–71) and Dundee (USOR 15–71) were released by OSU. They were selected as chance interspecific hybrids in 1971 from a nursery planting comprised of several thousand open-pollinated seedlings of *C. colurna* (Lagerstedt 1990). Unfortunately, Newburg and Dundee are susceptible to EFB and have had poor success in Oregon, but their use is still under investigation in Europe (where EFB is not present).

Corylus americana, the wild American hazelnut, is adapted to a wide area of North America east of the Rocky Mountains spanning many climatic zones and soil types including some very cold regions. It is also the natural host of *Anisogramma anomala*, the pathogen that causes EFB, and has been shown to be resistant or highly tolerant to the disease. Fortunately, *C. americana* crosses readily with *C. avellana* and hybrids are highly fertile and amenable to advanced generation breeding. Efforts have been ongoing since the early 1900s to use *C. americana* in hybrids with *C. avellana* to develop EFB-resistant hybrids adapted to colder regions (Fig. 6.10), as discussed previously. The challenge to breeding improved plants is the tiny, thick-shelled nuts of the wild species, it is very shrubby, spreading growth habit, and its tendency to tightly hold the nuts in their thick, fleshy husks at maturity. Fortunately, interspecific hybrids are intermediate in most traits between the parental species, and backcross hybrids to *C. avellana* show continued progress in the improvement of production traits and nut quality. However, recent studies show challenges exist in maintaining EFB resistance in advanced generation backcross hybrids, except for the case when single dominant R-genes are present, for example from *C. americana* cv. Rush (Bhattarai et al. 2017; Molnar and Capik 2012b). Studies to examine the transmission of EFB resistance from a wide diversity of *C. americana* parents as well as in the F2 generation are ongoing in multiple locations in the USA (Molnar et al. 2018b).

Corylus heterophylla is the most widely utilized wild *Corylus* resource in China. It crosses readily with *C. avellana*; therefore, it holds great value for use in interspecific hybrid breeding for enhancing climatic adaptation, especially

Fig. 6.10 Interspecific hybrid (*Corylus americana* x *C. avellana*) under selection in Wisconsin for expanding the climatic adaptation of hazelnut. (Photo by S.A. Mehlenbacher)

considering the cold and dry winters of eastern China to which it is native (Tables 6.8 and 6.9). Named the Ping'ou hybrids, crosses of *C. heterophylla* × *C. avellana* are considered the most important *Corylus* germplasm for cultivation in China. Five cultivars (Bokehong, Dawei, Jinling, Pingdinghuan, Yuzhui) and 14 breeding selections have been chosen for widespread planting in the region. They are divided into three groups according to their cold hardiness, but in general are reported to express strong cold tolerance (−20 °C to −38 °C) and a high yield potential (3–4 mt/ha) with individual nut weights in a range of 1.5–3.5 g, depending on the cultivar or selection. Currently, Ping'ou hybrids account for more than 90% of the hazelnut production in China, with the total cultivated area reported to be about 50 thousand ha in 2016 (Wang et al. 2018).

6.4.3 Clonal Selection

The assumption of clonal selection is that point and indel mutations have accumulated in clonally propagated old cultivars, and that superior plants can be identified among the different clones surveyed. The variability observed includes differences in yield, phenology and nut traits, such as percent kernel, ease of pellicle removal and presence of double seeds.

Clonal selection has been carried out with success in several leading hazelnut cultivars: Gironell and Negret in Spain (Rovira et al. 1997); Tonda Gentile delle Langhe (Valentini et al. 2001b), Tonda Romana (Tombesi et al. 2017) and Tonda di Giffoni in Italy (Petriccione et al. 2010); in Imperiale de Trebizonde, Tonda Gentile delle Langhe, Red Lambert and Cosford in Romania (Botu et al. 2005); in Uzunmusa, Palaz, Kalınkara, Çakıldak and Tombul in Turkey (Balık et al. 2018; Islam 2003; Islam and Ozgüven 2001).

6.4.4 Role of Biotechnology

Biotechnology offers a wide range of techniques to support breeding. In the case of hazelnut, advancements in this field have been slower than for other nut crops. One reason for this is due to the limited number of genetic studies and their dispersion in the world, hindering the possibility of building large consortia for sharing the cost of complex projects, such as the fine mapping of traits and genome sequencing. Another reason is the recalcitrant nature of the species when grown in vitro for obtaining somatic embryogenesis and as a precursor for subsequent genetic engineering.

In recent times, the reduction of associated costs and widespread access to the technologies has favored genome sequencing and development of SNP (single nucleotide polymorphism) markers for fine mapping. The role of biotechnology is thus becoming more relevant in molecular breeding of hazelnut with interesting perspectives for the development of MAS (marker-assisted selections) for a range of traits and the identification and manipulation of genes of resistance or susceptibility to pathogens. Building this capacity is a fundamental step needed to expedite research on adaptation to stressful environmental factors and for transitioning into the use of genetic engineering.

6.5 Molecular Breeding

In perennial tree species a major constraint for breeding is the long juvenility period that delays the observations of reproductive traits. Other difficult traits to detect are those for disease resistance/susceptibility that require standardized methods and

replicates. In these cases, molecular breeding can provide tools to accelerate and improve accuracy.

6.5.1 Molecular Marker-Assisted Breeding

The first efforts in molecular breeding of hazelnuts were carried out at Oregon State University to develop maps and molecular markers associated with resistance to EFB, a trait controlled by a dominant allele at a single locus found in the obsolete pollinizer cv. Gasaway. To identify the gene responsible for resistance, a map-based cloning effort was initiated in a population of 138 seedlings from the cross of OSU 252.146 × OSU 414.062, which segregates for resistance (Table 6.10). The first map published by Mehlenbacher et al. (2006) placed the S (incompatibility) locus and Gasaway R-gene on LG (linkage group) 5 and LG 6, respectively. This map was further developed in subsequent years until obtaining highly saturated maps for the two parents containing: 270 SSR/RAPD markers and 1741 SNPs (maternal map) and 199 SSR/RAPD markers and 1468 SNPs (paternal map) (Rowley et al. 2018). Chromosome walking was also initiated using primers designed from 8 RAPD markers linked to EFB resistance, which utilized screening a bacterial artificial chromosome (BAC) library and a polymerase chain reaction-based pooling and subpooling strategy. From this, a high-resolution genetic map and a physical map of the resistance region were constructed, including 51 markers. The region was sequenced and the coding sequences annotated. In 7 contigs <1 cM from the resistance locus, 233 genes were predicted and blasted against sequences in GenBank (BLASTP). Two of the putative genes were identified as members of the p-loop NTPase and F-box superfamilies, which were previously shown to have disease resistance properties. The work produced 44 new DNA markers linked to the EFB resistance gene and narrowed the resistance region to a single BAC in which five genes had a 100% match in the transcriptome (Sathuvalli et al. 2017). Further mapping, complementation and expression tests of the genes in the BAC sequences are needed to confirm which confer resistance to EFB.

Using common SSR markers, the maps for Tonda Gentile delle Langhe × Hall's Giant (Beltramo et al. 2016) and for a progeny from southern Russia segregating for EFB resistance (Honig and Molnar pers. comm), were aligned with the reference map for OSU 252.146 × OSU 414.062.

Beltramo et al. (2016) and Torello Marinoni et al. (2018) developed their map on the same progeny for the detection of quantitative trait loci for vegetative traits (Table 6.10). Torello Marinoni et al. (2018) constructed saturated maps of the two parent cultivars, including in each map about 1200 SNPs and a set of SSR markers for LG identification (Fig. 6.11). From this study, a strong QTL region was detected for time of vegetative budburst. Further work has also been done for QTL analysis of other characters, including male and female flowering time and nut and seed traits (unpublished data).

Table 6.10 Molecular linkage maps and association maps developed in hazelnut

Reference	Pedigree or type of material used	Markers	Agronomic trait targeted	Main Results
Mehlenbacher et al. (2006)	OSU 252.146 × OSU 414.062 (138 individuals)	RAPD and SSR (maternal map: 249 RAPD and 20 SSR markers; paternal map: 271 RAPD and 28 SSR markers)	Pollen-stigma incompatibility. Resistance to eastern filbert blight (EFB)	The S-locus was placed on chromosome 5S, where 6 markers linked within a distance of 10 cM were identified. A locus for resistance to EFB was placed on chromosome 6R, and 2 markers strictly linked were identified
Gürcan et al. (2010a)	OSU 252.146 × OSU 414.062 (138 individuals)	SSR		81 new SSR markers from enriched libraries were added to the map published by Mehlenbacher et al. (2006)
Beltramo et al. (2016)	Tonda Gentile delle Langhe × Hall's Giant (163 individuals)	SSR (152)	Vigor. Sucker habit. Time of leaf budburst	15 QTLs were identified for the examined traits. Of them, 10 were *major* QTLs, including a stably expressed region on LG_02 of maternal map for time of leaf budburst
Colburn et al. (2017)	OSU 252.146 × OSU 414.062 (138 individuals)	SSR		53 new SSR markers from transcriptome sequences were added to the map published by Mehlenbacher et al. (2006)
Bhattarai and Mehlenbacher (2017)	OSU 252.146 × OSU 414.062 (138 individuals)	SSR		101 new tri-nucleotide SSR markers from the *C. avellana* cv. Jefferson genome sequence were added to the map published by Mehlenbacher et al. (2006)

(continued)

Table 6.10 (continued)

Reference	Pedigree or type of material used	Markers	Agronomic trait targeted	Main Results
Öztürk et al. (2017)	40 wild accessions and 24 cvs. from the Slovenian national hazelnut collection	AFLP (11) SSR (49)	Nut and kernel traits (length, thickness, nut caliber, kernel weight, shape uniformity)	Association mapping of wild and cultivated hazelnut accession grown in Slovenia; 49 SSR markers were significantly associated with nut and kernel traits
Torello Marinoni et al. (2018)	Tonda Gentile delle Langhe × Hall's Giant (213 individuals)	SSR and SNP (maternal map: 20 SSR markers and 1216 SNPs; paternal map: 19 SSR markers and 1192 SNP)	Time of leaf budburst	29 QTLs for time of leaf bud burst were detected, confirming the stably expressed region on LG_02 of the maternal map detected by Beltramo et al. (2016). A reciprocal translocation was detected in cv. Tonda Gentile delle Langhe between two non-homologous chromosomes
Rowley et al. (2018)	OSU 252.146 × OSU 414.062 (138 individuals)	SSR, RAPD and SNP (maternal map: 270 SSR/RAPD markers and 1741 SNPs; paternal map: 199 SSR/ RAPD markers and 1468 SNPs)	Pollen-stigma incompatibility. Resistance to eastern filbert blight	Two high-density genetic maps representing a five-fold increase in marker density over previous map. Various major genes have been mapped, including resistance to EFB and genes related to self-incompatibility
Bhattarai and Mehlenbacher (2018)	OSU 252.146 × OSU 414.062 (138 individuals)	SSR		132 new nucleotide SSR markers with repeat motifs of 4, 5 or 6 bp from the *C. avellana* cv. Jefferson genome sequence were added to the map published by Mehlenbacher et al. (2006)

(continued)

Table 6.10 (continued)

Reference	Pedigree or type of material used	Markers	Agronomic trait targeted	Main Results
Frary et al. (2019a)	390 accessions (16 cvs., 232 landraces and 142 wild plants) from the Turkish national hazelnut collection	SSR (30)	Agro-morphological traits (44)	A total of 145 QTLs were detected with the largest proportions identified for involucre (26%) and inflorescence (14%) morphology
Frary et al. (2019b)	390 accessions (16 cvs., 232 landraces and 142 wild plants) from the Turkish national hazelnut collection	SSR (30)	Nut and kernel traits (25)	A total of 78 significant marker-trait associations for nut (24%) and kernel (26%) appearance, nut quality (19%), shell thickness (16%) and yield-related (15%) traits were detected in the population

The first association mapping effort was carried out by Öztürk et al. (2017) on 54 wild accessions and 48 cultivars from the Slovenian hazelnut national collection using AFLP and SSR markers. A total of 64 accessions were also characterized for 10 nut and 7 kernel traits. Phenotypic and molecular data were related to identify markers linked to the traits. Their results showed 49 SSR markers significantly associated with nut and kernel traits.

Further association mapping work was carried out to determine the genetic control of 44 agro-morphological traits (Frary et al. 2019a) and 13 nut and 12 kernel traits (Frary et al. 2019b) in 390 *Corylus avellana* accessions from 9 provinces in the Black Sea region of Turkey. Loci associated with these characteristics were mapped using a set of 30 simple sequence repeat loci.

6.5.2 Functional Genomics

Transcriptome studies and genome sequencing were carried out at OSU using the EFB-resistant cv. Jefferson (OSU 703.007) (Rowley et al. 2012). In total, 6.8 Gb of hazelnut transcriptome data obtained by Illumina RNA-seq technology from leaves, catkins, bark and seedlings of Jefferson, were assembled de novo into 28,255 contigs with an average length of 532 bp and an N50 of 961 bp. Sequence comparisons using BLASTX and gene ontology (GO) showed high similarity of the predicted proteins to sequences in related plants, with 80.8% having similarity to

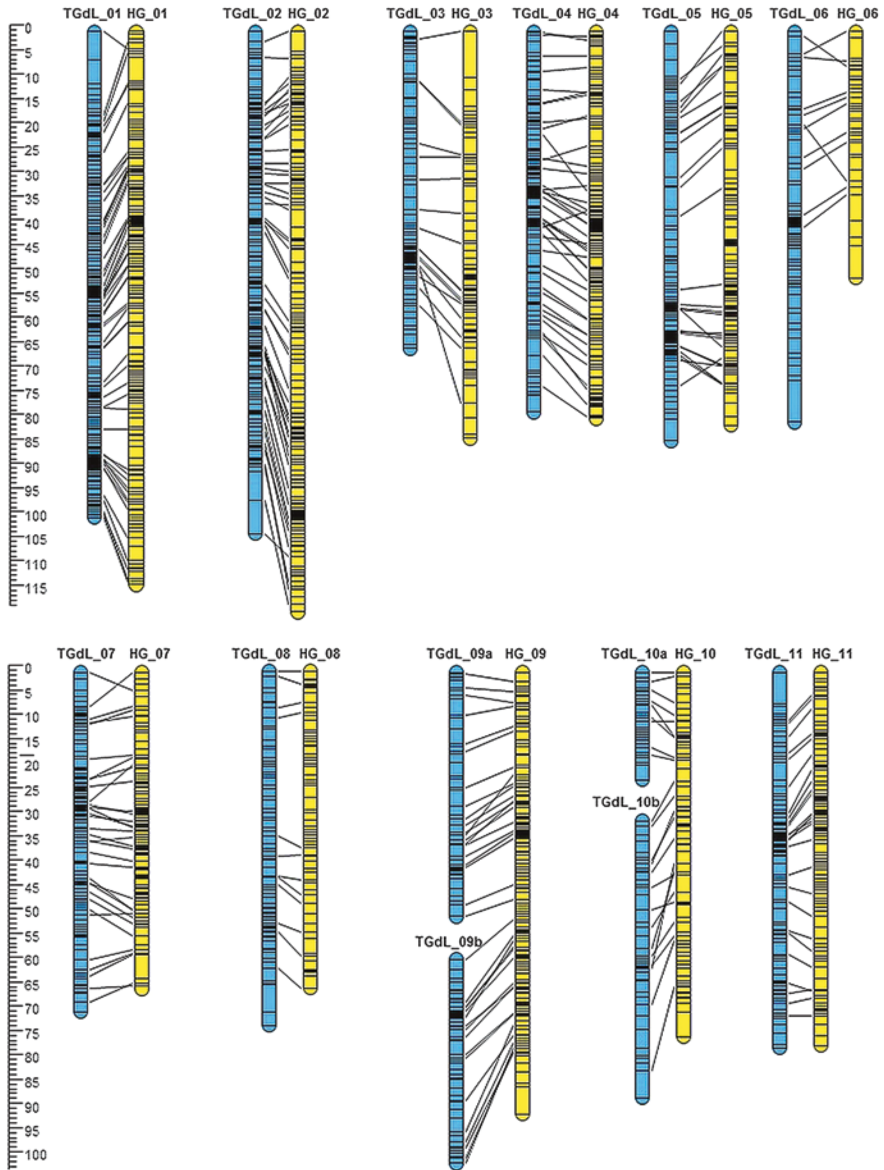

Fig. 6.11 Genetic maps of the *Corylus avellana* cvs. Tonda Gentile delle Langhe (TGdL) and Hall's Giant (HG), aligned on the basis of markers developed on common scaffolds. (Torello Marinoni et al. 2018)

sequences of grape (*Vitis vinifera* L.), poplar (*Populus trichocarpa* Torr. & A. Gray) and castor bean (*Ricinus communis* L.).

Further transcriptomic studies were carried out in *Corylus heterophylla* and were aimed at the identification of cold tolerance genes in floral buds (Chen et al. 2014) and to detect genes involved in ovule abortion and development during fruit growth in hazelnut (Cheng et al. 2015).

The *Corylus heterophylla* floral bud transcriptome was sequenced using Illumina sequencing technology, de novo assembled and annotated. A total of 40,941 unigenes were identified, and 30,549 were annotated in the NCBI Non-redundant (Nr) protein database. A total of 17,207 unigenes were mapped onto 128 pathways using the KEGG database. A set of candidate genes potentially involved in cold tolerance were identified. The expression patterns of the selected genes during four stages of cold acclimation suggested that these genes might be involved in different cold responsive stages in *C. heterophylla* floral buds. The work provides abundant information and material for future studies on the molecular mechanism of hazelnut floral bud's response to cold stress.

The presence of blank nuts across species of the genus *Corylus* is a very common phenomenon, often associated with embryo abortion that may cause serious yield losses in *C. heterophylla* and *C. avellana*. In a study by Cheng et al. (2015), de novo transcriptome sequencing and RNA-seq analysis were conducted in *C. heterophylla* using the Illumina HiSeq 2000 technology. Two digital gene expression libraries were constructed, one for a normally developing ovule and one for an empty (abortive) ovule. Transcriptome sequencing and assembly results revealed 55,353 unigenes. After annotation gene expression differences in developing and abortive ovules were identified using digital gene expression profiling. In abortive ovules, compared with developing ovules, 1637 and 715 unigenes were significantly upregulated and downregulated, respectively. The results provide fundamental genetic information that will improve the understanding of the molecular mechanisms of abortive ovule formation in hazelnut.

More recently, the *Corylus avellana* genome of cv. Jefferson was sequenced at 93× coverage using Illumina technology (Rowley 2016; Rowley et al. 2018). The draft genome assembly produced 36,641 genomic contigs and 36,090 predicted genes. Approximately 91% (345 Mb) of the flow-cytometry determined genome size was captured and 34,910 putative gene loci were functionally annotated. The majority of the annotated genes shared homology with genes of the related genera *Vitis*, *Prunus*, *Populus*, and *Ricinus*. The genomes of 7 other cvs. (Barcelona, Tombul, Tonda Gentile delle Langhe, Tonda di Giffoni, Daviana, Hall's Giant, Ratoli) were sequenced at lower coverage (~20).

The hazelnut genome and transcriptome data produced at OSU are available through a basic local alignment search tool (BLAST) portal and web resources (https://www.cavellanagenomeportal.com).

Based on recent improvements in sequencing abilities by Pacific Biosciences (PacBio) technology, the genome of cv. Jefferson was sequenced and assembled into longer contigs. Compared to the Illumina sequence, the PacBio sequencing produced fewer contigs (1865), and they are much longer (1.03 Mb). The work of

alignment of contigs with the linkage map is in progress and results will be shared with other research teams in the near future.

The Illumina re-sequencing of two clones of Tonda Gentile delle Langhe and two clones of its mutation Tonda di Biglini was carried out by the University of Torino at 80x coverage. The goal was to study clonal variation and detect polymorphisms able to identify and differentiate material obtained by clonal selection. The amount of variability was higher than expected with about 130,000 SNPs/INDELs differences between any two sets of clones (unpublished data).

Altogether, these resources will greatly aid future efforts to identify important genes and will be of importance to breeders for marker-assisted breeding efforts and for developing knowledge useful for genetic engineering strategies.

6.6 Micropropagation and Genetic Engineering

To date, biotechnological techniques other than micropropagation remain limited in respect to supporting hazelnut breeding. Micropropagation is now used for commercial purposes, especially when new cultivars are released and there is a need to rapidly increase the number of trees in a short time (Fig. 6.12). Akın et al. (2017) optimized a tissue culture medium for *Corylus avellana*. The propagation of *C.*

Fig. 6.12 Growth chamber of hazelnuts propagated by tissue culture. (Photo by R. Botta)

americana × *C. avellana* hybrids is more challenging but significant progress has been made in recent years at the University of Guelph, Ontario, Canada (Latawa et al. 2016).

Only a small amount of research has been published on protoplast culture and somatic embryogenesis of *Corylus*. Gametic embryogenesis was attempted and homozygous early embryos were obtained through isolated microspore culture in five hazelnut genotypes (Gniech Karasawa et al. 2016). The response to culture, however, was highly genotype-dependent and full plantlet development was not observed. No reports of successful embryogenesis from somatic tissues, other than young cotyledonary explants (Aygün et al. 2009; Berros et al. 2005), have been reported. Although a recent paper on organogenesis showed the possibility of obtaining morphogenesis from callus in cv. Tonda Romana (Silvestri et al. 2016), the lack of an efficient regeneration protocol significantly hinders the development of genetic engineering techniques. Further research is currently being carried out at the University of Torino with the aim of obtaining embryogenesis and subsequent use of genome editing for the modification of target genes.

Corylus avellana cell suspension cultures may also be a promising approach for in vitro production of metabolites such as paclitaxel, an antimitotic agent with activity against a range of cancers, and linoleic and oleic acids, used in clinical and economical applications such as cosmetic and pharmaceutical products. However, fast-growing callus is a prerequisite for the success. Recently, a modified MS medium (M10) was developed to increase callus fresh weight and paclitaxel concentration (55.8 and 37.2%, respectively). Cell suspension of *C. avellana* can be effectively used in paclitaxel production, reducing the high cost of drug-therapy (Salehi et al. 2017).

6.7 Mutation Breeding

Naturally occurring clonal variants are common in traditional cultivars and, in some cases, they bear distinctive traits in comparison with the standard variety (Valentini et al. 2014). They can be selected as plus variant clones or as new cultivars, depending on relevance of distinctive traits.

Conventionally-induced mutagenesis by Co_{60} irradiation at 8 kR was used to obtain suckerless plants of cv. Tonda Gentile delle Langhe (Me et al. 1988). However, the strategy was abandoned because the mutation was found to not be sufficiently stable following cycles of clonal propagation.

6.8 Conclusions and Prospects

Despite the tremendous amount of genetic diversity present in *Corylus* germplasm and the existence of about 500 cultivars described in the literature, currently there is a shortage of leading cultivars; only about 20 are widely grown and another 30 considered interesting for breeding.

The substantial economic value of hazelnuts and the strong demand for plants for orchard establishment in many regions worldwide indicate a need to increase investments in genetic improvement. Ten breeding programs have been active over the last 60 years in different countries. However, current breeding programs include only two in the USA that are relatively large, with smaller programs in Romania, Italy and China. Unfortunately, most previous breeding programs have been terminated, which is a cause for concern. In countries of traditional cultivation, such as Turkey, Italy and Spain, there is a strong link of historical cultivars with the territory and the tendency to use only the local cultivars in new plantings. For example, in Italy, geographical protections are in place for their four major cultivars. This scenario significantly reduces interest in planting new cultivars, and consequently the funding for breeding and along with it the introduction of innovations. World commercial production based on only a small varietal platform, and with few active breeding programs, is undoubtedly risky, especially in a scenario of climate change compounded by the possibility of introducing alien pests and pathogens (fortunately, EFB is currently present only in North America; however, consider the severe impacts of the Asian brown marmorated stink bug (*Halyomorpha halys*) in Italy, the Republic of Georgia, and elsewhere). There is a huge amount of genetic variability in *Corylus avellana* as well as in *C. americana*, *C. colurna* and *C. heterophylla*, that can be exploited for developing cultivars bearing enhanced adaptive and agronomic traits. On the other hand, the market is influenced by the quality standards of kernels requested by the confectionary industry that are difficult to match without several cycles of backcrosses requiring many decades of effort (and substantial funding).

Standard methods to breed hazelnuts have been developed and proven; however, it currently takes 17 years from the time of hand pollination until the resulting cultivar can be released. Using DNA markers and genomic technologies, we have increased our knowledge of genetic diversity and the control of traits in *Corylus*, but marker-assisted selection has not been widely developed and utilized beyond selection for EFB resistance related to *C. avellana* Gasaway. With the decreasing costs of DNA sequencing, it will become easier and faster to develop effective DNA markers. These tools will facilitate the study of genetics, allow for marker-aided breeding and support multiple levels of breeding decisions. Further, the development of genome editing technologies and successful protocols for somatic embryogenesis will favor the development of genetically-modified cultivars bearing improved traits while possibly maintaining the positive nut quality attributes of the original cultivars desired by the industry. This scenario will become much more feasible when our knowledge of the hazelnut genome becomes deeper.

Although a set of old and new cultivars are currently available, and breeding methods have been standardized, a larger and much more accessible genetic platform of *Corylus* germplasm should be created to support improvements in all geographic regions interested in cultivation and to face the emerging and future challenges of climate changes. National and international policies should consider the strategic importance of a crop like hazelnuts, which is under very high demand from the confectionary industry and still poorly utilized as a nutraceutical commodity (a place where additional value may also be exploited). When considering the historically low-input, sustainable nature of hazelnut production in combination with high demand from existing and developing industries, as well as the many stakeholders and downstream end users and consumers of hazelnut products, enhanced funding for research and support of *Corylus* breeding may yield considerable and long-lasting societal and economic benefits worldwide.

Appendices

Appendix 1: Major Hazelnut Germplasm Collections

Site	Institution	No. of accessions	Species
Corvallis, OR, USA	United States Dept. of Agriculture – Agricultural Research Service	607	*C. avellana* cultivars (187), *C. avellana* selections (164), *C. americana* (42), *C. californica* (40), *C. chinensis* (15), *C. colurna* (17), *C. cornuta* (14), *C. fargesii* (6), *C. ferox* (1), *C. heterophylla* (25), *C. jacquemontii* (4), *C. sieboldiana* (34), *C. yunnanensis* (2), interspecific hybrids (62)
Corvallis, OR, USA	Oregon State University	418	*C. avellana* cultivars (80), *C. avellana* selections (166 from nuts collected in 7 countries), *C. americana* (45), *C. californica* (1), *C. chinensis* (29), *C. colurna* (38), *C. cornuta* (28), *C. fargesii* (1), *C. ferox* (1), *C. heterophylla* (22), *C. jacquemontii* (7), *C. sieboldiana* (1)
Giresun, Turkey	Hazelnut Research Institute	390	*C. avellana*
Beijing	China Research Institute of Forestry, Chinese Academy of Forestry	378	*C. heterophylla* × *C. avellana* (43), *C. heterophylla* (220), *C. avellana* (35), *C. mandshurica* (18), *C. kweichowensis* (62)
Constantí, Tarragona, Spain	IRTA	253 (139 Spanish, 114 foreign)	*C. avellana*, *C. colurna*, *C. heterophylla*, *C. ferox* var. *tibetica*

(continued)

Site	Institution	No. of accessions	Species
Chieri (TO), Italy	University of Torino	86	*C. avellana*
Le Cese, Caprarola (VT), Italy	ARSIAL, Tuscia University	58	*C. avellana*

Appendix 2: Main Hazelnut Cultivars and Their Important Traits and Cultivation Locations

Cultivar	Country of Origin	Cultivation area(s)	Description of important traits
Allahverdi	Turkey	Not widespread yet	For kernel market. Released recently as a selection, small nut (1.8 g) with round shape, high percent kernel (49%) and pellicle removal (90%), early maturity (early August), late leafing, productive, yield variation is low between years
Barcelona (Fertile de Coutard, Castanyera)	Spain	USA (Oregon, Willamette Valley), Chile	For in-shell market. Large nut (3.8 g) with round shape (RI = 0.91), 43% kernel, half of pellicle removed by blanching. Vigorous tree, moderately productive, late maturity (early October in Oregon). Resistant to bud mites. Some tolerance to EFB; infected trees remain productive for several years. Incompatibility alleles S1 S2. The cv. Barcelona grown in Chile is similar but probably not genetically identical to the original
Çakıldak	Turkey	Ordu	For kernel market. Small nut (2.0 g) with round shape (RI = 1.1), high percent kernel (50–54%) and pellicle removal (85–87%), planted mostly at higher elevations, maturity mid-late August, late leafing, prone to alternate bearing, low flavor
Camponica	Italy, South	Italy, South	For in-shell market. Large nut (3.4–3.7 g) with round shape (RI = 0.92), 46–48% kernel, 70–80% of pellicle removed by blanching. Early mid-season maturity (August in Italy). Quantitative resistance to EFB. Incompatibility alleles S1 S2

(continued)

Cultivar	Country of Origin	Cultivation area(s)	Description of important traits
Clark	USA, Oregon	USA, Oregon	For kernel market. Small to medium nut (2.4 g) with round shape, 51% kernel, good pellicle removal by blanching. Small tree (TCA 60% of cv. Barcelona), very high yield efficiency. Moderate susceptibility to big bud mite, less susceptible to eastern filbert blight than Barcelona. Female receptivity late. Incompatibility alleles S3 S8
Corabel® Fercoril	France, Southwest	France, Southwest	For in-shell market. Very large nut (3.7–4.5 g) with round shape, 44–47% kernel, easy pellicle removal, low fiber. Very vigorous tree, productive, late nut maturity (late-September to early October in France). Very late female receptivity. Incompatibility alleles S1 S3
Dorris	USA, Oregon	USA, Oregon	For kernel and in-shell markets. Medium to large nut (3.3 g) with round shape, 43% kernel, good pellicle removal by blanching, excellent aroma. Small tree (TCA 59% of cv. Barcelona) with high yield efficiency. High resistance to big bud mite, high resistance to EFB from Gasaway. Incompatibility alleles S1 S12
Ennis	USA, Oregon	USA (Oregon, Willamette Valley); France, Southwest	For in-shell market. Very large nut (3.8–4.7 g), slightly long shape. Pellicle not removed by blanching. Moderately vigorous tree, productive, late nut maturity (early October in Oregon). Susceptible to big bud mite, highly susceptible to EFB. Very late female receptivity. Incompatibility alleles S1 S11
Foşa	Turkey	Trabzon, Akcakoca	For kernel and in-shell market. Small nut (1.7 g) with round shape (1.1), high percent kernel (50%) and pellicle removal (85%), early maturity (mid-August), good flavor, kernel cavity size of 2.2 mm
Giresun Melezi	Turkey	Not widespread yet	For kernel market. Recently released cultivar, small to medium nut (2.4 g) with round shape, high percent kernel (52%) and pellicle removal (90%), early maturity (mid-August), late leafing
Hall's Giant (Merveille de Bollwiller)	Germany/ France (Alsace)	France, Southwest	Used as pollinizer. Large nut (3.5–4.2 g) with conical shape, 36–41% kernel, easy pellicle removal, low fiber, suitable for in-shell market. Vigorous tree, not productive. Late nut maturity (late September–early October in France). Quantitative resistance to EFB. Incompatibility alleles S5 S15

(continued)

Cultivar	Country of Origin	Cultivation area(s)	Description of important traits
Jefferson	USA, Oregon	USA, Oregon	For in-shell market. Large nut (3.7 g) with round shape, 45% kernel, 70% of pellicle removed by blanching. Moderately vigorous tree (TCA 70% of cv. Barcelona) with upright growth. Nut maturity 3 days after Barcelona. Highly resistant to big bud mite, high resistance to EFB from Gasaway. Very late female receptivity. Incompatibility alleles S1 S3
Lewis	USA, Oregon	USA, Oregon	For kernel market. Medium nut (2.9 g), 48% kernel, good pellicle removal by blanching, good aroma, midseason nut maturity. Medium tree (TCA 78% of cv. Barcelona) with high yield efficiency. Moderate resistance to big bud mite, quantitative resistance to EFB. Occasional problem with kernel mold in cool, wet environments. Incompatibility alleles S3 S8
McDonald	USA, Oregon	USA, Oregon	For kernel market. Medium to small nut (2.5 g), 52% kernel, very good pellicle removal by blanching, excellent aroma. Nut maturity 2 weeks before Barcelona. Medium tree (TCA 70% of Barcelona) with high yield efficiency. Resistance to big bud mite, high resistance to EFB from Gasaway. Incompatibility alleles S2 S15
Mortarella	Italy, South	Italy, South	For kernel market. Medium to small nut (2.0–2.5 g) with long shape (RI = 0.80), 46–48% kernel, 70–80% of pellicle removed by blanching, excellent flavor (used for chopped kernels and industrial paste). Low vigor tree with high yield (2.5–2.8 t/ha). Early nut maturity (August in Italy). Often infected with apple mosaic virus. Incompatibility alleles S2 S17
Negret	Spain, Catalunya	Spain, Catalunya	For kernel market. Small nut (2.0 g) with ovoid shape (RI = 0.75), 48–50% kernel, very easy pellicle removal (90%), good flavor. Moderate tree vigor, high yield, mid-season to late nut maturity. Susceptible to EFB. Often infected with apple mosaic virus. Incompatibility alleles S10 S22

(continued)

Cultivar	Country of Origin	Cultivation area(s)	Description of important traits
Nocchione	Italy	Italy, Center and South	For pollinizer and kernel market. Medium to large nut (3.0–3.2 g) with round shape (RI = 0.92), 38–40% kernel, 80% of pellicle removed by blanching. Early to mid-season nut maturity. Moderate tree vigor and spreading growth habit, high yield. It is grown under many names as the main cultivar in Sicily and as the main pollinizer of cv. Tonda Romana in Latium. Incompatibility alleles S1 S2
Okay28	Turkey	Not widespread yet	For kernel market. Recently released cultivar, medium nut (2.85 g) with round shape, high percent kernel (54%) and pellicle removal (92%), early maturity (mid-August), late leafing
Palaz	Turkey	Ordu	For kernel market. Small nut (2.1 g) with round shape (RI = 0.9), high percent kernel (51%) and pellicle removal (92–94%), early maturity (early August), prone to alternate bearing, good flavor, large kernel cavity (3.25 mm)
Pauetet	Spain, Catalunia	Spain (Catalunya), France (Southwest)	For kernel market. Small nut (2.0 g) with ovoid shape (RI = 0.75), 48–50% kernel, 60–70% of pellicle removed by blanching, good flavor. Tree more vigorous and tolerant of high soil pH than cv. Negret. High yield, mid-season to late nut maturity (September). Incompatibility alleles S18 S22
Sacajawea	USA, Oregon	USA, Oregon	For kernel market. Medium nut (2.8 g), 52% kernel, very good pellicle removal by blanching, excellent aroma. Nut maturity 10 days before cv. Barcelona. Medium to vigorous tree (TCA 86% of Barcelona) with moderately high yield efficiency. High resistance to big bud mite, high quantitative resistance to EFB. Incompatibility alleles S1 S22
San Giovanni	Italy, South	Italy, South	For chopped kernel and industrial paste market. Medium nut (2.5–2.8 g) with long shape (RI = 0.80), 47–48% kernel, 70–80% of pellicle removed by blanching, good flavor. High yield (2.5–3.0 mt/ha) and early nut maturity (August in Italy). Incompatibility alleles S2 S8

(continued)

Cultivar	Country of Origin	Cultivation area(s)	Description of important traits
Segorbe (Comun Aleva)	Spain, Catalunya	France, Southwest	For kernel market and pollinizer. Medium to large nut (2.6–3.2 g) with round shape (RI = 0.86), 40–45% kernel, half of pellicle removed by blanching. Vigorous tree, late nut maturity (September–October). Tolerant of adverse conditions. Incompatibility alleles S9 S23
Tombul	Turkey	Giresun	Best Turkish cultivar for kernel market. Small nut (1.8 g) with round shape (RI = 1.1), high percent kernel (54%), high pellicle removal (94%), early maturity (Early August), excellent flavor, small kernel cavity (1.5 mm), medium productivity (1.5 mt/ha)
Tonda di Giffoni	Italy, South	Italy (Center and South), Spain, Chile and others	For kernel market. Medium nut (2.5–2.8 g) with round shape (RI = 0.90), 46–48% kernel, 80–90% of pellicle removed by blanching, excellent flavor. Mid-season to late nut maturity (September in Italy). Good quantitative resistance to EFB. High yield (2.5 mt/ha) and high adaptation to different environments. Problems with kernel mold in cool, wet environments. Incompatibility alleles S2 S23
Tonda Francescana®	Italy, Central	Not widespread yet	For kernel market. Small to medium nut (2.4 g) with round shape (RI = 0.94), 45–48% kernel, good pellicle removal by blanching, good flavor. Very early nut maturity (August in Italy), low susceptible to big bud mite. High productivity (3.0 mt/ha). Incompatibility alleles S2 S20
Tonda Gentile delle Langhe	Italy, Northwest	Italy, Northwest	For kernel market. Small to medium nut (2.2–2.4 g) with triangular-round shape (RI = 0.90), 46–48% kernel, 80–90% of pellicle removed by blanching, excellent flavor. Very early nut maturity (August in Italy). Susceptible to big bud mite, highly susceptible to EFB. Moderate productivity (1.8–2.0 mt/ha) in northwestern Italy but low in most other locations. Incompatibility alleles S2 S7
Tonda Pacifica	USA, Oregon	Chile, to be commercialized	For kernel market. Small nut (2.2 g), 47% kernel, excellent pellicle removal by blanching, excellent aroma. Nut maturity 8 days before Barcelona. Moderately vigorous tree (TCA 77% of Barcelona), moderately high yield efficiency. Moderate resistance to big bud mite, highly susceptible to EFB. Incompatibility alleles S1 S2

(continued)

Cultivar	Country of Origin	Cultivation area(s)	Description of important traits
Tonda Romana	Italy, Central	Italy, Central	For kernel market. Medium nut (2.5–2.7 g) with round shape (RI = 0.95), 45–47% kernel, 50% of pellicle removed by blanching, very good aroma. Nut maturity mid-season to late (September in Italy). Resistant to big bud mite, highly susceptible to EFB. High yield (2.0–2.5 mt/ha). Incompatibility alleles S10 S20
Wepster	USA, Oregon	USA, Oregon	For kernel market. Small nut (2.3 g), 46% kernel, very good pellicle removal by blanching, very good aroma. Nut maturity 10 days before cv. Barcelona. Vigorous tree (TCA 90% of Barcelona), high yield efficiency. High resistance to big bud mite, high resistance to EFB from Gasaway. Incompatibility alleles S1 S2
Yamhill	USA, Oregon	USA (Oregon), Chile	For kernel market. Small nut (2.3 g), 49% kernel, 50% of pellicle removed by blanching, little fiber on pellicle. Nut maturity 10 days before cv. Barcelona. Low vigor tree (TCA 60% of Barcelona) with spreading growth habit, very high yield efficiency. High resistance to big bud mite, high resistance to EFB from Gasaway. Incompatibility alleles S8 S26

TCA trunk cross-sectional area (30 cm above soil line), *RI* roundness index

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Chapter 7
Macadamia (*Macadamia* spp.) Breeding

Bruce L. Topp, Catherine J. Nock, Craig M. Hardner, Mobashwer Alam, and Katie M. O'Connor

Abstract Macadamia breeding is in its infancy with most cultivars only two generations removed from their wild progenitors. Two active breeding programs have released cultivars in the past decade and several others are in progress. Until recently, the majority of cultivars have been selected from commercial seedling orchards. Breeders are hindered by the large size of the trees which can grow to 15 m, long juvenile phases (fruiting after 5–7 years), and a poor correlation (r = 0.1) between annual nut-in-shell yield at 4 and 10 years. Current programs involve controlled cross-pollination to produce biparental families, progeny trials planted in randomized replicated designs, prediction of clonal and breeding values and use of a multi-trait weighted selection index. Strategies that allow rapid phenotyping are being examined. Selection for small trees and reduced juvenility will accelerate breeding by improving trait prediction accuracy through replication and reducing selection cycle periods, respectively. We discuss the potential to improve breeding efficiency and reduce cultivar release time by incorporation of genomic selection methods. There may be genetic variation in wild germplasm for dwarfing, resistance to major pests and diseases and adaptation to a wider range of climates. Genomic and phenotypic characterization of wild germplasm to identify material of highest conservation value and to identify useful traits has commenced on an ex situ collection of over 300 accessions of the 4 species.

Keywords Breeding · Crop improvement · Horticulture · Nut tree · Perennial

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7.1 Introduction

The genus *Macadamia* F. Muell., includes subtropical rainforest trees belonging to the Proteaceae family. The four species are *M. integrifolia* Maiden & Betche, *M. tetraphylla* L.A.S. Johnson, *M. ternifolia* F. Muell. and *M. jansenii* C.L. Gross & P.H. Weston. All are native to the east coast of Australia in northern New South Wales and southern Queensland at about 26–29° S lat. (Fig. 7.1). *Macadamia tetraphylla* is the southernmost species and most populations are located in New South Wales. It is a large tree commonly with four leaves per whorl and produces a distinctively wrinkled or rough-shelled nut with an edible kernel (Fig. 7.2). *Macadamia integrifolia* is a large tree with three leaves per whorl and produces a smooth-shelled nut with edible kernel. It has the largest latitudinal range extending north from the border of Queensland and New South Wales where its distribution overlaps with *M. tetraphylla*. The range of *M. ternifolia* extends from the Samford Valley north of Brisbane and overlaps with *M. integrifolia*. *Macademia jansenii* is the most northern species and is known to exist in only one location north of Bundaberg (Fig. 7.1). *Macadamia ternifolia* and *M. jansenii* are small trees which produce inedible nuts containing cyanogenic glycosides (Dahler et al. 1995).

In Australia, macadamia flowering occurs from August to early October. Each long, pendant raceme has 100–300 florets. Macadamia is predominantly outcrossing and this is promoted by protandry with the pollen released prior to stigma

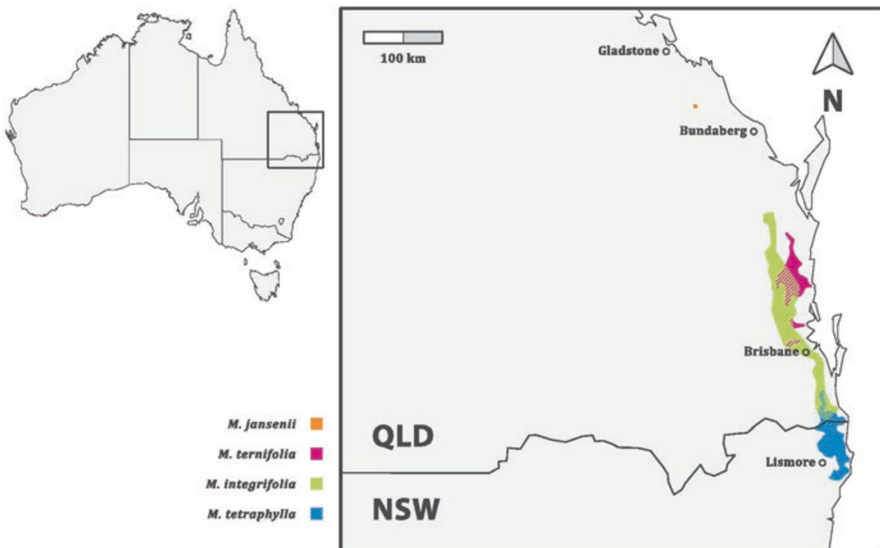

Fig. 7.1 Distribution limits for the four species of *Macadamia* across Queensland (QLD) and New South Wales (NSW) in Australia. Species location data collated from Peace (2005), Costello et al. (2009), Powell et al. (2010), Powell et al. (2014) and Shapcott and Powell (2011). (Image courtesy of Todd Fox)

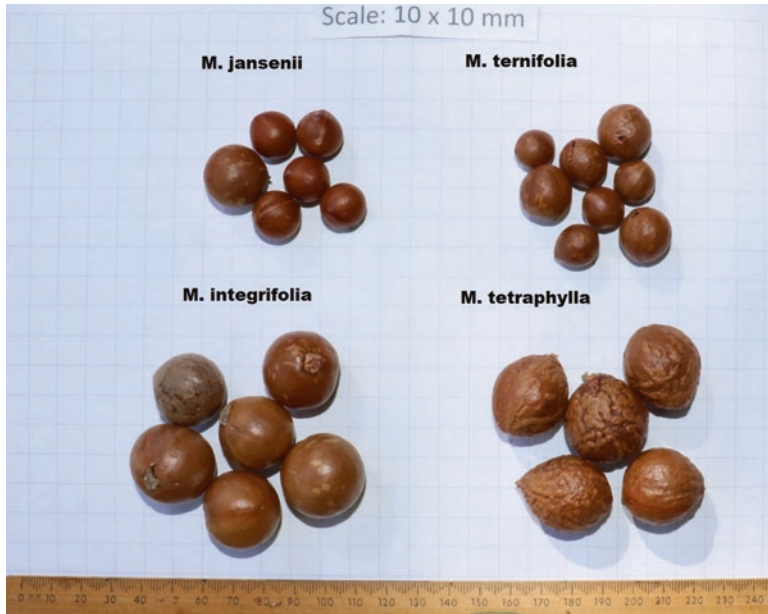

Fig. 7.2 Nuts of the four species of *Macadamia* native to south east Queensland and northeast NSW, Australia. (Image courtesy of Thuy Mai and edited by Nik Nieuwenhuis)

receptivity (Trueman 2013). The fruits mature in March to April (28–32 weeks after flowering) but may not drop from the tree until as late as August. Botanically, the fruit is classified as a follicle rather than a nut (Strohschen 1986). It is a dry, dehiscent fruit developed from one carpel. The green husk is the outer pericarp, which splits along a single longitudinal suture as it dries after maturity. The shell is the hard seed coat or testa, which encloses the kernel comprised of two large cotyledons and a small embryonic axis.

Macadamia integrifolia and *M. tetraphylla* and their hybrids are cultivated around the world for their high quality kernel which is eaten raw, roasted or in baked goods and confectionary. The oil is used for cooking, salads and cosmetics. There are also industrial uses of the kernel for biodiesel production (Azad et al. 2017) and the shell for composite timber products, biochar and nanosorbents (Girdis et al. 2017; Wongcharee et al. 2017). Macadamia is the highest oil-yielding commercial nut with about 75% oil by fresh weight (Strohschen 1986). The oil is mostly monounsaturated fats (about 80%) primarily oleic but with significant amounts of palmitoleic acid (Saleeb et al. 1973). It is a rich source of nutrients with several health benefits including reducing the risk of coronary disease and obesity, as well as having antibacterial properties (Cock 2008; Garg et al. 2007; Lima et al. 2014; Ros 2010).

Gross (1995) reports that Aborigines in the Mt. Bauple region of Australia called macadamia fruit *jindilli* and in northern New South Wales the local name was *kindal kindal*. Around the turn of the century, the first Australian orchards were established near Lismore, NSW and later in Queensland (Hardner et al. 2009). Cultivation

began in the late 1800s in Hawaii following importation of seed from Australia (Hardner 2016; Hardner et al. 2009; Peace et al. 2008). The Hawaii Agricultural Experiment Station (HAES) pioneered breeding and grafting, with seedlings being planted around 1920, followed by potential cultivar evaluation and selection in the mid-1930s (Hamilton and Fukunaga 1959; Hardner 2016). The majority of recent commercial cultivars are derived from *M. integrifolia* by different breeding strategies (Hardner 2016). Chloroplast resequencing indicates that the Hawaiian cultivars in particular were derived from a very narrow genetic basis, while extensive variability was found in this species. This suggests that future breeding may benefit from the largely unexploited germplasm diversity (Nock et al. 2019).

This chapter describes current macadamia breeding strategies and opportunities for future improvement. The domestication is relatively recent, and due to a long selection cycle some breeding strategies are in their infancy. This chapter also provides an overview on the current knowledge and methods that may potentially be used in future.

7.2 Cultivation and Traditional Breeding

7.2.1 Current Cultivation

Macadamias are grown commercially in 18 countries with an estimated 209,721 ha in 2016 producing 186,460 mt of nut-in-shell at 3.5% moisture content. Australia and South Africa produce almost half the crop, with significant production in Kenya, USA (Hawaii) and China (Fig. 7.3). Production also occurs in Zimbabwe, Guatemala, Malawi, Brazil, Dominican Republic, Mexico, Columbia, Mozambique, Paraguay, Rwanda, New Zealand, Swaziland and Vietnam. China is predicted to

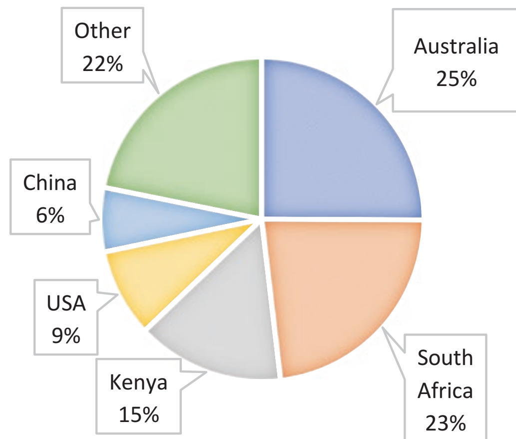

Fig. 7.3 World production (% of total) by country in 2016. (Source: International Nut Council estimates)

significantly increase its planting area from 65,000 ha in 2015 to 265,000 ha in 2020 and become a major producer (International Nut Council estimates). Australia has 17,000 ha, over 40,000 mt of nut-in-shell and 11,500 mt of kernel per year.

Although the trees are native to lowland subtropical rainforest, they can be cultivated across a range of environments and generally are adapted to climates where avocado and lemon are grown (Storey 1976). In Australia, they are grown commercially between Mackay in Queensland (21° S lat.) and Macksville in New South Wales (30° S lat.). In tropical regions, such as Vietnam, trees are grown at lower latitudes but at elevations of 300–700 m. Macadamia reportedly do not tolerate extreme temperatures. While mature trees can withstand mild frosts for short periods, young trees can be severely damaged or killed. Alternatively, high temperatures inhibit shoot growth and reduce kernel quality (Stephenson and Gallagher 1986; Trochoulias and Lahav 1983). Integrated management of nutrition, irrigation, weed control, soil erosion and pest/disease monitoring is now applied in orchards that are planted with cultivar scions grafted onto seedling rootstocks (O'Hare et al. 2004).

7.2.2 Current Challenges

Cultivars are estimated to be two to five generations from their wild progenitors. The cv. Keauhou (HAES 246) is still widely grown in Australia and was selected after only two generations of open-pollination from wild germplasm (Hardner et al. 2009). This recent domestication means that many traits of current cultivars remain similar to those in wild populations. Selective breeding can improve profitability; however, a number of significant obstacles may hinder genetic improvement. These include large plant size, a long juvenile period, time to peak production, and a poor correlation of traits on young and mature trees. These characteristics impede both commercial production and breeding efficiency.

Mature trees can be 15 m tall and 10 m wide. Planting at moderate densities of 300 trees ha^{-1} restricts canopy growth along the row, but the trees grow into the row and require pruning to maintain productivity. Cultivars with reduced tree size would enable dense plantings and allow higher early yields. Pruning or selective limb removal is common practice in mature orchards to allow machinery access, improve spray penetration and increase light distribution within the canopy. Access of light to the orchard floor is of particular importance for sward development and erosion prevention. Reduction of soil losses in conjunction with mulching improves subsurface root development and yield (Bright et al. 2016). Cropping efficiency may be increased by considering relationships between architectural traits in pruning and training (Toft et al. 2019), and by selecting for certain floral and architectural traits in breeding for new cultivars (Toft et al. 2018). From a breeding viewpoint, smaller trees would increase genetic gain by allowing increased replication and thus accuracy of trait predictions.

The time to first fruit production was similar in three breeding sites located in New South Wales and Queensland (Table 7.1). Seedling trees from multiple families

Table 7.1 Percentage of seedling trees producing fruit from ages 4–8 years at three Australian sites

	Percentage of population fruiting		
	Dunoon, NSW	Newrybar, NSW	Bundaberg, QLD
Tree age (years)	(n = 182)	(n = 126)	(n = 379)
4	38	13	33
5	62	57	72
6	79	78	89
7	94	88	97
8	99	93	96
Mean fruiting age	5.21	5.5	5.03

were planted at each site. There was no selection of parents with regard to precocity. The percentage of seedlings producing fruit was measured from 4–8 years from field planting. Precocity at 4 years of age varied with 13% fruiting at Newrybar and 38% at Dunoon. By the end of the trials at year 8, at least 93% of seedlings were fruiting, with the mean age to first fruiting of 5.03–5.50 years. Seedling trees were grown in a nursery for 2 years prior to field planting and so the time from nut-to-nut is about 7 years. Selection for increased precocity, which is moderately heritable in other fruit tree crops (Hansche 1983), will reduce selection cycle lengths and increase genetic gain per unit of time (O'Connor et al. 2018).

A medium-density orchard is typically planted at 8 × 4 m (312 trees ha^{-1}) and will commence fruiting after 4 years, but not reach peak production until 12–15 years (Fig. 7.4). Orchard establishment costs are high and a positive cash flow is not achieved for at least 8 years after planting (O'Hare et al. 2004). The long-term investment requires a low risk of new cultivars failing under commercial production. Hence, stringent evaluation is required and this increases the cost of breeding.

In Australia, nuts fall from March to August and growers mechanically harvest every 2–6 weeks. Delays in harvest can result in reduction of kernel quality. Cultivars vary in the duration and peak time of nut drop; a single cultivar may drop nuts over 4 months. The cv. Release drops its entire crop within 1 month. A series of cultivars with staggered but compact nut drop would reduce the cost of harvesting.

The length of selection cycle is determined by the length of the juvenile period but also by the time required to accurately evaluate the assessment traits. Moderate to highly heritable traits such as kernel recovery (KR; H = 0.6) can be evaluated as soon as nuts are produced and do not change with tree age (Hardner et al. 2001). Low heritability traits such as nut-in-shell (NIS) yield (H = 0.15) require more time to estimate because the yields of young trees do not reflect that of mature trees (Fig. 7.5). At year 4 cumulative NIS yield and annual NIS yield were poorly correlated with yield at year 10 (0.38 and 0.11, respectively). At year 8, the correlations were 0.94 and 0.97, respectively, but it could be argued that trials completed at age 7 would provide reliable estimates. It would be useful to know the correlations with trees at age 15 when they reach peak production.

Fig. 7.4 Macadamia orchard. (**a**) Growers evaluating new cultivars in a 7-year-old medium density orchard (300 trees ha^{-1}), (**b**) Controlled hand-pollination to produce hybrid seed for breeding, (**c**) Nut-in-husk 3 months after pollination. (Images courtesy of Bruce Topp)

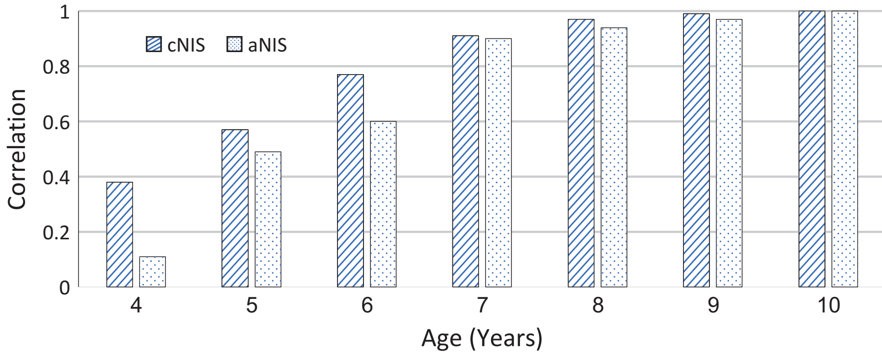

Fig. 7.5 The effect of tree age on the genetic correlation with mature (10 year) tree yield for cumulative nut-in-shell (cNIS) and annual nut-in-shell (aNIS). (Source: adapted from Hardner et al. 2002)

At the start of a new phase of Australian breeding, key stakeholders were consulted on future cultivar requirements. Growers, marketers and consultants identified the most important characteristics required in new macadamias as high kernel yield, resistance to husk spot (*Pseudocercospora macadamiae*) fungal disease, desirable consumer kernel traits, resistance to the fruit spotting bug (*Amblypelta nitida*) and smaller trees (O'Hare and Topp 2010).

7.2.3 Early Improvement Strategies

Macadamia had the reputation of being almost impossible to clonally propagate, but in 1927 Ralph Moltzau of the HAES was successful in grafting (Storey 1963). Girdling of the scion branch 6–8 weeks prior to grafting was an important addition to the process that allowed accumulation of starch above the girdle (Beaumont and Moltzau 1937). Until this time, all commercial orchards were planted using seedling trees. The ability to propagate clonally from large segregating populations provided an opportunity for selective breeding. HAES researchers recognized the possibilities and commenced the first major breeding program. More than 100,000 seedlings were evaluated and about 50 of the most promising were grafted and evaluated in regional field trials on the major Hawaiian Islands (Hamilton and Ito 1976). The cultivars released from this program account for the majority of world plantings and over 80% of those grown in Australia (Jones and Mayer 2009).

More recently, a similar example of mass phenotypic selection has been reported in Kenya (Gitonga et al. 2009). By 1974, over 800,000 ungrafted seedlings had been supplied to Kenyan farmers as an alternative cash crop to tea and coffee. Agronomic surveys identified 300 superior trees for yield and nut quality. From this population, 30 genotypes were clonally propagated for further evaluation and 7 cultivars were selected and released in the late 1980s (Gitonga et al. 2009).

In Australia, clonal propagation of superior open-pollinated seedlings commenced in the 1950s. The Queensland Department of Agriculture and Stock and the New South Wales Department of Agriculture systematically evaluated grower seedlings in much the same manner as had occurred in Hawaii. Sixteen selections were recommended as worthy of propagation based on nut characteristics considered suitable for processing. The selections were named and divided into categories of thin-shelled nuts for table purposes and thick-shelled nuts for processing (Ross et al. 1961).

Breeding commenced in Brazil at the Institute of Agronomy (IAC) in Campinas in the 1940s with the introduction of seed from HAES cultivars (Aguiar et al. 2014). Several cultivars were released and are grown commercially including IAC 4-12B, IAC 9–20, Campinas-B, Keaumi (IAC 4–20), Keaudo (IAC 2–23), Kakere (IAC 5–10) and IAC 1–21. These cultivars tend to be spreading and round-shaped trees compared to the more upright growing HAES cultivars (Aguiar et al. 2014).

7.2.4 Recent Improvement Strategies

A private breeding program at Hidden Valley Plantations (HVP) at Beerwah, Queensland, commenced in 1972 (Bell et al. 1987) and subsequently released the A series of cultivars including A4 and A16, which were the first to receive Plant Breeders Rights protection. This program started with progeny populations derived from open-pollinated seed from high-yielding seedlings and named parents. More recently, progeny have been produced by semi-controlled cross-pollinations using insect proof enclosures, and double-grafted trees and pollen cultivars grown in containers. HVP uses rooted cuttings for clonal propagation of all their elite selections (Bell et al. 1987).

An Australian industry-funded breeding program commenced in 1996, with an initial population of about 2000 seedlings planted in 1997–1998 at Bundaberg, Queensland and Alstonville, New South Wales (Hardner et al. 2002). The seedlings were created by controlled hand-pollination (Fig. 7.4b) involving 14 parents to produce 130 families (Topp et al. 2016). The program followed a two-stage selection process with an 8 year seedling progeny trial in stage 1 followed by 8 year regional testing in replicated variety trials in stage 2 (Fig. 7.6). Selection in the progeny experiment predicted genetic values for NIS yield, KR, tree height and canopy width. An economically weighted selection index was used to combine all selected traits (Hardner et al. 2006). Four cultivars were released in 2017.

In China, the Yunnan Institute of Tropical Crops (22°0′53.83″ N lat., 100°46′52.10″ E long., elev. 560 m) commenced breeding in 2012. They have about 10,000 plants under evaluation that were derived from the Australian cvs. Own Choice, Hinde (H2), Daddow and A4, and the Hawaiian cvs. Purvis (HAES 294), Pahala (HAES 788), Makai (HAES 800), Kau (HAES 344), Keaau (HAES 660), Kauka (HAES 741), Dennison (HAES 790), HAES 783, HAES 900 and HAES

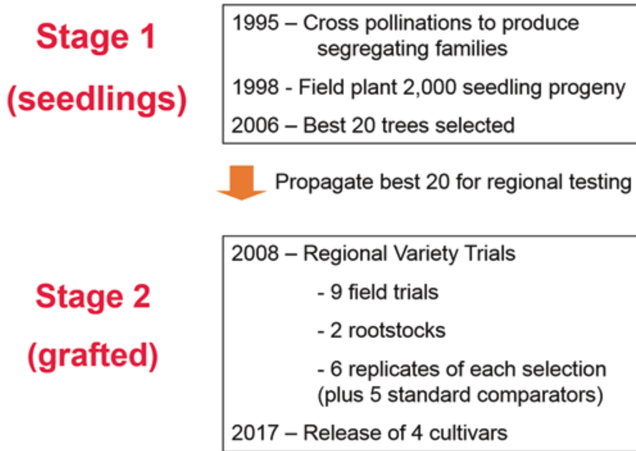

Fig. 7.6 Timeline for traditional Australian national breeding program with two stages of selection

932. The program aims to produce cultivars with high yield and quality nuts that are adapted to cool mountainous environments (X.Y. He, pers. comm. 2018).

In South Africa, the Agricultural Research Council – Tropical and Subtropical Crops campus (25°27’05.30” S lat., 30°58’09.60″ E long., elev. 670 m) has established seed orchards, consisting of 18 cultivars, in preparation for future breeding. The goals are to improve yield and quality and to reduce stink bug predation. Longer term goals include breeding for rootstocks that are dwarfing and resistant to *Phytophthora*. Evaluation in an abandoned seedling orchard in the 2010s resulted in selection of approximately 50 elites. These are to be culled to five elites that will be propagated for regional evaluations (M. Penter, pers. comm. 2018).

7.2.5 Limitations

Large populations are required to generate the unique outliers that combine appropriate levels of all target traits. The following is a simplified example where it is assumed all traits are inherited independently. In the Australian industry breeding plots, the following frequencies (in brackets) of seedlings have occurred for five target traits:

(a) Cumulative NIS yield >20 kg at year 8 (1.9%),
(b) Total KR >40% (13.1%),
(c) Tree height <4 m (11.1%),
(d) Reject kernel <5%, (40.5%)
(e) Ratio of whole kernels >50% (21.4%)

Thus, a segregating population of 42,169 trees (1/0.019 × 0.131 × 0.111 × 0.40 5 × 0.214) would on average produce one seedling with all five traits at the desired level. At commercial planting density, this would require 135 ha maintained for 8 years. This illustrates the high cost of breeding using a conventional strategy and a strong argument for exploring alternative strategies. Topp et al. (2012) compared four breeding strategies in terms of time to release new cultivars, breeding costs and expected genetic gain determined by stochastic modelling. A two-stage strategy that selected for highly heritable traits such as KR (Hardner et al. 2001) followed by clonally replicated trials to measure low heritability traits such as NIS yield (Hardner et al. 2002) was more efficient than a conventional strategy, as outlined in Fig. 7.6.

7.3 Germplasm Biodiversity and Conservation

7.3.1 Germplasm Diversity

Despite the relatively recent European settlement of Australia, the natural distribution of macadamia overlaps with an area that has experienced significant impact from agriculture, land clearing and development due to productive soils, proximity to the coast and high rainfall. The lowland rainforest of subtropical Australia is currently listed as a critically endangered ecological community, and it is estimated that over 70% of macadamia habitat has been cleared since European settlement began in 1824 (Costello et al. 2009). *Macadamia jansenii* is known from a single isolated population (Shapcott and Powell 2011), while the distribution of *M. integrifolia*, *M. ternifolia* and *M. tetraphylla* populations is fragmented and wild trees are rare (Powell et al. 2010, 2014). All four species are listed as threatened under Australian Commonwealth and State legislation and in the IUCN Red List (Costello et al. 2009; EPBC Act 1999).

A number of studies have investigated genetic diversity, gene flow and the impact of habitat fragmentation in wild populations. Flowering, fruit production and seedling emergence in small fragments exceeded those in continuous *Macadamia integrifolia* habitats, suggesting that the population viability is not affected by fragmentation (Neal et al. 2010). A network of remnant fragments was identified through spatial modelling, and it was proposed that long-term connectivity could be maintained in this species through in situ conservation of remnant populations (Powell et al. 2010). Recent studies using chloroplast genome sequencing support earlier evidence for strong latitudinal population structure in *M. integrifolia* (Nock et al. 2019; Ahmad Termizi et al. 2014; Peace 2005). Some domesticated germplasm could not be traced back to current wild populations, suggesting that some of the diversity in the wild has been lost (Nock et al. 2019).

Over 99% of the Big Scrub*,* Australia's largest area of continuous subtropical lowland rainforest, was cleared during the nineteenth century and much of the original habitat of *Macadamia tetraphylla* in New South Wales was lost (Parkes et al. 2012). Genetic diversity is correlated with population size in this species, particularly among juvenile cohorts, and there is evidence for inbreeding in some populations (O'Connor et al. 2015; Spain and Lowe 2011). Recently, pollen flow from orchards into wild *M. tetraphylla* populations was reported. Crop-wild hybrids were found up to 1600 m from orchards. Gravity, water or rodents may have also transported nuts from orchards into native vegetation, as pure *M. integrifolia* genotypes were found growing among wild *M. tetraphylla* trees (O'Connor et al. 2015).

7.3.2 Cultivar Characterization and Phylogeny

Genetic diversity in terms of heterozygosity and allelic richness is relatively high in *Macadamia integrifolia*, *M. tetraphylla* and cultivars (Nock et al. 2014b; O'Connor et al. 2015; Peace 2005; Peace et al. 2008; Spain and Lowe 2011). Genetic characterization has been recently conducted using high-throughput genotyping techniques in macadamia cultivars (Alam et al. 2018, 2019; O'Connor et al. 2019a). The source of improved germplasm used globally in commercial orchards is reviewed by Hardner et al. (2009). Global production comes primarily from cultivars developed in Hawaii (Hardner 2016). Most of the Hawaiian selections are derived from a wild population in the northern distribution of *M. integrifolia* (Ahmad Termizi et al. 2014). Very few *M. tetraphylla* cultivars were selected in Hawaii. This may have been because the kernel from this species was more variable than those from *M. integrifolia*, although subsequent studies suggest this may have been due to biased sampling (Hardner 2016). Multiple origins of other cultivars, including those developed in South Africa and Australia, have been identified (Aradhya et al. 1998; Peace 2005; Peace et al. 2005; Steiger et al. 2003). Germplasm from *M. tetraphylla* was important in the development of early selections in South Africa, and the industry is based on cultivars of *M. integrifolia*, *M. tetraphylla* and hybrids. Many Australian selections are also hybrids between *M. integrifolia* and *M. tetraphylla*, and a single Hawaiian cultivar HAES 791 includes *M. ternifolia* germplasm (Peace et al. 2005). Recent genetic analysis showed that progeny of pure *M. integrifolia* cultivars were distinct from two interspecific hybrid progeny groups (O'Connor et al. 2019a).

7.3.3 Genetic Resource Conservation Approaches

In Australia, in situ and ex situ conservation of macadamia is supported by the industry, government, universities and nongovernment organizations. In the 1990s, cuttings were propagated from 372 individuals sampled from 77 sites including remnant populations, old orchards and stands of unknown origin across the

geographic range of the remnant populations. This collection was established across three ex situ replicated plantings. This collection has been used for studies of genetic diversity (Ahmad Termizi et al. 2014; Peace 2005) and is being examined for potentially useful traits to use in breeding (Mai et al. unpublished data). A recovery plan for the *Southern Macadamia Species* has been adopted by the Australian Government, with the objective of protecting wild populations from decline and ensuring their long-term viability (Costello et al. 2009). The southern clade was recognized as the genus *Macadamia* including four species following a comprehensive morphological and molecular phylogenetic analysis of the tribe Macadamieae (Mast et al. 2008). Recognized threats to the genus include habitat fragmentation, land clearing, weed invasion, small population sizes, changed fire regimes and climate change (Costello et al. 2009; Powell et al. 2014).

7.4 Molecular Breeding

7.4.1 Molecular Marker-Assisted Selection

Employing molecular markers for genetic improvement through methods such as genome-wide association studies (GWAS), marker-assisted selection (MAS) and genomic selection is of interest in fruit-tree breeding. GWAS test genetic markers such as single nucleotide polymorphisms (SNPs) individually to determine if they are in linkage disequilibrium with genes controlling target traits (Balding 2006; Huang and Han 2014; Khan and Korban 2012). Markers significantly associated with the trait can then be used to select elite candidates using MAS based on predicted breeding values of individuals (Hayes and Goddard 2010; Iwata et al. 2016; Lynch and Walsh 1998; Muranty et al. 2014). GWAS and MAS are most effective and valuable for monogenic or oligogenic traits since the gain is proportionate to the variance captured by significant markers (Collard et al. 2005; Hayes and Goddard 2010; Huang and Han 2014; Luby and Shaw 2001).

There is limited research regarding GWAS in fruit trees. In apple, significant associations were found in 6 fruit quality traits using 1200 seedlings genotyped for 2500 SNPs (Kumar et al. 2013). Markers associated with fruit quality traits have also been found in pear (Iwata et al. 2013) and peach (Cao et al. 2012). GWAS and subsequent MAS for commercially important yield component traits would assist macadamia breeding, provided only a few genes control these traits, and that these are of moderate to large effects (O’Connor et al. 2018). Hardner et al. (2005) suggested that MAS may be most successful if applied across macadamia family groups compared to detection within a family, due to the outcrossing nature of the genus, power, and detection of QTLs across diverse and heterozygous genotypes. GWAS in breeding progeny from over 30 families has been conducted for the sticktight (Akinsanmi et al. 2012) trait that is associated with husk spot disease (O’Connor et al. 2017), and was more recently used to identify associations between SNP markers and nut weight, kernel weight and KR (O’Connor et al. 2019b).

Another method of employing genomics in breeding is with genomic selection. Genomic selection (GS) utilizes all available genetic markers (genome-wide) to estimate the effects of loci that affect a trait (Meuwissen et al. 2001). GS is therefore more effective than MAS and traditional phenotypic selection for quantitative traits such as yield, as it can explain more of the genetic variability (Grattapaglia and Resende 2011; Heffner et al. 2009; Meuwissen et al. 2001). GS is particularly attractive for horticulture tree breeding since selection cycles can be accelerated by selecting elite juveniles based on their genotype (Desta and Ortiz 2014; Heffner et al. 2010; Jannink et al. 2010; Meuwissen et al. 2001).

In simulations using GS for citrus quality traits, high prediction accuracies ($r > 0.7$) were achieved for 6 out of 17 traits (Minamikawa et al. 2017). In apple, Kumar et al. (2012) found model predictions to be high for fruit quality traits, at $r = 0.70–0.90$. In comparison, Muranty et al. (2015) obtained much lower accuracies for 10 traits in apple, with a maximum prediction accuracy of $r = 0.5$. Model accuracy increased with trait heritability in oil palm, which also has a long juvenile period and selection cycle (Kwong et al. 2017). Macadamia breeding could potentially benefit from GS due to the long juvenile period and selection cycle; however, no simulations have been published (O'Connor et al. 2018).

7.4.2 Functional Genomics

Since the release of the first plant genome sequences of *Arabidopsis* and rice in the early 2000s, identification and functional characterization of their genes continues to advance our understanding of the genetic basis of growth and development in plants (Buell and Voytas 2017; Mustafiz et al. 2016). Subsequent innovations in sequencing and bioinformatics have enabled genome sequence assembly from next generation sequence (NGS) reads and the identification and characterization of genes and gene families. Whole genome sequences are now available for many food crops. Determining the function of genes predicted in silico is the greatest challenge in crop genomics (Badenes et al. 2016; Pérez-Martín et al. 2017). Orthologous genes underlie some agriculturally-important traits, such as time of flowering and plant height in different species (Lenser and Theißen 2013; Van Nocker and Gardiner 2014). Gene function can be inferred in a newly-sequenced genome by identifying gene orthologs using comparative genomic approaches, although additional experimental evidence is required to validate these findings.

Until recently, limited sequence data for macadamia were available and very little was known about the functional content of the genome (Peace et al. 2008). Some early research identified two proteins in *Macadamia integrifolia* kernel with antimicrobial activity. The novel protein MiAMP1 showed broad-range antimicrobial activity and inhibited the growth of a range of plant pathogens, possibly through interaction with membrane surfaces. McManus et al. (1999) reported that the structure of MiAMP1 differed from other plant microbial proteins, and was similar to a yeast toxin. Further work isolated a new family of four antimicrobial peptides

related to the 7S globulin seed storage proteins found in other plants (Marcus et al. 1999). One of them, MiAMP2c inhibited pathogenic fungi in vitro, and two from kernel exudate isolations exhibited in vitro antimicrobial activity. More recently, using a combination of shotgun proteomics and verification with patient IgE recognition, 7S (MiAMP2) and 11S globulin were identified as putative allergenic proteins in macadamia kernel (Rost et al. 2016). Recent research investigated the biochemical mechanisms determining macadamia fatty acid composition. *Macadamia tetraphylla* genes encoding the acyltransferease enzyme DGAT1 and an acyl-ACP desaturase were sequenced and characterized. Expression analyses indicate that these genes are functional and involved in the fatty acid biosynthetic pathway with potential to alter fatty acid content and composition (Arroyo-Caro et al. 2016).

Following annotation of the first draft assembly of *Macadamia integrifolia* cultivar HAES 741, a total of 35,337 protein-coding genes were predicted. Most of these (31,908) were supported by gene expression evidence in the leaves, shoots or flowers used for transcriptome sequencing. Functional annotation and comparative genome analyses assigned putative protein-coding genes to orthologous protein domains, gene families, gene ontology terms and metabolic pathways. The results point to an expansion of genes involved in the recognition of microbial pathogens, plant defense response and synthesis of monoterpenes (Nock et al. 2016). Further research to determine the genetic basis of pathogen recognition and plant defense could assist in the development of resistant cultivars.

Cyanogenesis is one of the defense strategies employed by macadamia. The cyanogenic glycosides dhurrin and proteacin have been identified, and cyanide has been detected in *Macadamia ternifolia*, *M. tetraphylla* and *M. integrifolia* seedlings (Dahler et al. 1995; Swenson et al. 1989). Candidate genes with high sequence homology to those involved in the biosynthesis and catabolism of cyanogenic glycosides in the cyanogenic plants sorghum and clover were recently identified in *M. integrifolia* (Nock et al. 2016). Uncovering the genetic basis of cyanogenesis in macadamia and detection of natural variants may support the incorporation of *M. ternifolia,* a smaller tree with a bitter kernel, into breeding programs. Selection of mutant wild trees with sweet seed was pivotal in the domestication of cultivated almond, *Prunus dulcis* (Sánchez-Perez et al. 2008). Identification of the loci involved in cyanogenesis is enabling the development of markers for selection of seedlings with sweet genotypes for almond breeding (Sánchez-Pérez et al. 2010).

7.4.3 Bioinformatics

Macadamia genomic data were initially used for de novo assembly of the chloroplast genome, and to develop short sequence repeat (SSR) markers that have been used for cultivar identification, pollination and gene flow studies (Nock et al. 2014b; O'Connor et al. 2015) and recently for paternity analysis (Langdon et al. 2019). A chloroplast phylogenomic analysis including representatives of all major

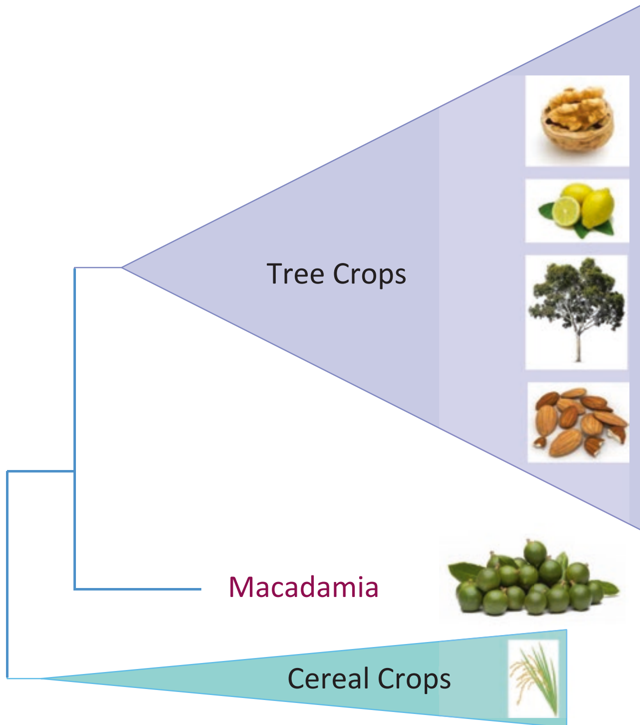

Fig. 7.7 Evolutionary relationship of macadamia to other crops including the core eudicot tree crops and monocot cereal crops based on chloroplast phylogenomic analysis of 83 genes and 87 taxa. (Source: Modified from Nock et al. 2014a)

angiosperm lineages confirmed the position of *Macadamia* (Proteaceae) in the basal eudicot order Proteales (Nock et al. 2014a). Macadamia is distantly related to other fruit and nut tree crops (Fig. 7.7).

For comparative genomic analyses, the genome of the sacred lotus (*Nelumbo nucifera*) is the closest available, despite divergence more than 125 million years ago (Ming et al. 2013; Moore et al. 2010). The macadamia genome project is an initiative of an Australian consortium and aims to develop a reference genome for macadamia. The first draft assembly of *Macadamia integrifolia* cv. HAES 741 was constructed using ~52 Gb of next generation sequence (NGS) data. Macadamia is diploid and has 14 chromosomes (Ramsay 1963; Storey and Saleeb 1970). Preliminary de novo assembly with Illumina sequence reads, followed by scaffolding with mate-pair reads produced 193,493 scaffolds [EMBL-ENA accession ERP015338] with an N50 of 4,745 bp. Transcriptome data from three cDNA libraries (flower bud, young leaf, shoot) generated 244,925 clustered transcripts. These were used as reference expressed sequence tags (EST) with *Nelumbo* and

Table 7.2 Summary of progress towards the development of a reference genome for macadamia

	Scaffolds			Assembly			Genes	
Version	Number	Longest (Mb)	Gaps (Mb)	Length	N50 (kb)	% of Genome	% Complete	% Gene content
v1	193,493	0.64	70	518	4.7	79	60.6	77.4
v2	4,089	2.19	11	744	413	93	84.3	90.2

Arabidopsis proteins in the evidence-based gene model prediction pipeline MAKER (Nock et al. 2016). Subsequently, additional short-read, PacBio long-read and RNA-seq data (>200 Gb in total) have been assembled and annotated. The improved v2 assembly is substantially more complete (4,416 scaffolds, 414 kb N50). It covers 93% of the revised kmer-estimated genome size of 800 Mb (Table 7.2). A SNP-based genetic linkage map is under construction, and will be used to order genes and markers and anchor assembled scaffolds to linkage groups (Langdon et al. unpublished data 2018). A reference genome sequence for macadamia will enhance molecular marker-assisted breeding using GWAS, MAS and GS that should accelerate the development of new cultivars.

7.5 Tissue Culture and Genetic Engineering

Tissue culture has been successfully used to propagate numerous crops. In vitro propagation has been widely used in tree and nut crops such as chestnut (Chevre and Salesses 1987; Cuenca et al. 2017; Marie Chevre et al. 1983; Piagnani and Eccher 1988; Roussos et al. 2016; Vidal et al. 2015), walnut (Hassankhah et al. 2014; Höltken et al. 2015; Licea-Moreno et al. 2015; Lone et al. 2017), hazelnut (Koyuncu et al. 2017), pistachio (Benmahioul 2017), oil palm (Parveez et al. 2012), poplar (Confalonieri et al. 2003) and apple (Huh et al. 2017). There are few studies of tissue culture in macadamia, and the success rate is very low. To our knowledge, no genetic modification has been applied to macadamia.

7.5.1 Tissue Culture

As employed in other tree species, tissue culture could potentially be used in macadamia to fast-track cultivar multiplication, generate disease-free plants, and to facilitate mutation and genetic modification. Tissue culture has been attempted in macadamia; however, few references on macadamia tissue culture protocols are available. New rooted plants can be developed in 5 months (Fig. 7.8).

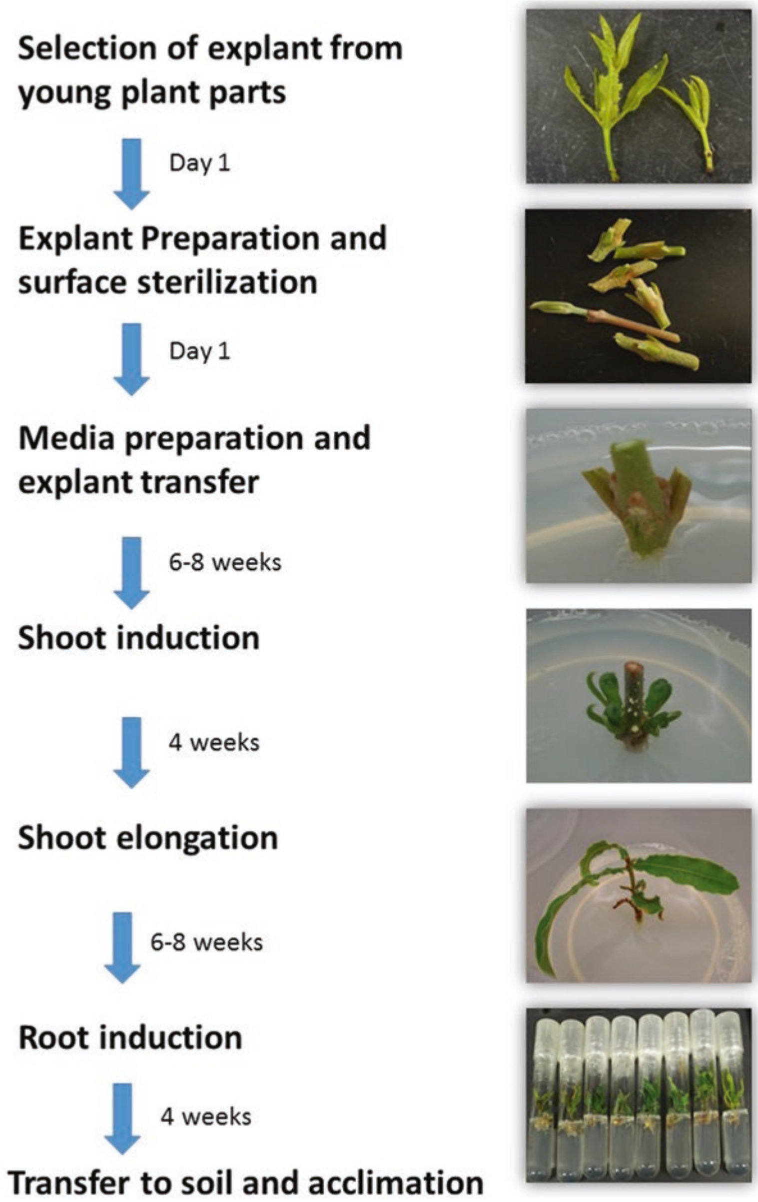

Fig. 7.8 Steps in the tissue culture of macadamia. Image: Provided by M. Orozco-Cárdenas, Plant Transformation Research Center, University of California Riverside, CA

7.5.1.1 Explant Selection

Selection of a suitable explant is the preliminary tissue culture step. For some crops, such as tobacco and carrot, any part of the plant can be used (Abdin et al. 2017), while successful regeneration for others depends on the particular part of the explant used. Success is also dependent on the age of the material, as young, fresh growth regenerates better than older leaves or stems (Hu and Wang 1983). Shoots tips, apical/axillary buds, nodal cuttings and seeds/fruits have been used as explants for successful in vitro regeneration of macadamia. Young nodal explants from shoots of different cultivars of *Macadamia integrifolia* and *M. tetraphylla* (Bhalla et al. 2003; Mulwa and Bhalla 2000) were used for shoot regeneration. Mulwa and Bhalla (2006) regenerated shoots from immature cotyledons. Mature kernels were used in a patented tissue culture medium (Atehortua et al. 2009).

7.5.1.2 Aseptic Culture

The most important step of tissue culture is to provide an aseptic environment. Explants are the initial source of contamination, hence the need to use a surface disinfectant such as alcohol and/detergents. Schroeder (1961) sterilized nuts (explants) using calcium hypochlorite and/or flaming after immersion. Bennell (1984) used 1% V/V solution of sodium hypochlorite containing 0.1% Tween 20 as a wetting agent. Cuthbertson (1991) introduced a different method of sterilization of apical buds/shoots/axillary buds. Explants were immersed in 70% ethyl alcohol containing 0.01% Tween 20 (1 min) followed by treatment with 10% calcium hypochlorite containing Tween 20. A standard method for macadamia nodal explant sterilization was developed by Mulwa and Bhalla (2000). Single node explants were washed with 1% Tween 20 followed by running tap water for 30–45 min. Three different treatments were compared, with the optimal decontamination method a treatment for 15 min with 0.5% SDS in 0.1% Triton X-100 followed by 20 min in 1% NaOCl in 0.1% Tween 20 solution. In later experiments, the surface of developing cotyledons was disinfected with 0.125% (v/v) NaOCl containing 0.01% (v/v) Tween 20 for 20 min (Mulwa and Bhalla 2006). Gitonga et al. (2008) used 0.35% NaOCl for surface sterilization of nodal explants. Cha-um et al. (2011) successfully sterilized single node cuttings by dipping explants in 70% alcohol for 15 s and 0.15% NaOCl for 20 min. Further investigations are required to develop protocols for sterilization.

7.5.1.3 Shoot Proliferation and Root Formation

Micropropagation has potential for clonal propagation of macadamia. Schroeder (1961) was the first to report in vitro callus formation in macadamia. However, successful in vitro shoot regeneration in *Macadamia integrifolia* cultivars was not achieved until 1984. Shoots were regenerated from nodal explants in Nitsch and Nitsch basal medium culture containing 10 μM benzyladenine (Bennell 1984).

Later, Cuthbertson (1991) produced shoots in low salt (30%) MS medium. A rapid (12 fold in 12 weeks) protocol for in vitro shoot multiplication using half-strength MS medium has been described (Mulwa and Bhalla 2000). Other factors such as sucrose and hormone content are also important for shoot regeneration (Gitonga et al. 2008). Atehortua et al. (2009) patented a new tissue culture medium for macadamia, which requires further evaluation for successful regeneration. Differences in shoot generation across different media may be related to different cultivars.

Poor in vitro regeneration in macadamia is likely due to a lack of standardized protocols. Some studies report poor rooting in cultured shoots, browning of the media and leaf necrosis (Gitonga et al. 2008; Mulwa and Bhalla 2000). Root organogenesis in macadamia was first recorded by Cha-um et al. (2011) who observed 100% root induction in shoots in CO_2-enriched phototrophic conditions in a vented vessel containing vermiculite as supporting media.

7.5.2 Genetic Engineering

Genetic modification (GM), often referred to as genetic engineering, can accelerate genetic gain (Gasser and Fraley 1989). It has been used to alter pest and disease resistance (Bliffeld et al. 1999; McDowell and Woffenden 2003; Tripathi et al. 2017), tolerance to herbicides and abiotic stresses (Marco et al. 2015; Shah et al. 1986; Umezawa et al. 2006), nutritional quality (Mandal and Mandal 2000; Matas et al. 2009), and the times of flowering and ripening (Jung and Müller 2009; Park et al. 2004). The use of genetic engineering does not remove the requirement for field evaluation of the new material for uniformity, stability and environmental adaptation. The use of GM has been restricted due to ethical, environmental, health and social concerns raised by its critics. There have been no attempts to genetically modify macadamia cultivars, although a trial of *Agrobacterium* transformation investigated the effect on proteoid root formation (Cuthbertson 1991). There may be potential for use of GM as a tool to reduce breeding cycles. Several genes that promote early and continuous flowering have been genetically manipulated into tree crops reducing the time to first flowering (Iwata et al. 2016). These may be combined with strategies that allow segregation for nontransgenic plants prior to cultivar release.

7.6 Mutation Breeding

Mutation occurs naturally or artificially (Lodish et al. 1995). Both physical and chemical agents are used to induce mutation in plants. Although induced mutation has been used for decades in plant breeding (Lapins 1983), there are only two reports in macadamia.

With the aim of developing dwarf genotypes, Bennell (1984) experimented on different cultivars, including Own Choice, HAES 660, HAES 344 and HAES 741.

To induce mutations in scions and nodal explants of the cultivars, γ-irradiation was used. The irradiated samples were cultured in vitro and grafted onto seedling rootstocks; however, details of the generation and selection of dwarf plants were not reported. In another experiment, Bell (1996) irradiated macadamia pollen with short wave (254 nm) ultra-violet (UV) light and a mutation protocol was described.

7.7 Hybridization

7.7.1 Conventional Hybridization

Several researchers (Hardner et al. 2000; Saleeb et al. 1973; Smith 1956; Storey 1959) have reported on interspecific hybrids with intermediate adult morphology, particularly among *Macadamia integrifolia* and *M. tetraphylla* in wild and cultivated germplasm. However, the detection of hybrids based on juvenile morphology is difficult (Hardner et al. 2009).

Molecular and artificial hybridization studies have confirmed the opportunity for interspecific hybridization within the genus. Natural hybrids have been confirmed where the southern populations of *Macadamia integrifolia* and the northern populations of *M. tetraphylla* overlap (Peace 2005; Fig. 7.1). In contrast, although the distribution of *M. integrifolia* and *M. ternifolia* are sympatric, few apparent hybrids of these species have been observed in the wild (Hardner et al. 2009). Artificial hybrids between *M. integrifolia* and *M. tetraphylla* are productive, and crosses among *M. ternifolia* and *M. integrifolia* (Fig. 7.9) and *M. jansenii* produce viable progeny (Hardner et al. 2000).

To date, there are no reports of somatic cell hybridization in macadamia.

7.7.2 Hybrid Cultivars

While *Macadamia integrifolia* cultivars were preferred in Hawaii, there are a number of hybrid cultivars from selection programs in Australia, California and South Africa (Hardner et al. 2009). The use of molecular markers indicate that the primary factor defining structure of domesticated germplasm is species composition, with cultivars exhibiting a range of *M. integrifolia* × *M. tetraphylla* hybridity and a trispecies (*M. integrifolia*, *M. tetraphylla*, *M. ternifolia*) hybrid (HAES 791) that is used in commercial production (Peace 2005). However, these cultivars appear to be derived from ex situ hybridization, as allelic contributions did not match those found in natural hybrid zones (Peace et al. 2002, 2005, 2008). Some of the most successful Australia cultivars, such as A4 and A16, are hybrids between *M. integrifolia* and *M. tetraphylla* (Hardner et al. 2009).

Fig. 7.9 Left to right: Nuts of *Macadamia ternifolia*, *M. ternifolia* × *M. integrifolia* hybrid and *M. integrifolia* (cv. HAES 344) illustrating intermediate nut size of the hybrid

Development of hybrid cultivars offers the opportunity to incorporate desirable characteristics from different species; however, no data are available on hybrid vigor in the crop. *Macadamia tetraphylla* × *M. integrifolia* hybrids are more productive in cooler environments in comparison to *M. integrifolia* (Hardner et al. 2009). It has been also suggested that pure *M. tetraphylla* and hybrid germplasm produce poorer quality kernel as compared to pure *M. integrifolia* cultivars (Moltzau and Ripperton 1939). However, a review of controlled experiments (Hardner et al. 2009) did not detect species differences in kernel size, oil content, color following roasting, or sucrose content. The reduced stature of *M. ternifolia* and *M. jansenii* may offer a pathway for reducing the vigor through incorporation of these characteristics into commercial germplasm by the development of hybrids. However, as these species produce bitter kernels with high levels of cyanoglucosides, further information is required on the inheritance of this trait and opportunities to reduce expression in interspecific hybrids.

7.8 Conclusions and Prospects

Cultivation and breeding of macadamia are in their infancy compared with many horticultural tree crops. Breeding programs have progressed to second generation populations in the Hidden Valley Plantations and Australian industry programs,

while most other programs are selecting from first generation populations. There has been significant impact from simple innovations such as clonal propagation by grafting and budding; technologies taken for granted in other crops. There is scope for use of biotechnologies that are more economical than conventional methods, but these must be coupled with methods of rapid and cost-effective phenotyping. The major limitations of large plant size, long generation time, long juvenile period and low young to mature tree yield correlations remain as obstacles for effective phenotypic characterization. A reference genome sequence for macadamia will inform and enhance molecular marker-assisted breeding approaches, such as GWAS, MAS and GS, that are anticipated to accelerate the development of new cultivars.

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Appendices

Appendix I: Research Institutes with Activities Related to Breeding, Genetics or Cultivar Development of Macadamia

Institute	Specialization and research activities	Contact information and website
The University of Queensland, Queensland, Australia	Breeding, genomics, nut quality, consumer preferences	A/Prof. Bruce Topp, b.topp@uq.edu.au, https://qaafi.uq.edu.au/
Department of Agriculture and Forestry, Queensland, Australia	Regional variety trial, industry benchmarking, orchard agronomy	Mr. Dougal Russell, Dougal.Russell@daf.qld.gov.au, www.daf.qld.gov.au
Southern Cross University, New South Wales, Australia	Genomics	Dr. Catherine Nock, cathy.nock@scu.edu.au, https://www.scu.edu.au/southern-cross-plant-science
Department of Primary Industries, New South Wales, Australia	Regional variety trial, orchard management	Mr. Jeremy Bright, Jeremy.Bright@dpi.nsw.gov.au, www.dpi.nsw.gov.au
Hidden Valley Plantations, Beerwah, Queensland, Australia	Breeding	Mr. David Bell, davidbell@hvp-macadamias.com, http://www.hvp-macadamias.com/

Institute	Specialization and research activities	Contact information and website
Yunnan Macadamia (Lincang) Biotechnology Development Co. Ltd., Lincang, China	Breeding	Mrs. Chen Yuxiu, ynmacltd@163.com
Yunnan Institute of Tropical Crops, Yunnan, China	Breeding, genomics	Prof. He Xiyong, heda0691@163.com
Institute of Forest Tree Improvement and Biotechnology, Vietnamese Academy of Forest Sciences, Hanoi, Vietnam	Variety evaluation, breeding	Dr. Nguyen Duc Kien, nguyen.duc.kien@vafs.gov.vn, http://vafs.gov.vn/en/vietnamese-academy-of-forest-science-vafs/
Macadamia Conservation Trust	Conservation of genetic resources	Mr. Paul O'Hare, wild@macadamias.org, http://www.wildmacadamias.org.au/
Agricultural Research Council – Tropical and Subtropical Crops Campus, South Africa	Genomics	Mr. Mark Penter, infoitsc@arc.agric.za, http://www.arc.agric.za/arc-itsc/Pages/ARC-ITSC-Homepage.aspx
Pesquisadora Centro APTA-Frutas/Instituto Agronômico, Campinas, Brazil	Cultivar evaluation	Dr. Graciela da Rocha Sobierajski, sobierajski@iac.sp.gov.br, http://www.iac.sp.gov.br/areasdepesquisa/frutas/
University Hawaii – Manoa, College of Tropical Agriculture and Human Resources	Cultivar evaluation	Dr. Alyssa Cho, acho@hawaii.edu, https://cms.ctahr.hawaii.edu/tropfruitnut/Home.aspx

Appendix II: Genetic Resources of Macadamia Cultivars Grown in Australia

Cultivar (synonym)	Released by	IPP*	Comment
A4	HVP	Yes	Precocious
A16	HVP	Yes	Small tree; late dropper
A29	HVP	Yes	Planted in southern NSW
A38	HVP	Yes	Susceptible to husk spot
A203	HVP	Yes	Compact flowering; popular in Bundaberg, QLD
A268	HVP	Yes	Large nuts
A376	HVP	Yes	Recent release; high KR
A403	HVP	Yes	Recent release; all rounder
A447	HVP	Yes	Recent release; 70% size of standard tree
A538	HVP	Yes	Recent release; small tree precocious
Beaumont (HAES 695)	EAS	No	Late dropper; used as seedling rootstock
Daddow	EAS	No	Very dense canopy
H2 (Hinde)	EAS	No	Used as seedling rootstock
HAES 246 (Keauhou)	HAES	No	40 year old standard in NSW
HAES 333 (Ikaika)	HAES	No	Thick shell; not currently planted

Cultivar (synonym)	Released by	IPP*	Comment
HAES 344 (Kau)	HAES	No	30 year old standard in NSW
HAES 508 (Kakea)	HAES	No	Large tree; poor performance in warm locations
HAES 660 (Keaau)	HAES	No	Early dropper, small nuts; some pre-germination
HAES 741 (Mauka)	HAES	No	Early dropper; small nuts; not precocious
HAES 781	HAES	No	Very large tree
HAES 783	HAES	No	High % whole kernels
HAES 791 (Fuji)	HAES	No	Ancestry contains *M. ternifolia*
HAES 800 (Makai)	HAES	No	Spreading habit; prone to wind damage
HAES 814	HAES	No	Small, precocious tree; yield drops in older trees
HAES 816	HAES	No	High KR; light colour kernel
HAES 842	HAES	No	High NIS and KR; darker kernel colour
HAES 849	HAES	No	Light and late flowering
MCT1	MCT	Yes	Recent release; high KR
MIV1-G	DAF	Yes	Recent release; precocious; high KR; mid-late dropper
MIV1-J	DAF	Yes	Recent release; large nut; high KR suited to Bundaberg, QLD
MIV1-P	DAF	Yes	Recent release; late dropper; small tree
MIV1-R	DAF	Yes	Recent release; suited to northern NSW
NG8	EAS	Yes	Small tree
Own Choice	EAS	No	Prone to sticktights and pre-germination
Own Venture	EAS	No	Late nut drop

Abbreviations: *DAF* Department of Agriculture and Fisheries (Queensland); EAS Early Australian selection; HAES Hawaiian Agricultural Experiment Station; *HVP* Hidden Valley Plantations; *IPP** Intellectual Property Protection by license or Plant Breeders Rights; *KR* Kernel recovery;, *MCT* Macadamia Conservation Trust; *NIS* Nut-in-shell; *NSW* New South Wales; *QLD* Queensland

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Chapter 8
Peanut (*Arachis hypogaea* L.) Breeding

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Abstract Cultivated peanut (*Arachis hypogaea* L.), a vital source of proteins and nutrient-rich fodder for livestock, is considered globally as a major oilseed crop. Being a segmental allopolyploid with AABB genome conformation, the cultivated peanut is considered to have evolved through single interspecific hybridization amid two diploid species. A number of biotic and abiotic forces restrict the production and productivity of peanut. Intensive attempts to develop superior peanut varieties with inherent tolerance/resistance and enriched nutritional components were executed to combat stress factors in fulfilling the requirements of farmers and consumers. Breeding objectives in the past were achieved mainly through mass and pure-line selections. Subsequently to accomplish breeding objectives, peanut breeders employed backcross and pedigree approaches followed by inter- and intra-specific hybridization in a considerable way. Simultaneously, peanut breeding through the mutagenic approach played a noteworthy part during the development of multiple

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propitious high-yielding varieties. Traditional breeding approaches helped in identification and advancement of cultivars with inherent resistant traits, but such resistance traits are tightly linked with inferior pod and kernel characteristics that are extremely challenging to break. Under non-conventional approaches, several molecular breeding techniques were successfully attempted to break this barrier. Marker-assisted selection (MAS) and transformation of genes coding the traits of interest, overlaying the way of gene insertion, assisted significantly in establishing superior varieties of peanut with inherent resistance and enhanced pod and kernel features. Among all efficient markers, microsatellite markers were extensively employed in constructing linkage maps, genotyping as well as MAS, owing to the distinguishable and co-dominance nature of these markers. A number of reproducible molecular markers were developed that are associated with salinity and drought tolerance, as well as resistance to biotic stresses like rust, and leaf spots, and to a certain extent *Sclerotinia* blight etc. *Agrobacterium*-mediated genetic transformations, via in planta or particle-bombardment approaches, have resulted in development of transgenic peanuts with enhanced yield attributes and inherent resistance against a few biotic and abiotic stresses. Such genetically transformed peanut populations could also be employed as donor parents in traditional breeding system to develop fungal and a few virus disease tolerant varieties. Nevertheless, it could be suggested that a combination of breeding and biotechnological tools and approaches, might deliver an inherent, cost-effective, as well as eco-friendly solutions in developing better peanut varieties globally.

Keywords Allopolyploidy · Bioinformatics · Genetic transformation · Interspecific hybridization · Marker assisted selection · Mutation breeding

8.1 Introduction

Peanut is an annually grown oilseed crop. It can be an erect type with ~60 cm height or can be horizontal with ~30 cm height (Fig. 8.1a). Inflorescences of peanut comprise 3 perfect flowers that bloom in the axils of the leaves (Fig. 8.1b). Peanut varies from the majority of angiosperms, since its fertilized ovary is carried on a peg (gynophore) into the ground, where pod development begins and the fruits are produced (Fig. 8.1a) (Burow et al. 2008). Leaves of peanut are tetrafoliate and grow in an alternative fashion on the central stem as well as on the lateral branches (Fig. 8.1b). Leaflets of peanut are mostly oblong to lanceolate. It possesses green angular stems that may be pubescent or glabrous in texture (Sharma et al. 2003). Peanut is self-pollinated and the flowers are perfect, comprising both the female and male reproductive organs. The flowers are papilionaceous carrying 5 vibrant petals. Out of the 5 petals, the largest one is *standard*; 2 of them are *wings* and the other 2 united *keels* (Fig. 8.2). The calyx is 5-lobed having 1 lobe opposite the keel, while the other 4 are united at their base at the rear edge of standard. The androecium is monadelphous having the filaments merged for 2/3 of their length and comprises 8 active and 2 sterile stamens (Stalker et al. 2016).

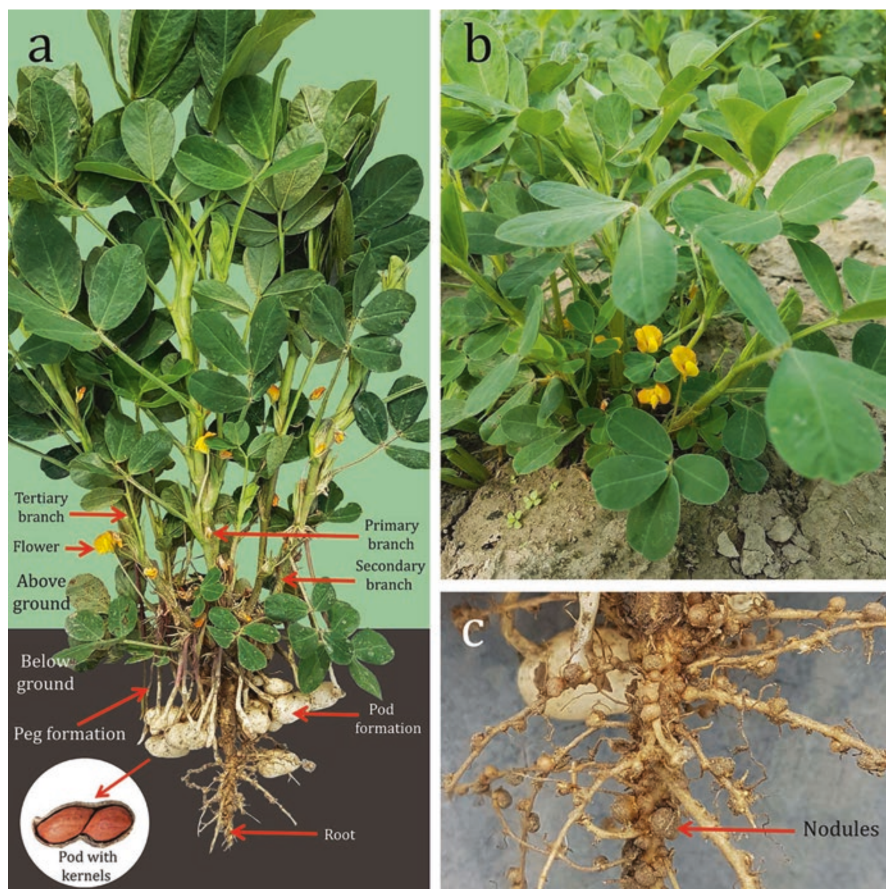

Fig. 8.1 Botanical features of peanut (*Arachis hypogaea* L.). (**a**) Full grown plant showing branching pattern, peg formation and pod development, (**b**) Full grown erect type plant showing flowers, tetrafoliate leaves and typical branching pattern, (**c**) Distinctive characteristics of root and root nodules. (Photos by S. Gantait)

In-depth analyses of peanut, starting from its classification, distribution, importance, domestication, selections, pre-breeding, early improvement, and cultivation practices may help in exploring the possibilities of increasing peanut production, solving the biotic and abiotic stress management issues of the crop and tackling the cost of cultivation towards more economic gain in a sustainable way.

This chapter addresses current problems and challenges in genetic improvement of peanut, its advanced breeding methodologies and modern biotechnology, and also the implication of bioinformatics tools towards food and nutritional security that stands as an immediate fundamental need of global humankind. Over time, the demand for peanut has escalated owing to a drastic change in population, urbanization, income level and consumption pattern. Hence, to meet the balance between demand and supply in the near future, proper methodologies must be implemented with the objective to increase the production and productivity of peanut.

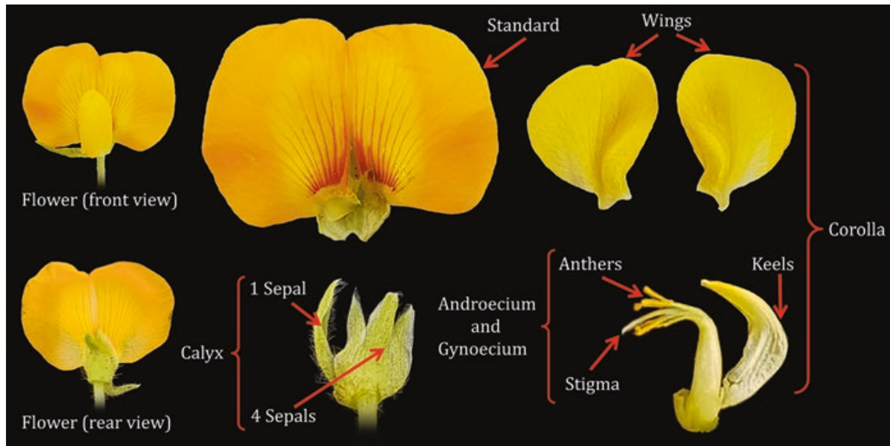

Fig. 8.2 Floral morphology (intact and dissected) of peanut (*Arachis hypogaea* L.). (Photo by S. Gantait)

8.1.1 Botanical Classification and Distribution

8.1.1.1 Botanical Classification

Kingdom: Plantae
Division: Tracheophyta
Subdivision: Spermatophytina
Class: Magnoliophyta
Order: Fabales
Family: Fabaceae
Subfamily: Faboideae
Tribe: Aeschynomeneae
Genus: *Arachis*
Species: *hypogaea*
Common names: Groundnut, peanut

Arachis hypogaea L. (Linnaeus 1753) with paripinnate leaves, tubular hypanthium and straight embryo, with a distinctive feature of geocarpic peg leading to a subterranean fruiting habit, belongs to the family Fabaceae (Rudd 1981).

Krapovickas (1968) divided the cultivated species into 2 subspecies, each of which has 2 botanical varieties. This classification is based on growth habit, branching pattern, inflorescence, pod and seed characters, seed dormancy, etc. This classification is more conveniently used in many literature sources because of the ease of grouping. Based on subspecific nomenclature and the varietal associations, Krapovickas and Gregory (1994) further classified the 2 subspecies of peanut into 6 botanical varieties, which freely exchange genes within and among them:

1. Subspecies *hypogaea*: Dark green foliage; alternate arrangement of flowers on lateral branches; main stem unaccompanied by flowering; simple inflorescence and usual presence of seed dormancy.
   (a) Var. *hypogaea* (Virginia and runner types): Glabrous leaflets on ventral side; pods may be very small or large; 2 seeded; less hairy and intermediate to late maturity.
   (b) Var. *hirsuta* (Peruvian humpback and Chinese dragon): Ventral side with hairy leaflets; 2–4 seeded with very late maturity.
2. Subspecies *fastigiata*: Flowers on central stem; no sequential arrangement of flowers; either simple or compound inflorescence; foliage is light green in color and lack of seed dormancy.
   (a) Var. *fastigiata* (Valencia type): Glabrous leaflets on ventral side; 2–4 seeded (rarely 5); short and slender reproductive branches.
   (b) Var. *peruviana*: Leaflets glabrous as in *fastigiata*; long and robust reproductive branches seen both on the central stem and branches.
   (c) Var. *aequatoriana*: Hairy leaflets on ventral side; long hairy reproductive branches; reticulated deep pods; highly branched and erect purple stems.
   (d) Var. *vulgaris* (Spanish type): Hairy leaflets on ventral side; highly branched and upright branches; 2 seeded; base of the plant with clustered pods (Singh and Simpson 1994).

8.1.1.2 Geographical Distribution

Bolivia and adjoining regions of South America are considered to be the center of origin for cultivated peanut, which is now cultivated throughout the warm temperate and tropical territories of the world; however, commercial cultivation is confined to 40 °N and 40 °S latitudes. China and India from Asia; Indonesia, Myanmar and Vietnam from Southeast Asia; African countries such as Sudan, Nigeria, Chad, Democratic Republic of Congo, Zimbabwe, Mozambique, Uganda, Burkina Faso and Mali; the Caribbean; USA; Brazil, Argentina and Mexico in Latin America are the major peanut producing countries. A total of 81 *Arachis* species have been reported in the literature (Krapovickas and Gregory 1994; Valls et al. 2013), including annual and the perennial forms of cultivated peanut. Depending on the extent of diversity in cultivated peanut germplasm, Krapovickas (1968) recognized 5 centers and, Gregory and Gregory (1976) identified 6 centers of genetic diversity; while recent explorations added Ecuador as the seventh center with distinct group of landraces referred as var. *aequatoriana* (Singh and Nigam 1997). These centers are: (1) The eastern foothills of the Andes in Bolivia, (2) The Guarani region, chiefly Paraguay, (3) Goiás and Minas Gerais (Brazil), (4) Rondônia and northwest Mato Grosso (Brazil), (5) Peru, (6) Northeastern Brazil and (7) Ecuador.

8.1.2 Importance

Peanut is an economically-important legume, the root of which fixes nitrogen and is used as a food-feed-fodder crop in the semiarid tropics of India and Africa. It is mainly cultivated for extraction of its seed oil (44–56%) and also used in confectionaries (Gantait et al. 2017). The oil is mainly used for cooking because of its flavor and a relatively high smoke point (232 °C for refined oil). Due to its high monounsaturated fatty acid content it is considered healthier than most of the other saturated oils and is resistant to rancidity. There are several grades of peanut oils in the USA including aromatic roasted peanut oil, refined peanut oil, extra virgin or cold pressed peanut oil and peanut extract. Refined peanut oil is exempt from allergen labeling laws of the USA. Peanut seeds contain high quality digestible protein (22–30%), and minerals (phosphorus, calcium, magnesium and potassium) (Savage and Keenan 1994; Asibuo et al. 2008). Seeds can be processed and used in confectioneries or consumed directly, either boiled or roasted, or ground to make peanut butter (Fig. 8.3). The young pods, young leaves and leaf tips may be consumed as a vegetable and green leafy vegetable, respectively (Martin and Ruberte 1975). Peanut seeds are very important sources of vitamins E, K, B, and fiber. Furthermore, peanuts are good source of thiamine and niacin compared to cereal crops (Kassa et al. 2009). Peanut also forms a part in folk medicine and used in the treatment of many diseases.

Fig. 8.3 Uses of peanut (*Arachis hypogaea*). (**a**) Peanut oil, (**b**) Peanut butter, (**c**) Roasted peanuts (as snacks), (**d**) Fried peanuts, (**e**) Boiled peanuts, (**f**) Chikki (peanut sweet made from peanuts and jaggery). (Source: https://en.wikipedia.org/wiki/Peanut)

8.1.3 Domestication, Selection and Early Improvements

Cultivated peanut has a long and relatively complex history, which involves natural evolution and human domestication. Ecologically diverse countries from the continents of Asia, Africa and America have been cultivating peanuts as a major oilseed crop for millennia (Holbrook and Isleib 2001). Besides, these regions possess morphologically diverse landraces of peanut (Krapovickas and Vanni 2010). According to Bonavia (1982), the Huarmey Valley, near the Peruvian coast possesses the most ancient record of *A. hypogaea* from roughly 3500–4500 years ago. Ancient people of Northwest Peru (approximately 3500–3800 years ago) used peanut pods, which revealed the evidence of peanut cultivation along with some wild *Arachis* species. Radiocarbon-dated macrobotanical remains from about 7840 years ago appear to have morphological resemblance to a wild *Arachis* species. Peanut is an allopolyploid with two different genomes *AABB* (Stalker 1992), and any one of the *A* genome species, *A. cardenasii*, *A. chacoense, A. correntina, A. duranensis*; and *A. villosa* and *A. batizocoi* of the *B* genome have been hypothesized as possible progenitors of *A. hypogaea* (Singh and Smart 1998). However, Paik-Ro et al. (1992) reported that *A. batizocoi* is not closely related to *A. hypogaea* and hence cannot be considered as the *B* genome donor. Based on restriction fragment length polymorphism (RFLP) studies (Kochert et al. 1991); chromosome mapping of r-DNA loci by FISH (Seijo et al. 2004) and GISH (Seijo et al. 2007) analyses suggest that *A. ipaensis* could be the *B* genome donor which has been subsequently accepted as the progenitor species of *B* genome.

The degree and magnitude of genetic variability, the nature of variability, determines the efficiency of peanut germplasm/segregating population in terms of improvement for a particular trait of interest in breeding programs.

8.2 Cultivation and Traditional Breeding

8.2.1 Current Cultivation Practices

A number of features are involved in successful cultivation of peanut with good yields. Loose and friable soil, uniform seedbed and planting depth, spacing, weed control and irrigation are the few among those features. Depth of sowing should preferably be in the range of 5–7 cm when the soil temperature remains 18 °C or above, for better germination. Plant-to-plant 4–7 cm and row-to-row with 30–35 cm spacing is suitable for both rainfed and irrigated conditions. Application of basal N has to be avoided but P may be incorporated through green manure. Seeds treated with suitable N fixing and P solubilizing bacterial cultures yield higher than using chemical fertilizers. The crop prefers residual phosphorus and requires adequate levels of potassium for normal growth and development, and calcium (Ca) for pod and seed development. Boron (B) and molybdenum (Mo) are generally applied as a

mixture during early vegetative growth. To maximize yield and peanut quality, protective/supplementary irrigation at critical stages of crop growth (flowering, pod and seed formation, maturation) is generally provided in areas with low rainfall. Excess moisture is detrimental to the crop and should be avoided where scheduled surface irrigation is practiced. However, during flowering and pod filling a good amount of moisture is needed. The various physical parameters such as light, space, soil nutrients, available soil moisture, etc., make peanut more susceptible to a wide range of weeds that results in the plants being turned into host for several pests and diseases. During the entire period of crop growth, three weeding operations should be carried out if herbicides are not applied. The first weeding is done during pre-emergence of seedlings (within 7 days after planting), the second between 21–28 days after sowing, while the last weeding must be done within 40–60 days after sowing, depending on weed intensity.

8.2.2 Current Agricultural Problems and Challenges

Several challenges and developments faced by the peanut crop in different parts of the world presently are: high domestic demand, changing agro-climatic conditions, large domestic market, declining soil fertility, and enterprising farming community, access to global markets, as well as well-equipped laboratories and scope for increasing cropping intensity. The major thrust areas are to improve farming in drylands, use of wastelands, commodity exchanges/future markets, supply chain, storage, processing, and value addition activities, grading and standardization of products etc., in addition to the major abiotic and biotic stresses. There are growing concerns over new and emerging insect pests (white grubs and storage beetles in India) and diseases (soil borne and virus diseases), leading to significant yield losses (Porter et al. 1990). In Asia and Africa, losses are severe due to early and late leaf spots caused by *Cercospora arachidicola* Hori and *Cercosporidium personatum* (Berk. and Curt.) Deighton and also by leaf rust (*Puccinia arachidis* Speg.). Chemical control of these diseases is expensive, which the resource-poor smallholder farmers of the semiarid tropics cannot afford.

8.2.3 Improvement Strategies

For peanut, little attention has been paid to take advantage of environmental factors and available germplasm resources. Improved cultivation practices coupled with growing improved cultivars make it possible for a synergistic yield enhancement. Farmer participatory varietal selection (FPVS), water and nutrient management, and consideration of the environmental and edaphic factors should be helpful in crop-stress management. Strategies to ensure maximum yield from a promising genotype should exploit the proper abiotic and biotic stress management,

acclimatization to agro-climatic zones and use modern genetic tools for the betterment of the crop (Johansen and Rao 1996). The physical factors of the soil such as temperature, ambient radiation and carbon dioxide regimes also affect productivity in the peanut.

8.2.4 Traditional Breeding Methodologies and Limitations

The main objectives of peanut breeding programs are to enhance yield per unit area or to improve a particular trait of interest. Although several improved varieties of peanut have been developed throughout the world, only a few are in the limelight and are commercially successful. Selection of desirable agronomic traits in plant breeding programs has traditionally been performed at the phenotypic level, which requires evaluation either in the field or under controlled conditions to assess them for traits, such as disease resistance, drought tolerance or yield parameters. This process is time consuming and often restricted by growing seasons or the limited available resources. Field screening of plants for diseases may result in the loss of plant material for further evaluation or inadequate disease pressure which leads to *escapes*. Wild species of peanut offer a vast reservoir of valuable genes that are not available in the cultivated species. Interspecific hybridization in this crop was attempted as early as 1938 between *A. hypogaea* and *A. glabrata* but without success (Hull and Carver 1938). Similarly, attempts to cross *A. hypogaea* × *A. villosulicarpa* and *A. hypogaea* × *A. diogoi* were also unsuccessful (Gregory 1946). However, a very few varieties Tamnut-74, and Spancross in USA and one in India (GPBD-4) have been released for commercial cultivation.

8.2.5 Role of Biotechnology

Applications of biotechnological tools aim to improve traits like higher yield and nutritive quality that can be sustained for long periods. Recent advances in modern techniques like molecular marker and gene sequencing have the capability to improve the disease and/or insect resistances and other agronomic traits in peanut. Marker-assisted selection (MAS) can significantly improve the efficiency of new and enhanced cultivar development. One key advantage of molecular markers over the traditionally used phenotypic markers is that they are unaffected by environmental conditions and are not dependent upon plant growth stage. In general, MAS is a non-destructive process that allows the identification of plants with desirable traits for advancement in a breeding program. Techniques that are common in developing molecular markers comprise random-amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs), amplified polymorphic DNAs (AFLPs), sequence characterized amplified regions (SCARs) and simple sequence repeats (SSRs) (Charcosset and Moreau 2004; Collard et al. 2005; Mohan et al.

1997). The availability of markers for a specific crop can determine the success of MAS in breeding programs. Important agronomic traits such as yield and yield components, plant height, days to maturity, and disease resistance are frequently influenced by multiple genes or quantitative trait loci (QTL). However, the number of markers associated with QTLs is rapidly increasing for crop plants. For peanut alone, molecular markers have been identified for maturity (Bland and Lax 2000), nematode resistance (Garcia et al. 1996), late leaf spots resistance (Luo et al. 2005a) and resistance to the aphid vector of peanut rosette disease, *Aphis craccivora* (Herselman et al. 2004).

To facilitate breeding strategies further, transformation of peanut with desired genes has provided immense advantages. Genetic transformation offers the proficiency to develop a transgenic peanut plant, following the insertion of prospective gene(s), which could express scores of advantageous traits associated with tolerance to environmental stress factors like salinity, drought, cold as well as metals/metalloids, and biotic stress like insect pests, pathogenic fungi, viruses, etc. Nevertheless, the success of genetic transformation by and large depends oregeneration efficiency of transgenic crops. Since peanut is a genotype-specific oil-seed crop, *in vitro* regeneration has proved to be quite tedious and requires defined protocols for each genotype, even those belonging to the same species. An ample number of successful attempts have been made to standardize regeneration protocols through multiple shoot culture, callus culture and somatic embryogenesis that certainly have assisted propagation of peanut cultivars on a large scale (Kundu and Gantait 2018).

8.3 Germplasm Biodiversity and Conservation

8.3.1 Germplasm Diversity

The great strength of peanut as a crop is its huge number of germplasm accessions (Holbrook and Stalker 2002). These nurturing centers of alleles have been underutilized. There are five primary peanut germplasm repositories in the world. The largest one contains 14,966 and 453 accessions of cultivated and wild *Arachis* species, gathered from 93 countries; this repository is housed at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. Next is the Indian National Gene Bank, ICAR-NBPGR with 13,819 peanut accessions. The third major holder with 9027 accessions is the USDA Southern Regional Plant Introduction Station, Griffin, GA, USA. Fourth is the collection of 7935 accessions at the National Research Centre for Groundnut, Junagadh, India (Figs. 8.4 and 8.5). Fifth largest is represented by the 5890 accessions deposited at the Chinese National Collection.

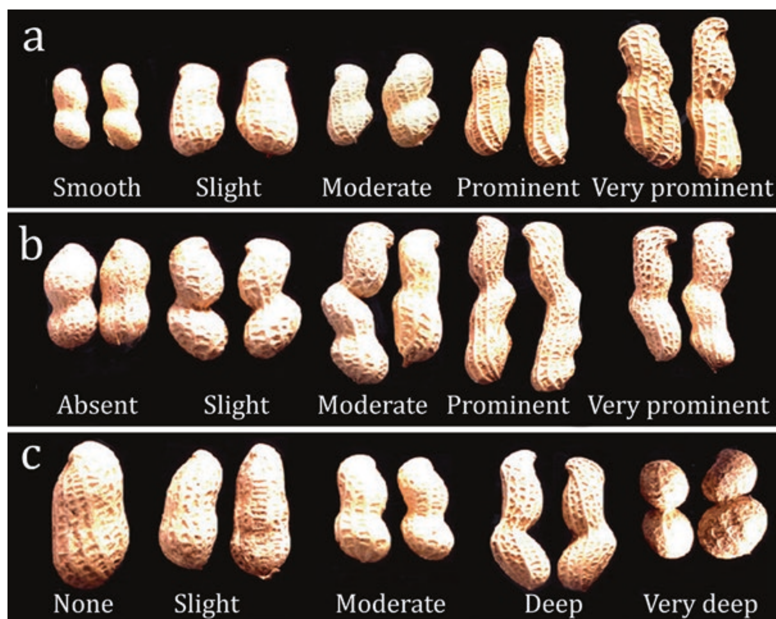

Fig. 8.4 Diversity of peanut (*Arachis hypogaea* L.) germplasms based on pod morphology. (**a**) Pod reticulation, (**b**) Pod beak, (**c**) Pod constriction. (Photos by A.L. Rathnakumar)

Fig. 8.5 Diversity of peanut (*Arachis hypogaea* L.) germplasms based on testa color. (**a**) White, (**b**) Off-white, (**c**) Tan, (**d**) Rose, (**e**) Rose with off-white, (**f**) Salmon, (**g**) Salmon with purple flecks, (**h**) Light red, (**i**) Red, (**j**) Red with white flecks, (**k**) Light purple, (**l**) Purple. (Photos by A.L. Rathnakumar)

8.3.2 Cultivars Characterization and Phylogeny

More efficient methods for evaluating and maintaining genetic diversity in germplasm collections are much needed. One possible method is the development and use of subsets of germplasm collections, called *active working collections* or *core collections*. A core collection would minimize repetitiveness within the collection and should, to the extent possible, represent the genetic diversity of a crop species. The core collection would serve as a working collection, which could be extensively examined, and the accessions excluded from the core collection would be available from the total collection (Brown 1989a, b).

Out of the huge germplasm repository collections, few core collections have been developed regionally viz. China (582 accessions), US (831 accessions), and ICRISAT (1704 accessions) (Holbrook et al. 1993; Jiang et al. 2004; Upadhyaya et al. 2003). Trait-based cores of a core collection i.e. mini-core collections have also been developed, which include early leaf spot (*Cercospora arachidicola* Hori); cylindrocladium black rot [*Cylindrocladeum crotalaiae* (Loos) Bell and Sobers] (Isleib et al. 1995); tomato spotted wilt (Anderson et al. 1996); reduced preharvest aflatoxin contamination and sclerotinia blight (*Sclerotinia minor* Jagger) (Holbrook et al. 1998); rhizoctonia limb rot (*Rhizoctonia solani* Kuhn) (Franke et al. 1999); and root-knot nematode (*Meloidogyne arenaria* (Neal) Chitwood race 1 (Holbrook et al. 2000), and the resistant accessions have been identified for each of these traits.

8.3.3 Genetic Resources Conservation Approaches

In lieu of all the species recounted so far (reviewed by Stalker et al. 2016), the Embrapa Genetic Resources and Biotechnology, Brasilia, Brazil holds the largest wild germplasm collection (over 1200 accessions) followed by the ICRISAT gene bank, which maintains 453 accessions. A large number of wild *Arachis* species are also preserved at Texas A&M University, College Station TX and North Carolina State University, Raleigh, NC. Several accessions were procured from local vendors wherein the seeds diverge in shape, size, color, and other features, and many outlines are seed combinations that are marked by plant size, seed colors and growth habit (Figs. 8.4 and 8.5). Therefore, regeneration plots should be of adequate size to assure that all variations perpetuate (Fig. 8.6). Keeping the cultivated species in a reasonably organized manner still remains the biggest concern due to vulnerability to diseases, especially accessions of var. *vulgaris*, and, in addition, multiplication of seeds may adversely be affected in a few localities.

It is remarkably more challenging to maintain the wild *Arachis* species than a cultivated peanut due to difficulties in little or no seed yield; 25% of wild species are grown in greenhouses. Even section *Rhizomatosae* accessions are grown through vegetative means. Less than 50% of wild species accessions are maintained in situ via vegetative propagation. Overspreading of branches in most of the species may

Fig. 8.6 Maintenance of peanut (*Arachis hypogaea* L.) germplasm in experimental fields. (Photo by S. Gantait)

lead to genetic impurity. To avoid this outcrossing condition, separate facilities with distinctive plants must be provided under greenhouse condition, which is both labor-intensive and expensive. The alternative to this cumbersome approach is to grow them in nurseries to produce large numbers of seeds for germplasm conservation and for further evaluation-based research. To avoid outcrossing and seed contamination, North Carolina State University has been growing peanut with never-tried seedlings into 2 × 3 m blocks and at a distance of 5 m. Moreover, the reproductively incompatible species are grown together, and there is possibility of sterile plant production due to outcrossing. For 10 or more years of storage, large numbers of seeds are produced, and the wild species accessions are grown under field conditions and seeds of the same can later be maintained in a −20 °C freezer. Many species could produce rare flowers though via self-pollination and form pegs under short-day conditions (Stalker and Wynne 1983); while the flowers with few pegs and seeds were produced under long-day conditions. Seeds of *A. guaranitica* and *A. tuberosa* enter into a state of permanent dormancy upon drying that restricts their long-term storage (Stalker and Simpson 1995).

8.3.4 Cytogenetics

8.3.4.1 Chromosome Number

Badami (1928) reported a chromosome number of 2n = 20 in the cultivated peanut species. Apart from this count, the chromosome number assigned to cultivated peanut types is 2n = 40. Husted (1936) was the first to study the morphology of somatic chromosomes and reported a chromosome number of 2n = 40 in cultivated types. Kawakami (1930) was the first to determine the true chromosome number of

Arachis hypogaea to be 2n = 40. The first chromosome count reported for a wild species was 2n = 40 for *A. glabrata* (section *Rhizomatosae*) (Gregory 1946). Most species in the genus *Arachis* are diploid (2n = 20). However, tetraploid species with chromosome numbers 2n = 40 have also been reported in section *Arachis* (*A. montocola*) and section *Rhizomatosae.* However, these species are highly cross-incompatible. This indicates that polyploidy may have arisen at least twice in this genus, once in the section *Arachis* and the other in section *Rhizomatosae,* each independently.

Lavia (1996, 1998) reported a chromosome number of x = 9 for *Arachis palustris*, *A. praecox* and in *A. decora* of the section *Arachis*. Among the two series of chromosome numbers that appear to occur in the genus *Arachis* (2n = 2x = 20 and 2n = 4x = 40) the diploid forms are more predominant and hence the basic chromosome number is believed to be x = 10. The proposed basic chromosome number of x = 9 in the species *A. palustris* and *A. praecox* may have originated by the selective elimination of a single chromosome from the other species having x = 10. On the other hand, Bera et al. (2002) proposed that the reverse might be true and species with chromosome number x = 10 may have originated by selective duplication of a single chromosome. The presence of two basic chromosome numbers (x = 9 and x = 10) and the lesser existence of polyploid species in the genus *Arachis* clearly indicates that aneuploidy has played a key role in the evolution and speciation of *Arachis* species, rather than polyploidization. Therefore, the species diversity of *Arachis* may be mainly due to structural chromosomal rearrangements and thus supports the theory that peanut is a *segmental polyploid* and that section *Arachis* represents the most advanced traits within the genus.

8.3.4.2 Chromosome Size, Types and Pairing Behavior

The chromosomes of peanut are small, ranging from 1.4 to 3.9 μm in length, mostly metacentric and difficult to karyotype. The chromosomes of most species of the section *Arachis* are symmetrical with median chromosomes. However, Husted (1936) analyzed somatic chromosomes of several cultivars and distinguished a pair of small chromosomes termed *A* chromosomes and another pair with a secondary constriction, termed *B* chromosomes. The few karyotype studies attempted in the species of the section *Arachis* (Singh and Moss 1982; Stalker 1991; Stalker and Dalmacio 1986) indicated the presence of a pair of small *A* chromosomes in most species; and is absent in the following species, *A. batizocoi*, *A. cruziana*, *A. magna*, *A. williamsii* and *A. ipaensis*. Instead they have a pair of satellite chromosome called *B* chromosomes (Smartt et al. 1978). Babu (1955) reported several types of secondary constriction in *A. hypogaea*, and proposed that cultivars could be distinguished based on karyotypic differences.

The meiotic chromosomes of *Arachis hypogaea* pair mostly as 20 bivalents, but a few multivalents have also been reported (Husted 1936). Hybrids among subspecific types exhibit mostly bivalents at metaphase I, but few univalents also exist at a low frequency. Husted (1936) concluded that structural differences in chromosomes exist between the two subspecies of *hypogaea* and *fastigiata*.

8.4 Molecular Breeding

8.4.1 Molecular Marker-Assisted Breeding

Peanut breeding aims to improve yield, quality, earliness, tolerance or resistance to disease and pests, and other desirable agronomic traits. The peanut germplasm consists of the cultivated allotetraploid species *Arachis hypogaea* and a large number of wild species (Halward et al. 1991). Molecular breeding constitutes genetic manipulation performed at DNA levels for crop improvement (Jiang 2013). An effective tool to access genetic diversity of peanut is molecular marker technology. Molecular markers can be used at any stage of growth; they are stable and versatile and not adversely influenced by the environment. These traits outweigh its cost compared to morphological markers that are inexpensive, but limited in number and highly influenced by environmental factors. Molecular markers can be used to develop genetic maps of peanut populations. These are used to estimate genetic diversity for identification of desirable and diverse parents to improve traits of interest. Molecular markers, used in peanut research are RFLP, RAPD, AFLP and SSRs. In peanut, codominant marker RFLP was used to identify both the recessive genes and multiple alleles (Stalker and Mozingo 2001). RAPD markers are dominant with primers, consisting of 10 nucleotides. Previously, genetic diversity among 13 mutant species was studied using 12 random primers (Bhagwat et al. 1997). A polymorphism of 5.5% was obtained with an average of 1.51 polymorphic bands and only one primer OPJ06 exhibited high polymorphism. Subsequently, 70 selected genotypes, representing variability for several morphological, physiological, and other characters, were studied for polymorphism with 48 oligonucleotide primers (Subramanian et al. 2000). Seven primers displayed 14.6% polymorphism with a total of 408 bands. Genetic similarity among 26 accessions studied, using 8 primers of 10-mer, ranged from 59.0 to 98.8% with an average of 86.2% (Dwivedi et al. 2001). Similarly, Raina et al. (2001) employed RAPD and ISSR fingerprints to identify polymorphism between cultivated peanut and wild species of *Arachis.* Thirteen species of *Arachis* and one species each from sections *Heteranthae*, *Rhizomatosae*, and *Procumbentes* showed polymorphism of 42.7% and 54.4% for RAPD and ISSR fingerprints, respectively. Genetic variability within and among species belonging to section *Rhizomatosae* was analyzed with 10 random primers, and 113 polymorphic bands were observed (Nóbile et al. 2004). Mallikarjuna et al. (2005) examined 32 accessions of wild species belonging to 25 species, using 29 primers wherein the observed polymorphic banding range was 5–33 with a similarity value range of 0–49% with an average of 15% similarity. He and Prakash (1997) utilized PCR-based AFLP markers in peanut, wherein, 28 primers were used to generate 111 AFLP markers in cultivated peanut, with only 3% of them exhibiting polymorphism. Herselman et al. (2004) used 308 AFLP primer combinations to identify markers associated with aphid vector resistance in peanut rosette disease; 20 markers were identified, of which 12 were mapped to 5 linkage groups covering a map distance of 139.4 cM to construct the first partial genetic linkage map for cultivated peanut.

SSR markers are codominant and easy to identify from a minimal amount of DNA. Hopkins et al. (1999) used 6 such SSR markers to detect polymorphisms among peanut DNA. It was observed that 17 unique genotypes representing the 19 accessions, tested with 2 to 14 DNA fragments, were amplified per SSR marker. Twelve SSR markers were used by Moretzsohn et al. (2004) to discriminate 60 cultivated genotypes and 36 wild accessions belonging to 8 sections. Up to 76% of microsatellite marker transferability was exhibited by species of section *Arachis*. Conversely, other *Arachis* sections exhibited only 45%. Subsequently, 14 microsatellite primer pairs detected 18 putative loci in cultivated peanut. A mean polymorphic percentage of 33% was observed (Gimenes et al. 2007). Tang et al. (2007) observed a maximum genetic distance of 0.992 when 34 SSR markers were utilized for genetic diversity studies, involving 4 botanical varieties of cultivated peanut. Another study was reported on the diversity and genetic relationships of 201 accessions of *A. hypogaea* and 13 accessions of wild *Arachis* species using 13 SSR markers. The primer pair amplified 108 polymorphic alleles in *A. hypogaea* (Naito et al. 2008). A sum of 249 alleles was found in the 15 analyzed loci and a high degree of intra- and inter-specific polymorphism was detected among 77 accessions of 4 species from section *Rhizomatosae* (Angelici et al. 2008). A total of 4000 SSR primers were also developed using next-generation sequencing technology, termed RNA-seq, for a global analysis of the peanut transcriptome during seed development (Zhang et al. 2012). Ren et al. (2014) assessed 196 peanut cultivars in China for genetic diversity and population structure using 146 highly polymorphic SSR markers. A total of 440 polymorphic bands with an average of 2.99 were amplified and showed an average gene diversity index of 0.11. This indicated the obvious genetic variations between cultivars from southern and northern regions of China. Subsequently, 111 SSR markers were selected for analysis of genetic diversity in peanut cultivars and breeding lines from China, India and the USA.

Mean values of gene diversity and polymorphic information content (PIC) were 0.480 and 0.429, respectively, but country-wise analysis showed mean gene diversity in the USA, China and India of 0.363, 0.489, and 0.47 with an average PIC of 0.323, 0.43 and 0.412, respectively. Genetic analysis also divided these peanut lines into 2 populations (P1, P2) that coincided with peanut market types and the geographic origin (Wang et al. 2016). Expressed sequence tags (ESTs) from public databases were used to develop EST derived SSR markers to increase the number of functional markers for peanut crop. A total of 2456 EST-SSR primer pairs were successfully designed with 340 (92.62%) primer pairs yielding clear and scorable PCR products, and 39 (10.66%) primer pairs exhibited polymorphisms. These markers may facilitate interspecific studies and genetic diversity analysis in peanut (Bosamia et al. 2015). Recently, 554 single locus SSR markers were used to identify molecular markers associated with seed-related traits in 104 peanut accessions. Out of these 554 markers, 30 major SSR markers were detected to be associated with 4 seed-related traits ($P < 1.81 \times 10^{-3}$) in different environments. Specifically, the marker AHGA44686 was simultaneously and repeatedly associated with seed length and 100-seed weight in multiple environments with large phenotypic variance (26.23% 32.30%). The favorable alleles of these associated markers revealed potential utili-

zation for peanut breeding programs (Zhao et al. 2017). Similarly, in another recent study, 204,439 SSR markers were developed in diploid genomes. Twenty-five QTLs for shelling percentage were also identified in a recombinant inbred line (RIL) population across 4 years. Shelling percentage is an important economic trait for peanut breeding. Five consistent QTLs were detected in at least two environments. These consistent and major QTL regions provide an opportunity for further gene discovery and development of functional markers for breeding (Luo et al. 2017). Genomic research of peanut is improving with the various types of genetic markers that can be used for genetic mapping, marker-assisted selection and gene discovery for molecular breeding. Even though scores of QTL were identified for distinctive agronomical and economically important traits, the breeding of peanut is still hindered due to its narrow genetic base and lack of significant polymorphisms.

8.4.2 Functional Genomics

Identification and understanding of gene function and phenotype is part of functional genomics. The large genome size of peanut (2800 Mb/1C) makes it unlikely to be entirely sequenced in the near future (Arumuganathan and Earle 1991). In 2012, 252,832 *Arachis* expressed genes (ESTs) were deposited in GenBank, compared to 12,781 ESTs in 2006 (Feng et al. 2012; Jesubatham and Burow 2006). ESTs from all primary organs of cultivated peanut are available and can be used to construct informative gene networks that impact productivity (Clevenger et al. 2016). Considerable quantities of genetic information on protein-coding genes for relative and functional genomics studies are provided by ESTs. Opposed to this, genes of low expression levels that are under represented in cDNA libraries can be detected by the sequencing of gene-rich islands of hypomethylated DNA. Besides such approaches, the macroarray (nylon-based) and microarray screening techniques are useful for studying gene expression. Several researchers have attempted to associate genes with phenotype using these techniques of gene expression analysis. First, 43 differentially regulated transcripts were identified by differential display in the response of peanut to drought stress (Jain et al. 2001). Microarray technology was demonstrated by EST-derived microarrays of around 400 unigenes probed under several conditions. Twenty-five ESTs, possibly linked with drought stress and response to *Aspergillus parasiticus* were recognized and 56 up-regulated transcripts were detected and established by real-time PCR, following contamination with *Cercospora arachidicola* (Luo et al. 2005a,b).

As many as 17,000 ESTs were sequenced and 5066 EST sequences were selected to make a cDNA microarray, which displayed variable expression patterns in seeds, leaves, stems, roots, flowers and gynophores of peanut (Bi et al. 2010). In another study, microarray expression profiling exhibited that 62 genes in resistant cultivars were up-expressed in response to *Aspergillus* infection (Guo et al. 2011). Several kinds of mutational analysis or gene traps can be employed to study the gene function.

Preliminary research was executed to establish the promoter traps (Anuradha et al. 2006). Targeting induced local lesions in genomes (TILLING) in peanut was commenced with the primary objective of knocking out *Arah2* (allergen) genes. It was observed that the significantly reduced amounts of *Arah2* could be achieved by RNA silencing in transgenic peanut, but a high rate of mutagenesis made it a nonviable approach (Ozias-Akins et al. 2009). TILLING was consequently used to identify mutations affecting seed traits in peanut. Mutations in fatty acid desaturase AhFAD2, was predicted to restore function to the normally inactive enzyme (Knoll et al. 2011). The TILLING by sequencing approach was adapted in another study, wherein mutations were identified for the gene *AhLOX7* (Guo et al. 2015). TILLING may have potential usefulness in breeding programs.

8.4.3 Bioinformatics

Recent advances in peanut molecular breeding have amassed large amounts of genomic data. PeanutMap, a map database for peanut, has been available for a decade (Jesubatham and Burow 2006). PeanutMap offers a web-based interface presenting the definite linkage groups from a map set. It comprises the available maps of the peanut genome, and reduced map sets of markers linked with the important attributes. This can be utilized to detect associations amid linkage groups in a map, display marker connections amid homoeologous chromosomes, as well as among the maps reported in several publications. A legume genome database, the Legume Information System, was announced to incorporate species-specific data and allows cross-legume assessments (Gonzales et al. 2005). The Legume Information System will allow evaluation of synteny amid several species and also includes sequence data, making it conceivable to explore the genes, expressed in diverse tissues and at several physiological circumstances. Ideal use of these data would assist the advancement of instruments eventually to simplify comparative analysis.

8.5 Genetic Engineering

8.5.1 Methodologies

Introgression of unique genes into the peanut genome through genetic transformation methods has created a new policy for the advancement of agronomic attributes or functional confirmation of the genes. Scientific investigations for the past 10 years were directed at the establishment of gene-transfer and in vitro regeneration protocols for peanut (Kundu and Gantait 2018). Particle bombardment and *Agrobacterium*-mediated approaches are extensively utilized for transgenic peanut plant development (Fig. 8.7).

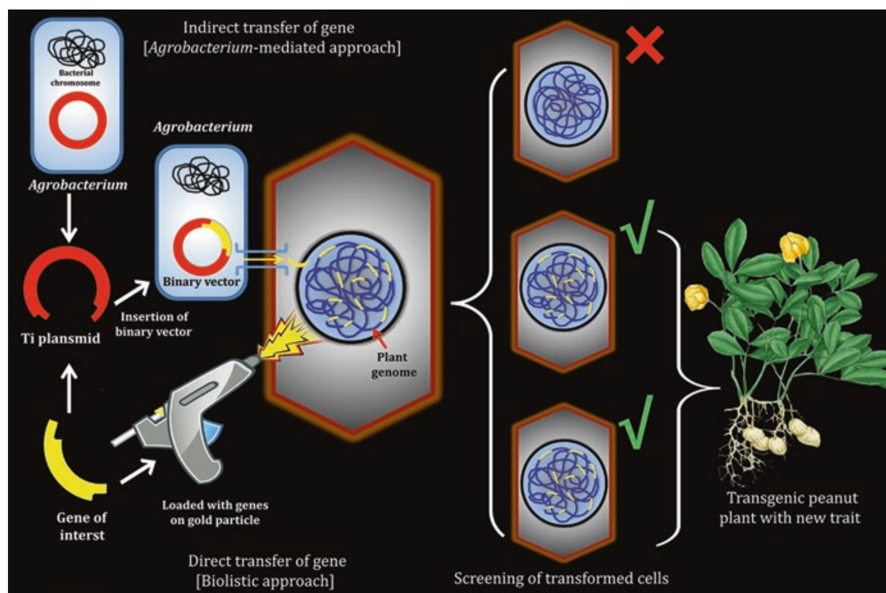

Fig. 8.7 Brief account on different modes of genetic transformation in peanut (Diagram by S. Gantait)

Besides this, direct regeneration of plantlets via in planta transformation approaches displayed huge benefits by escaping genotypic influence (Mallikarjuna et al. 2016).

8.5.1.1 *Agrobacterium*-Mediated Genetic Transformation

In contrast to the other alternative practices, *Agrobacterium*-mediated gene transmission is a broadly-acknowledged approach for peanut transformation. *Agrobacterium tumefaciens*-intervened transformation primarily relies on in vitro plant cell, tissue and/organ culture that takes a minimal period (typically 16–20 weeks) to develop initial putative genetically-transformed plants. *Agrobacterium*-based transformation provides exclusive benefits, for instance, superior proficiency of steady transformation counting mono-copy introgression, minimum incidence of gene silencing, transmission of concerned gene associated to transformation marker and capacity to transmit lengthier T-DNA sections (Mallikarjuna et al. 2016). Several *Agrobacterium* strains like A281, C58, EHA101, EHA105 and LBA4404 were employed to develop a transgenic peanut. *Agrobacterium*-based transformation procedures were substantiated involving several explants like cotyledonary nodes, hypocotyls, leaf segments and zygotic embryonic axes (Anuradha et al. 2008; Li et al. 1997; Tiwari and Tuli 2012). Successful genetic transformation in peanut (New Mexico Valencia variety) was reported by Cheng et al. (1996), who used the EHA105 strain to infect the leaf segments and

observed 10% *gus* positives *in vitro* plants (on screening medium) that exhibited 0.2–0.3% of transformation frequency and secured the incorporation of *uid A* and *npt II* genes. In *Agrobacterium* (C58)-intervened transformation of peanut (cv. JL-24), de-embryonated cotyledon explants when inoculated with *DREB1A* displayed 75% positive PCR, confirming the successful gene integration (Bhatnagar-Mathur et al. 2007). Notwithstanding the benefits of this process, frequent issues, such as low occurrence of gene incorporation and limited regeneration of transformed tissue/organ could give *false positive* results or *transgene escape* or even infertile regenerants (Tiwari et al. 2011).

8.5.1.2 In Planta Genetic Transformation

In planta genetic transformation is a tissue culture-free approach, selected for the development of transgenic plants. Embryo axes of peanut are isolated and subsequently injured by piercing with a fine needle to inoculate with *Agrobacterium* and the plants were raised in ex vitro environments. For instance, Rohini and Rao (2000) excised and wounded the embryonic axes and inoculated the same with *Agrobacterium* followed by incubation with leaf juice of tobacco; such explants, via histochemical assay and PCR, exhibited 3.3% of *gus* positive reaction. Notably, this in planta methodology was experimented with in an extensive way in peanut to authenticate the unique genes that are linked with abiotic and/or biotic stress (Keshavareddy et al. 2013; Manjulatha et al. 2014; Pandurangaiah et al. 2014). Integration of *AdSGT1* gene in the JL-24 cultivar of peanut via the in planta approach was described by Kumar and Kirti (2015a), who developed genetically-transformed peanut plants resistant to the late leaf spot pathogen. A consistent and competent in planta genetic transformation method is quite beneficial in comparison to the orthodox in vitro plant tissue culture-oriented approach, because it is not influenced by the genotype and pertinent to diverse crop species. In addition, the obligation of prolonged shoot and root regeneration phases and consequent *in vitro* culture-derived somaclonal variations can be avoided during in planta approach. Lately, extensive application of the in planta approach of gene transfer is showing significant success in several plant species. Nevertheless, the delectation of confirmed transgenics, developed through the in planta approach, necessitate a high-performance assessment system.

8.5.1.3 Particle Bombardment (Biolistics) Transformation

The *Agrobacterium*-free DNA transferal method was achieved by several approaches, such as electroporation, microinjection and particle bombardment (Li et al. 1995). Amid such approaches, the particle bombardment technique was the most extensively adopted mode for transgenic development. In this technique, insertion of a desired gene into a plant genome is done through high-speed dispersion of microprojectiles, layered by marked plasmid DNA (Klein et al. 1987). The three

key factors are: low genotypic influence, no influence of recalcitrance in explants, and no *Agrobacterium* interference, which establish this technique as the most efficient for gene delivery. Ozias-Akins et al. (1993) recorded as high as 12% of transformation frequency when peanut embryogenic calli were bombarded with 35S *gus* gene to develop genetically-transformed peanut plants. An approach to develop productive peanut plants was reported by Schnall and Weissinger (1993), employing zygotic embryonic axes in highly concentrated agar medium. Bombardment of juvenile cotyledon-derived somatic embryos of peanut with codon regulated *cry1Ac* accompanied by hpt antibiotic marker resulted in transgenic peanut which exhibited resistance to cornstalk borer, having an effectiveness of 0.85–2.3/bombardment attempts (Singsit et al. 1997). While the microprojectile method offers several benefits, simultaneously it carries some obvious constraints. The rate of transformation is a controlling feature and gene incorporation is a chance factor and the course of gene transfer itself could trigger DNA injury in the process of transformation. This procedure frequently induces insertion of transgenes into the target genome in manifold copies; therefore, resulting in uncontrolled or suppressed gene expression (Livingstone et al. 2005; Singsit et al. 1997). In addition to these reports, the approach is extremely expensive and the skill is difficult to acquire by researchers intending to develop transgenic peanuts.

8.5.2 Enhanced Traits

Transgenic plant development could assist prospective gene insertion into plant cells and influence a number of advantageous characters linked to the advancement of crop plants. Genetic transformation mechanisms establish a route for integration of vital candidate genes within the peanut genome for improving endurance to biotic stress like insect pests, pathogenic fungi, viruses and abiotic stress like salinity and drought, in addition to suppressing unfavorable genes for the enhancement of nutrient uptake. To date, a number of genes activated in response to abiotic and biotic stress conditions, have been detected, successfully cloned and transformed. The transgenic stress-tolerant peanut varieties acquire the potential to be used as donor parents in conventional breeding approaches for developing superior varieties tolerant to biotic and abiotic stresses (Gantait and Mondal 2018).

8.5.2.1 Tolerance to Abiotic Stress: Drought and Salinity

In plants, reactions against abiotic stress include stimulation and synchronization of stress reactive genes and their arrangements that offer improved tolerance to dry spells, salinity, chilling stress along with many additional unfavorable environmental conditions via transformation of enzymes and proteins that regulate the production of galactinol, glycine betaine, mannitol, proline and trehalose, as well as polyamines (Krishna et al. 2015).

Peanut is grown predominantly in rainfed areas, wherein considerably hot weather with lengthy drought phase persists, resulting in restricted yield of peanut pods. Establishment of cultivars, tolerant to drought stress proved to be indispensable to alleviate the abiotic stress situations (Chu et al. 2008). As an example, *AtNHX1* (a vacuolar Na^+ /H^+ antiportar) overexpression resulted in enhanced tolerance in peanut versus salt and water stress environments by grouping of Na^+ ions in the vacuoles, as reported by Asif et al. (2011). Genetically transformed peanut plants exhibited better revival, chlorophyll capacity and recovered under PEG (polyethylene glycol) induced desiccation stress. Resistance against drought in peanut was developed via the transformation of *rd29A*:*AtDREB1A* that exhibited a 40% rise in transpiration competence in water-scarce situations when compared to the non-transformed plants. Peanut seeds expressing *rd29A*:*AtDREB1A*, displayed regular germination and progressive growth, whereas seeds expressing 35S:*AtDREB1A*, presented late germination with restricted plant growth (Bhatnagar-Mathur et al. 2007, 2014).

Two other analyses, Pandurangaiah et al. (2014) and Patil et al. (2014), developed genetically-transformed peanut with transcription factors like MuNAC4 and AtNAC2, which exhibited significant endurance against salinity and drought stress, while retaining superior crop production in reduced irrigation condition. The peroxisomal ascorbate peroxidase genes of the herb *Salicornia brachiate* exhibited tolerance to both drought and salinity in tobacco and it was validated in peanut (Singh et al. 2014). Osmoprotectants and osmolytes crucially function as shields for plant cells via scavenging free radicals. The osmoprotectant mannitol plays a significant part in scavenging hydroxyl radicals, produced during abiotic stresses. Bhauso et al. (2014) developed transgenic peanut (expressing *mtlD* gene) that showed tolerance against drought stress with superior relative water content in comparison to non-transformed plants. Ramu et al. (2016) via *Agrobacterium*-mediated (in planta) successfully introduced and expressed the *PDH45*, *Alfin1*, and *PgHSF4* genes originating from *Pisum sativum*, *Medicago satifa* and *Pennisetum glaucum*, respectively, and the resultant transgenic peanut plants exhibited a high degree of tolerance to drought stress. In the same year, Sarkar et al. (2016) developed a genetically transformed peanut that showed comparable level of tolerance in drought environment, following successful transformation with *AtDREB1A*. Recently, the development of transgenic peanut (transformed with *AtHDG11* gene originated from *Arabidopsis thaliana*), tolerant against both salinity and drought stress was reported by Banavath et al. (2018).

8.5.2.2 Enhancement of Biomass Production

Plant growth and development, and consequent production of plant biomass, are significantly influenced by phytohormones. Cytokinins, among these phytohormones, perform a crucial part in plant biomass production via mass proliferation of plant tissue. The definite tissue site for cytokinin biosynthesis is yet to be confirmed, but the location of high concentrations of cytokinin is considered as the putative site

for cytokinin synthesis that might be fluctuating for obvious reasons. In addition, apart from cell cycle activity, abiotic stress conditions may influence the cytokinin concentrations that reportedly decrease with leaf senescence (Noodén et al. 1990). Iso pentenyl transferase (IPT) is an enzyme involved in the pathway of cytokinin production and its expression in peanut improved endurance to drought stress (Qin et al. 2011). As described by Qin et al. (2011), the genetically-transformed plants that expressed IPT under the control of the drought inducible SARK promoter, exhibited sustainable and superior biomass production under drought conditions prevailing in a greenhouse and experienced around 60% increase in production under in vivo environments.

8.5.2.3 Resistance to Biotic Stress

Microorganisms, such as bacteria, fungi, and viruses, as well as various other pathogens, drastically affect peanut production and have proved to be vulnerable to these biotic stress factors as well. Amid these, *Aspergillus flavus*, *Cercospora* leaf spot, tomato spot wilt virus and white mold are the main agents that are extensively dispersed in the atmosphere and instigate acute infection in peanut and eventually curtail the production of peanut pods (Brar et al. 1994). Detection of particular genes, from wild relatives, which provide tolerance to stress, and utilization of those genes for the development of genetically-transformed plants that would express the tolerant genes, may be the best alternative for genetic improvement of peanut.

8.5.2.3.1 Resistance to Fungi

Pathogenic fungi drastically affect the production and quality of peanut via synthesis of aflatoxin, which was detected as the strong carcinogen, by *Aspergillus* species. Via the genetic transformation methods, multiple genes were transferred into the peanut genome to offer disease resistance caused by pathogenic fungi. Stilbene phytoalexins are produced in the peanut kernel when infested by pathogenic fungi, and these stilbene phytoalexins effectively hinder growth and formation of fungi and its spores. Hain et al. (1990) isolated the peanut stilbene synthase gene and expressed the same in tobacco that ensued resveratrol synthesis. On the other hand, hydrolytic enzymes like chitinases and glucanases disintegrate cell walls of fungi and stop the formation of fungal spores. The genes encoding these enzymes have proved to be the salient candidate genes for establishment of transgenic plants, and resistant to pathogenic fungi. As reported by Sundaresha et al. (2010), resistance against *Aspergillus flavus* and *Cercospora arachidicola* was developed in JL 24, ICGV 89104 and ICGV 86031 cultivars of peanut via overexpressing the tobacco glucanase gene. Earlier, Rohini and Rao (2001) observed the resistance activity of the tobacco chitinase gene against peanut leaf spot or tikka disease (*C. arachidicola*), which drastically reduces the pod yield of peanut. It was only in 2012, when Iqbal et al. (2012) detected suitable interrelationships between chitinase activity and

fungal resistance when they developed a transgenic peanut via overexpressing rice chitinase gene to develop resistance against *C. arachidicola*. Anuradha et al. (2008) integrated the *RsAFP-2* gene inside the peanut genome and the ensuing genetically-transformed plants exhibited superior response to severe late leaf spot disease, conjointly triggered by *C. arachidicola* and *Pheaoisariopsis personata*. A recent report (Kumar and Kirti 2015b) indicated the upregulation of a number of genes which occurred in the wild peanut *A. diogoi* when the plants were exposed to the late leaf spot pathogen. Immanent expression of *AdSgt1* gene in genetically-transformed peanut plants acquired via in planta transformation, exhibited improved resistance against late leaf spot pathogen.

8.5.2.3.2 Resistance to Viruses

To confer resistance against viral diseases, researchers engineered *coat protein* (CP) *mediated resistance* for several groups, showing a remarkable achievement thus far. In general, peanut bud necrosis virus (PBNV), peanut mottle virus (PMV), peanut stripe virus (PSTV), groundnut rosette virus (GRV), Indian peanut clump virus (IPCV), and tobacco streak virus (TSV) restrict peanut pod yield to a great extent (Reddy et al. 2002). According to Gonsalves and Slightom (1993), for significant reduction of the virus-triggered diseases, the CP genes proved their efficiency in several instances. Subsequently, Sharma and Anjaiah (2000) inserted the gene of IPCV-CP in peanut via *Agrobacterium*-intervention, wherein the acquired transgenic plants proved to be resistant against IPCV. Mehta et al. (2013) established transgenic peanut plants, genetically transformed with TSV-CP gene which displayed resistance until the T3 generation to PSND virus under ex vitro environments. In addition, the least signs of disease were exhibited by the transgenic lines, which signified their strong tolerance to infestation of TSV as well.

8.5.2.3.3 Resistance to Insect Pests

A huge obstacle that still persists is to deal with the insect pests of peanut. Crystal (*Cry*) genes obtained from *Bacillus thuringenesis* were extensively employed to acquire plant resistance against insects. *CryIA* gene was primarily inserted by Singsit et al. (1997) in the peanut genome resulting in enhanced proficiency in defense of cornstalk borer. Resistance to *Spodoptera litura* was observed in peanut by Tiwari et al. (2008) and Keshavareddy et al. (2013) through the expression of chimeric *Btcry1AcF* and artificial *Cry1EC* genes. Peanut embryonic axes and cotyledons explants were inoculated with a solution of K599 strain of *Agrobacterium rhizogenes*, carrying an artificial *Cry8Ea1* gene (that is effective against the larvae of *Holotrichia parallela*) and consequently expressed by Geng et al. (2012) in the roots of peanut, displayed significant insecticidal properties.

The details of gene transformation to develop peanut with the advanced/enhanced traits are listed in Table 8.1.

Table 8.1 List of genes, mode of their transformation and their use to enhance several traits of peanut

Name of the gene	Mode of gene transformation	Enhanced trait	References
Enhanced resistance to abiotic stress			
merA	Microprojectile bombardment	Mercury stress	Yang et al. (2003)
AtDREB1A	*Agrobacterium*-mediated	Drought stress	Bhatnagar-Mathur et al. (2007)
AtDREB1A	*Agrobacterium*-mediated	Drought stress	Vadez et al. (2007)
AtNHX1	*Agrobacterium*-mediated	Salinity and drought stress	Asif et al. (2011)
IPT	*Agrobacterium*-mediated	Drought stress	Qin et al. (2011)
AtNHX1	*Agrobacterium*-mediated	Salinity and drought stress	Banjara et al. (2012)
AtDREB1A	*Agrobacterium*-mediated	Drought stress	Vadez et al. (2013)
AtDREB1A	*Agrobacterium*-mediated	Drought stress	Bhatnagar-Mathur et al. (2014)
mtlD	*Agrobacterium*-mediated	Salinity and drought stress	Bhauso et al. (2014)
PDH_{45}	*Agrobacterium*-mediated (in planta)	Drought stress	Manjulatha et al. (2014)
AtNAC2 (*ANAC092*)	*Agrobacterium*-mediated (in planta)	Salinity and drought stress	Patil et al. (2014)
AtDREB2A, *AtHB7* and *AtABF3*	*Agrobacterium*-mediated	Salinity and drought stress	Pruthvi et al. (2014)
AtDREB1A	*Agrobacterium*-mediated	Salinity and drought stress	Sarkar et al. (2014)
SbpPAX	*Agrobacterium*-mediated	Salinity stress	Singh et al. (2014)
MuNAC4	*Agrobacterium*-mediated	Drought stress	Pandurangaiah et al. (2014)
SbASR-1	*Agrobacterium*-mediated	Salinity and drought stress	Tiwari et al. (2015)
Alfin1, *PgHSF4*, and *PDH45*	*Agrobacterium*-mediated (in planta)	Drought stress	Ramu et al. (2016)
AtDREB1A	*Agrobacterium*-mediated	Drought stress	Sarkar et al. (2016)
AtHDG11	*Agrobacterium*-mediated	Salinity and drought stress	Banavath et al. (2018)
Enhanced resistance to virus			
TPWVN	*Agrobacterium*-mediated	Tomato spotted wilt virus	Li et al. (1997)

(continued)

Table 8.1 (continued)

Name of the gene	Mode of gene transformation	Enhanced trait	References
TPWVN	Microprojectile bombardment	Tomato spotted wilt virus	Yang et al. (1998)
TPWVN	Microprojectile bombardment	Tomato spotted wilt virus	Magbanua et al. (2000)
CP	Microprojectile bombardment	Peanut stripe potyvirus	Higgins et al. (2004)
Enhanced resistance to fungi			
AGLUI	Microprojectile bombardment	*Sclerotinia minor*	Chenault et al. (2003)
RsAFP-2	*Agrobacterium*-mediated	*Cercospora arachidicola*, *Pheaoisariopsis personata*	Anuradha et al. (2008)
BjNPR1 and *Tfgd*	*Agrobacterium*-mediated (in planta)	*Aspergillus flavus*, *C. arachidicola*	Sundaresha et al. (2016)
SGT1	*Agrobacterium*-mediated (in planta)	Induces cell death and enhanced disease resistance	Kumar and Kirti (2015a)
Enhanced resistance to insects			
crylA(c)	Microprojectile bombardment	Lesser cornstalk borer	Singsit et al. (1997)
Barley oxalate oxidase gene	Microprojectile bombardment	*Sclerotinia minor*	Livingstone et al. (2005)
cry1EC	*Agrobacterium*-mediated	*Spodoptera litura*	Tiwari et al. (2008)
cry1AcF	*Agrobacterium*-mediated (in planta)	*Spodoptera litura*	Keshavareddy et al. (2013)

8.6 Mutation Breeding

The existing variation among cultivated types of peanuts decreased during domestication. The difference between the A and B genomes are too narrow to be considered to an extent as A1 and A2 genomes (Smartt and Stalker 1982). Hybridization between different species is difficult to evolve as they produce sterile progenies. Much of the domesticated peanut collections were assessed for resistance to the leaf spots and rusts (Holbrook and Stalker 2002) and although adequate levels of resistance were recognized, the high level of resistance is absent in the germplasm collection. Resistance has been detected in other members of the genus but not in *Arachis hypogaea.* Mutation played a key role in developing and identifying better lines for cultivation in peanuts. The International Peanut Genome Initiative (http://www.peanutbioscience.com; https://peanutbase.org/) provides a valuable platform to explore genomic resource; whereas, the genomics of peanut mutants are yet to be explored. With a genome size of 2890 Mbp (cultivated) and 1260 Mbp (diploid genome) there is still a significant gap and opportunities to improve the available resources in exploring coding, non-coding regions, transposable elements, SSRs and SNP marker variations in developing saturated maps.

8.6.1 Conventional Mutagenesis (Seeds)

Although chemical mutagens are convenient and easy to handle, irradiation treatments were found to be efficient in generating mutants. Peanut seeds were subjected to mutagens like EMS, gamma rays and multi particle radiation fields in generating variations. EMS-induced mutants derived from Zhonghua 16 showed narrow *pod width* (*PW*) trait. This narrow pod with mutant was found to differ in CAD and ACS expression when compared with its wild type during 20 DAF and 40 DAF in developing peg tissues (Wan et al. 2017).

Researchers performed a combination of methods to induce variation through mutagenesis. Mutant lines were generated and selected for favorable traits. In India, the Bhabha Atomic Research Centre (BARC) has released a series of varieties through mutagenesis and selection (Table 8.2). Hybridization of selected and released mutants was also practiced in generating new varieties. TLG-45 was developed through hybridization of mutant derivatives TG-17 and TG-1.

Seeds of VRI 2 peanut variety (developed by Tamil Nadu Agricultural University, India) were treated with gamma rays and EMS for selection of better performing lines from M4 and M5 generations. TFDRG-5 derived from progeny of TAG-24 × VG-9514 cross hybrid was irradiated with gamma rays, sodium azide and a treatment combination of both in developing mutants for better seed quality and seed coat color variations (Mondal et al. 2007).

Table 8.2 List of peanut varieties released in India by BARC

Variety	Year of release	Salient features
TG-47 (RARS T1)	2011	Large seed, 115 days maturity, more 3-seeded pods
TDG-39	2009	Large seed, medium maturity
TBG-39	2008	High oleic acid, more branches
TG-51	2008	Early maturity, high shelling %
TLG-45	2007	Large seed, medium maturity
TG-38	2006	High shelling %, more 3-seeded pods, stem rot tolerance
TG-37A	2004	High yield, collar rot, drought tolerance
TPG-41	2004	Large seed, medium maturity, high oleic acid
TG-26	1996	Earliness, high harvest index, 20 days seed dormancy
TKG-19A	1996	Large seed, 30 days seed dormancy
TG-22	1992	Medium large seed, 50 days seed dormancy
TAG-24	1992	Earliness, high yield, high harvest index, partitioning %
Somnath (TGS-1)	1991	Large seed, semi-runner type
TG-3	1987	High yield
TG-17	1985	No secondary branches, 30 days seed dormancy
TG-1	1973	High yield, large seed, more branches, 50 days seed dormancy

8.6.2 In Vitro Mutagenesis and Selection

In vitro mutagenesis has been carried out; however, no reports have appeared on somaclonal variation and in vitro selection. Mixed high energy particle field irradiated peanut variety Luhua11 embryonic leaflets were cultured in MS, supplemented with B5 vitamins and 3% sucrose fortified with 10 mg/L 2,4-D. Grafting of regenerated plantlets on root stock Huayu 23 resulted in mutants performing better for protein content, oil content, oleic, lenoleic and palmitic acids content (Wang et al. 2015a,b).

8.6.3 Molecular Analysis

Analyses of mutants for seed testa color revealed the presence of two sets of genes regulating testa color. Genes *F1F2* and *D1D2* were found to be responsible for testa pigmentation. If either of the genes is recessive the result is white testa (Mondal et al. 2007).

Jung et al. (2000) identified *ahFAD2A* and *ahFAD2B* genes as recessive genes segregating in 3:1 and 15:1, depending on the parental lines used in the crossing program. A reduced level of *ahFAD2A* transcript and the difference between high oleic and the normal cultivar is a mutant. Genotypes of normal oleic peanuts are designated as AABB and high oleate peanut as aabb. Later, this was revalidated by Fang et al. (2012) by cloning and sequencing of *FAD2B* from LF2 and E2-4-83-12 that led to the identification of a mutation causing H105Y substitution at C313T in coding region. In that experiment, parental line LF2 a high-yielding Virginia type, led to the development of mutant E2-4-83-12 through EMS treatment. Then these results were reflected by Janila et al. (2016) which established that both fatty acid mutant alleles were contributing to healthy oil and prolonged shelf life of peanut. These alleles are present in linkage group a09 and b09 controlling the composition of oil quality. Substitution of G::C to A::T in *ahFAD2A* and insertion of A in *ahFAD2B* created the phenotype of high oleic acid content. Competitive allele specific PCR was designed to test the presence of alleles. This led to the development of the allele specific CAPS marker (Zhao et al. 2017). There are multiple seed storage proteins that give rise to allergens with the major ones being *Arah1, Arah2* and *Arah3* (Burks et al. 1998). *Arah2* is most important in causing human allergens.

Although only two genes were reported so far in enhancing oil quality, in-depth network analyses of these two genes revealed the presence of efficient nodes in network rewiring. Analyses of model oil seed plant *Brassica* proteins revealed the presence of gene homologs for targeted mutagenesis through CRISPR technology or transgenics (Fig. 8.8).

Fig. 8.8 Network analyses of FAD through model oil seed *Brassica* proteins. (Diagram by J.K. Yasin)

8.6.4 Enhanced Traits and Improved Cultivars

Mutations were intentionally carried out through mutagen treatments with the objective of generating variations for selection of better performing lines. Although the targeted traits are of commercial importance and health benefits, it was ensured that the farmers benefitted from those impacts. Mutants of flavorunner 458 with elevated levels of oleic acid replaced its parental line Florunner and other TSMV susceptible cultivars in parts of Texas. Subsequent to its selection, Florman

occupied 80% of area in the 1990s. This improved parental line was used to develop 12 cultivars in production in the USA. The first high oleic cultivar released in the USA was a runner type named Sun Oleic 95R (Gorbet and Knauft 1997), since then that trait has been incorporated into Virginia and Spanish types (Simpson et al. 2003). Using Sun Oleic 95R as a parent in MABC, high oleic genotypes ICGV 06110, ICGV 06142 and ICGV06420 were developed in India (Varshney 2016).

Developing resistance is a rare phenomenon in crop plants. Available germplasm sources were found to have narrow genetic variations and hence induced variations for screening disease tolerance were attempted. *Aspergillus*-resistant mutant lines were developed by irradiating peanut varieties through gamma rays and somaclonal variations (Azzam and Khalifa 2016). Markers linked to *Aspergillus* were also developed. Early leaf spot, late leaf spot, rust and peanut rosette diseases cause up to 50% yield loss in peanut. Under moisture stress conditions *Aspergillus* infections increase and cause aflatoxin contamination of seeds. Terminal heat stress reduces the productivity and increases the chances of aflatoxin infection in the field. Rust is a major biotic stress of peanut causing about 50% yield loss. This is more prevalent in the Deccan Plateau of India. Introgression of rust resistant QTL from GPBD 4 in popular farmers' cultivars, namely ICGV 91114, JL 24 and TAG 24, resulted in rust-resistant peanut lines, IPAHM103 with one dominant marker and GM2079, GM1536, GM2301 with three codominant markers (Varshney 2016).

High-yielding peanut varieties in India were achieved by consistent and continuous development of new varieties. By cultivating mutant varieties, peanut productivity in major peanut producing states, such as Gujarat, Andhra Pradesh, Maharashtra, Karnataka, Orissa and Rajasthan, have significantly increased. A drought-tolerant variety, TG-37A for Gujarat and Rajasthan and bold-seeded mutant varieties like TPG-41 and TLG-45 are most preferred for their earliness, seed dormancy and superior productivity. Among the peanut varieties, TAG-24, TG-26, TG-37A in the normal seed class, and TKG-19A and TPG-41 in the large seed class are the prominent varieties in India (*http://www.barc.gov.in/pubaware/agri_social_inpact.html*).

8.7 Hybridization

8.7.1 Conventional Hybridization

Van der Stok (1910) described the first artificial hybridization of peanut. Generally, emasculation is done during late afternoon or evening before maturity. Pollination is carried out the next morning as soon as the stigma becomes receptive and the pollen forms (Fig. 8.9). However, the procedure being labor intensive, consequent low yield results and less hybrid seeds are produced. During the reproductive stage, development incompatibility is observed among *Arachis* species, which may be due to genetic or physiological barriers. However, the stigmatic surface of the pistil is

Fig. 8.9 Artificial hybridization of peanut (*Arachis hypogaea* L.). (**a**) Flower bud with lower lip of the calyx, wings, standard, and keel pulled back to expose anthers and stigma to remove all eight anthers surrounding the stigma, (**b**) Flower bud with keel, wings and banner returned to their natural positions, (**c**) Picking a male flower, removing wings and squeezing pollen onto the flat end of the tweezers, (**d**) Applying pollen onto the tip of the stigma of an emasculated female flower and then placement of keel and wings of the female flower back to their normal positions, (**e**) Marking the pollinated flower with a nylon thread, (**f**) Large-scale practice of artificial hybridization of peanut, (**g**) Harvesting the marked pods developed after hybridization, (**h**) Harvested pods and kernels. (Photos by S. Gantait)

the first barrier to interspecific hybridization. Varied stigmas are observed in the species of *Arachis* and section *Rhizomatosae* (Akromah 2001; Lu et al. 1990). The large globular stigmas of annual peanuts lack hairs, while in perennial peanuts, small stigmas contain hairs that make it difficult for the pollen grains to adhere. This was the reason behind the success of producing more seeds when selfed, than the perennials, at the time of experimenting with interspecific hybridization. Furthermore, Halward and Stalker (1987) suggested that an alternative cause of incompatibility may be due to delimited pollen tube growth after pollen germination. For example, 6*x* interspecific *A. hypogaea* hybrids, when crossed with the two diploid species *A. cardenasii* and *A. batizocoi*, hindered hybridization. The fertilization was delayed when *A. stenosperma* was crossed with *A. hypogaea* (Pattee and Stalker 1992). It took more time for pollen to germinate when *A. hypogaea* was crossed with *A. glabrata*, this was due to blockage by callose plugs present in the pollen tubes (Shastri and Moss 1982). Crossing tetraploid species of *A. hypogaea* with diploid ones, resulted in a successful interspecific hybrid, since the embryo sac possessed a high amount of starch in the cultivated peanut (Pattee and Stalker 1991). It was observed that when *A. duranensis* was taken as a female parent for a cross between *A. duranensis* × *A. hypogaea* and × *A. stenosperma*, it led to embryo abortion. This may be due to endosperm degradation (Pattee and Stalker 1992). Moreover, when the species of *Arachis* was crossed with section *Erectoide*, the resultant plant grew with a normal peg and pod, without any endosperm

development (Singh 1998). Hormonal imbalances, or instance, a rise in abscisic acid (ABA) level and decline in gibberellic acid, zeatin riboside, indole acetic acid and dihydrozeatin riboside levels in normally-developing tissue, resulted in embryo abortion, as reported by Wang et al. (2012).

8.7.2 *Somatic Cell Hybridization*

In vitro culture techniques include protoplast fusion, haploidy, and *Agrobacterium*-mediated gene transfer, in planta techniques and somaclonal variation that have the potency to improve the crop. In the protoplast fusion technique, cells are isolated prior to the protoplast formation. Cell membranes are removed by the enzymatic plus osmotic stabilizer or by physical means. There are various means of protoplast fusion: (1) chemically induced ($NaNO_3$ treatment, application of high pH and high Ca^{+2} ion treatment and the most accepted polyethylene treatment) and (2) the electrical fusion method. The protoplast fusion technique is one of the ideal experimental systems; still it has not so much been applied in peanut. However, one case of protoplast fusion method was applied in *Arachis paraguariensis*, where protoplasts were isolated from leaf-derived callus, embedded in agarose blocks and cocultured with their own nurse cells (Li et al. 1993). The success in cultivated peanut is rather limited.

8.8 Conclusions and Prospects

Since peanut is not native to India, breeding efforts were initiated by introducing the popular varieties under cultivation from other countries. The then Madras state government, towards the end of nineteenth century, made purposeful introductions of improved peanut varieties. These varieties gradually spread over various parts of the country and formed the basis of peanut improvement programs and research in India. Since then the methods of breeding, common to self-pollinated crops, like introduction, mass and pure line selection, hybridization and selection (pedigree, modified and bulk pedigree, single seed descent, back cross methods), mutation breeding and interspecific hybridization were employed in developing 211 cultivars spread over more than a century (Table 8.3). A few novel breeding approaches like multiline, synthetics, modified composite cross, convergent crosses and selective diallel mating system have also been attempted but with limited success.

A high degree of sterility was encountered among interspecific crosses with compatible wild species of section *Arachis*, cross-incompatibility between cultivated and the wild species of sections other than *Arachis.* Very low seed set in F_1s coupled with high frequency of aneuploid individuals in the subsequent segregating generations and the consequent time lag in developing varieties with acceptable pod and seed characters have restricting genetic enhancement of peanut. In spite of these

Table 8.3 Breeding methods employed in developing peanut varieties in India

Breeding method	No. of varieties released
Introduction	3
Mass selection	29
Pure line selection	23
Pedigree	109
Bulk pedigree	22
Modified bulk pedigree	16
Single seed decent	1
Mutation	8
Total	211

drawbacks, peanut breeding and research have contributed substantially to overcome the challenges of yield-limiting abiotic and biotic stress factors, ensuring a momentous genetic improvement over recent decades. Superior peanut varieties with early maturity and short-term dormancy have been developed to fulfill farmers' requirements, with the aid of traditional breeding methods. However, this came with some undeniable limitations as well. Peanut being a self-pollinated species, conventional breeding approaches were adopted by scientists to upgrade these attributes (earliness and short-term dormancy), into improved varieties of peanut.

Owing to the inaccessibility of descriptive traits and their evolutionary background, breeders rarely make use of the wide-ranging, genetically-divergent accessions in peanut breeding programs. As a solution, mini-core collections and core collections were generated for the attributes such as early maturity, nutritive value, resistance against biotic/abiotic stress, etc. For promotion in commercial cultivation, genotypes selected from core/mini-core collections were exploited for the development of several promising varieties with high-yield. Only a limited number of genotypes are undergoing recurrent exploitation to develop improved varieties with attributes like early maturity and disease resistance; however, a more efficient utilization of genotypes belonging to the core/mini-core collections is needed for a comprehensive exploitation of such huge genetic resources.

Accelerated improvement in peanut genomics has been documented in the last 10–15 years in such a way that a large number of markers, such as, AFLP, RFLP, RAPD, and SSR including EST and SNP, have been developed that enriched peanut genomic resources. Such reproducible markers have fruitfully assessed the relations between traits and markers, the magnitude of genetic diversity, as well as being effectively utilized in genetic linkage map construction. Nevertheless, peanut breeders have identified important inadequacies in the genetic linkage map, since the polymorphic alleles in intraspecific crosses could not be identified by the RFLP loci positioned on the map. As a consequence, there was an exigent requirement to generate more PCR-based genetic linkage maps, because merely a small number of sporadically spaced AFLP- and SSR-based genetic maps were described for intra-

specific crosses in peanut thus far. Correspondingly, there is a necessity to furnish the preexistent genetic maps employing additional PCR-based markers. Substantial attempts have also been made to develop novel SSR markers; however, bulk generation of EST, RGA, and SNP markers would currently be of considerable significance.

Exhaustive selection of segregating populations are necessary for the breeding of peanut varieties with superior nutritive value and stress-resistant traits, and this process is challenging because it involves enormous genetic resources. MAS offers a technique to detect the concerned gene and assist in appropriate gene pyramiding of agronomically-improved genotypes. Identification of genes that express resistance to the key biotic and abiotic stress agents has already been accomplished. Via the intervention of genetic transformation technology, improved genotypes, with inherent resistance were developed. Nevertheless, it could be suggested that a comprehensive tactic, collaborating the traditional, mutation-based, MAS-based, and genetic transformation-based approaches, might deliver an inherent, cost-effective, as well as eco-friendly solution, following the requirements and preferences of consumers by diminishing the escalating risk and expense of peanut production.

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Appendices

Appendix I: Specialized Research Institutes on Peanut and Their Online Resources

Institution	Specialization Research Activities	Contact information and website
ICAR - Directorate of Groundnut Research (ICAR-DGR)	Conduct basic and strategic research to enhance production, productivity and quality of peanut; act as the national repository of working collection of peanut germplasm and information on peanut research; establish relevant institutional linkages; offer consultancy and training; and provide logistic support and coordination mechanism for generation of location-specific technology through the All India Coordinated Research Project on Groundnut.	www.dgr.org.in

(continued)

Institution	Specialization Research Activities	Contact information and website
International Crops Research Institute for the Semi-Arid Tropics	Perform fundamental and strategic research for the enhancement of peanut production, productivity and quality; act as an international repository of working collection of peanut germplasms; create pertinent institutional linkages; offer consultancy and trainings	http://www.icrisat.org
The Peanut Institute	It is a non-profit organization that supports nutrition research and develops educational programs to encourage healthful lifestyles that include peanuts and peanut products.	http://www.peanut-institute.org
Peanut Lab, College of Agricultural and Environmental Sciences, University of Georgia	Improvement of peanut production through the development and use of advanced genetic and genomic technologies and better crop protection measures	http://www.caes.uga.edud
Oil Crops Research Institute of Chinese Academy of Agricultural Sciences, Key Laboratory of Oil Crop Biology of the Ministry of Agriculture, Wuhan, China	OCRI has peanut as one of its major mandate crop with a mission to conduct basic and applied research that can lead to enhance the productivity and utilization of peanut. The current research fields cover germplasm, genetics, breeding, functional genomic, genetic engineering, safety assessment of genetic modified organisms (GMOS), plant nutrition and physiology, plant pathology, chemical analysis, food safety and product processing.	http://en.oilcrops.com.cn
Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, India	Development of new varieties of peanut using radiation-induced mutagenesis and hybridization, execution of basic and applied research for the enhancement of peanut production, productivity and quality	http://www.barc.gov.in/randd/hfa.html
Department of Field Crops, Plant Science Institute, ARO, Bet-Dagan, Israel	Introduction and breeding of peanut to expand the variety of products for farmers and consumers with improved yield and quality, development and application of agro-techniques to improve yield and quality traits of peanut, development of agricultural methodologies to accommodate climate change, food security and alternative energy sources, as well as training and education of next generation scientists in the above areas	http://www.agri.gov.il/en/units/institutes/1.aspx
Institut Sénégalais de Recherches Agricoles (ISRA)-CNRA, Bambey, Sénégal	Enhancement of peanut yield through the improvement and use of advanced genetic and genomic approaches and superior crop protection measures	http://www.isra.sn

(continued)

Institution	Specialization Research Activities	Contact information and website
USDA-ARS Wheat, Peanut and Other Field Crops Research Unit, Stillwater OK	Development of improved peanut cultivars and germplasm, evaluation of advanced breeding lines and current peanut varieties, integrated management of peanut diseases, and development of improved high-oleic peanut varieties	https://www.ars.usda.gov/plains-area/stillwater-ok/wheat-peanut-and-other-field-crops-research/
USDA-ARS, National Peanut Research Laboratory (NPRL), Dawson, GA	Developing strategies to identify useful genes in peanut and breeding high yielding peanut varieties and germplasm, enhancing the competitiveness of U.S. Peanuts and peanut-based cropping systems, as well as development of postharvest systems to assess and preserve peanut quality and safety	https://www.ars.usda.gov/southeast-area/dawson-ga/national-peanut-research-laboratory/

Appendix II: Genetic Resources of Peanut During the Last 10 Years

Cultivar	Important traits	Cultivation location
Habit group: Spanish Bunch		
GG-5 (Gujarat Groundnut-5)	Drought tolerant; leaflets stay green at maturity	Gujarat, India
Jawahar groundnut-3 (JGN-3)	Tolerant of drought, sucking pests and leaf spots (ELS, LLS)	Madhya Pradesh, India
R-9251	Early maturity; tolerant of PBND	Karnataka, India
Apoorva (R-8808)	Tolerant of LLS and PBND	Karnataka, Andhra Pradesh and Tamil Nadu, India
Phule Vyas (JL-220)	High oil content; early maturity	Maharashtra, India
ALR-3 (ALG-63)	Suitable for early sowing in south-west monsoon; tolerant of LLS and resistant to rust	Tamil Nadu, India
Co-3 (TNAU-256)	Tolerant of PBND	Tamil Nadu, India
VRI (Gn)-5	Resistant to rust and LLS	Tamil Nadu, India
Co (Gn)-4 (TNAU-269)	Resistant to rust and LLS; possesses high oil content	Tamil Nadu, India
GG-7 (J-38)	Early maturity; tolerant of LLS	Gujarat and southern Rajasthan, India
AK-159	Early maturity; possesses high oil content	Maharashtra and Madhya Pradesh, India

(continued)

Cultivar	Important traits	Cultivation location
GG-6	Early maturity; high shelling (73%)	Gujarat, India
TPG-41	Large seeds (>60 g/100 kernels); high O/L ratio (3.27), tolerant of rust; possesses fresh seed dormancy (up to 25 days)	Throughout India
TG-37A	Possesses fresh seed dormancy (up to 15 days); tolerant of collar rot, rust and LLS	Rajsthan, Uttar Pradesh, Punjab, Gujarat, Orissa, West Bengal, and NEH states, India
Vikas (GPBD-4)	Resistant to LLS and rust	Maharashtra, Karnataka, Andhra Pradesh, Tamil Nadu, Jharkhand, West Bengal, Orissa, and Assam, India
Prutha (Dh-86)	Semi-dwarf; high harvest index (HI); tolerant of LLS and sucking pests	Gujarat, southern Rajasthan, Maharashtra, Orissa and West Bengal, India
Kadiri-5	Tolerant of drought and leaf spots	Aandhra Pradesh, India
Kadiri-6 (K-1240)	Tolerant of leaf spots	Aandhra Pradesh and West Bengal, India
Pratap Mungphali-2 (ICUG-92195)	Tolerant of ELS, LLS and PBND and tolerant of *Spodoptera litura*, leaf miner and thrips	Rajasthan, India
GG-8 (J-53)	Tolerant of PBND, collar and stem rot diseases	Northern Maharashtra and Madhya Pradesh, India
TG-38B (TG-38)	Tolerant of stem rot	Orissa, West Bengal, Bihar and North Eastern states, India
Vasundhara (Dh-101)	Tolerant of stem rot, PBND, thrips and *Spodoptera litura*	West Bengal, Orissa, Jharkhand and Assam, India
Ratneshwar (LGN-1)	Tolerant of LLS, stem rot, rust and PBND	Maharashtra, India
TLG-45	Large seeded	Maharashtra, India
SG-99	Tolerant of PBND	Punjab, India
ICGV-91114	Early maturity; tolerant of rust and LLS and drought	Andhra Pradesh and Orissa, India
Phule Unap (JL-286)	Early maturity; tolerant of LLS, rust and stem rot, thrips, leaf miner and *Spodoptera litura*	Maharashtra, India
Abhaya (TPT-25)	Tolerant of early and mid-season moisture stress conditions; tolerant of LLS, sucking insects (jassids and thrips) and *Spodoptera litura*	Andhra Pradesh, India
TMV (Gn-13)	Tolerant of early and mid-season moisture stress conditions, tolerant of LLS, rust and PBND	Tamil Nadu, India
Kalahasti (TCGS-320)	Suitable for rabi situation for kalahasti malady endemic areas and also for kharif in North coastal and north Telengana of Andhra Pradesh; tolerant of PBND; resistant to jassids	Andhra Pradesh, India

(continued)

Cultivar	Important traits	Cultivation location
Narayani (TCGS-29)	Tolerant of mid-season moisture stress conditions	Andhra Pradesh, India
VL-Moongphali-1	Resistant to LLS and root rot diseases	Uttarakhand, India
TG-51	Tolerant of stem rot and root rot diseases	West Bengal, Orissa, Jharkhand and Assam, India
Ajeya (R-2001-3)	Resistant to PBND; drought tolerant; wider adaptability	Southern Maharashtra, Karnataka, Andhra Pradesh, and Tamil Nadu, India
VRI (Gn)-6	Tolerant of LLS, rust and PBND	Southern Maharashtra, Karnataka, Andhra Pradesh, and Tamil Nadu, India
Jawahar Groundnut-23 (JGN-23)	Tolerant of ELS, LLS and drought	Madhya Pradesh, India
Kadiri-9	Tolerant of early and end-of-season drought	Andhra Pradesh, India
Greeshma	Early maturity; tolerant of drought, high temperature and aflatoxin contamination	Andhra Pradesh, India
Vijetha (R-2001-2)	Resistant to PBND	West Bengal, Orissa, Jharkhand, Assam; southern Maharashtra, Karnataka, Andhra Pradesh, and Tamil Nadu, India
Girnar-3 (PBS-12160)	Tolerant of leaf miner and thrips	West Bengal, Orissa, and Manipur, India
Kadiri Haritandhra (K-1319)	Possesses fresh seed dormancy (up to 20 days); multiple diseases (rust, ELS, LLS, stem rot, PBND) and insect pests (thrips, *Spodoptera litura*, jassid, *Helicoverpa*) resistant	Karnataka and Maharashtra, India
GPBD-5	Tolerant of LLS and rust	Jharkhand and Manipur, India
JL-501	Early maturity	Gujarat and southern Rajasthan, India
RARS-T-1 and T-2	Bold seeded pods	Andhra Pradesh, India
Pratap Raj Mungphali	Early maturity; tolerant of ELS, LLS, PBND, jassids, thrips, leaf miner and *Spodoptera litura*	Andhra Pradesh, India
ICGV-00350	Resistant to LLS and rust; tolerant of stem rot	Tamil Nadu and Andhra Pradesh, India
GJG-31 (J 71)	Tolerant of stem rot	Gujarat, India
Habit Group: Virginia Runner		
DSG-1	Suitable for transitional zone for rain-fed conditions	Karnataka, India
GG-14 (JSP-28)	High oil content; tolerant of thrips, *Spodoptera litura* and leaf miner	Northern Rajasthan, Punjab, Haryana and Uttar Pradesh, India
Utkarsh (CSMG-9510)	Possesses fresh seed dormancy (up to 40–45 days)	Uttar Pradesh, Punjab and northern Rajasthan, India

(continued)

Cultivar	Important traits	Cultivation location
Durga (RG-382)	Suitable for sandy and loamy soils; resistant to jassids, leaf miner and thrips	Rajasthan, India
GG-16 (JSP-39)	Tolerant of PBND, root rot diseases, thrips, *Spodoptera litura* and leaf miner	Tamil Nadu, Andhra Pradesh, Kerala and southern Maharashtra, India
M-548	High protein content; bold kernel; tolerant of leaf spots and collar rot	Punjab, India
GJG-HPS-1 (JSP-HPS-44)	Bold kernels (76 g/100 kernels)	Gujarat, India
Divya (CSMG-2003-19)	Tolerant of PBND	Uttar Pradesh and Rajasthan, India
Raj Mungfali 1 (RG 510)	Bold kernels (76 g/100 kernels)	Rajasthan and Punjab, India
GJG-17 (JSP-48)	Tolerant of stem rot	Gujarat, India

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Chapter 9
Advances in Edible Pine Nut Trees (*Pinus* spp.) Breeding Strategies

Hanguo Zhang and Zhen Zhang

Abstract In response to the increasing demand for food and nutrition, there is an urgent need to improve the structure of grain production. The development and utilization of resources such as edible pine nuts will effectively alleviate the demand for grain crops. Edible pine nuts are an important food resources, are nutrient rich and may be a useful component and supplement of a healthy diet. In order to meet the growing demand for pine nuts, their economic value should be fully understood and existing wild pine nut resources should be sustainably managed to increase productivity. In order to achieve this, research into pine nut improvement and production should be strengthened; part of this process is the development of a selective breeding program. This chapter explains the collection, conservation, management, development and utilization of edible pine nut tree germplasm resources in a breeding strategy, and presents case studies where improvements to seed quality and pine nut production have been achieved.

Keywords Biotechnology · Conservation · Evaluation · Genetic improvement · Genetic resources

9.1 Introduction

Global human population is expected to reach 9 billion by 2050 and the Food and Agriculture Organization (FAO) believes that grain production will need to rise by 70% within the next 40 years to meet the associated increased demand for food. Thus, there is an urgent need to improve the structure of food production (Ronald 2014) to ensure an adequate supply of nutritious food.

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Advances in technology have contributed to the development of nuts as a healthy food source and appropriate methods of collection, conservation, management and utilization of these resources have been researched. Edible pine nut trees, which are an important food resource (Agrimi 2017; Nergiz and Donmez 2004; Pettenella et al. 2014), provide pine nuts that can effectively supplement diets. Pine nuts are considered to have high nutritional and health values and are featured in oriental and high-quality western cuisine (Kang et al. 2015; Park et al. 2013, 2016; Wang and Chen 2004; Xie et al. 2016).

Pine nut production is currently not meeting market demands and there are frequent seasonal availability issues, particularly prior to harvest, that drive high product prices. To meet the growing demand for pine nuts, it is important for research to engage with the pine nut industry to improve and increase stability of production, understand the economic value of the industry, ensure appropriate management of existing wild pine nut resources and improve the pine tree breeding process for enhanced seed production.

9.2 Distribution and Importance

9.2.1 Resource Distribution and Morphological Characteristics

Although theoretically all pine seeds (nuts) are edible, most are too small to harvest for food. Globally, there are more than 100 species of pine trees that produce sufficiently large seeds; of these, about 30 are suitable for consumption (Ren et al. 2006), including *Pinus koraiensis*, *P. pinea*, *P. sylvestris, P. cembra, P. edulis* and *P. monophylla*, and constitute the main sources of pine nut products in the world. These species have a wide global distribution, for example, *P. koraiensis* occurs in northeastern China, *P. pinea* and *P. sibirica* Du Tour in continental Europe, *P. edulis* in Canada, the USA and Mexico. There are broad morphological and ecological differences among the species, where plant height ranges from multi-stemmed bushes under 5 m (such as *P. pumila*) to trees with a main trunk and height of more than 30 m (such as *P. sibirica* and *P. koraiensis*) and habitat conditions range from barren and cold (−50 °C) in northeastern Siberia (*P. pumila*) to hot, semi-desert elevations over 1500 m in New Mexico, Nevada and Mexico and high plateau areas of 2000 m in China (*P. armandii*). Pine seed productivity varies among species, from up to 335 kg ha^{-1} for *P. coulteri*, to 100 kg ha^{-1} for *P. koraiensis* and 50–60 kg ha^{-1} for *P. sibirica*. Although some closely related species have similar biological and ecological characteristics, such as *P. sibirica* and *P. koraiensis*, they have morphological differences. For example, Mao et al. (2003) reported that compared *P. koraiensis*, *P. sibirica* seeds were narrower, the episperm was about two-thirds thinner, the stone

cell layer on the testa section was thinner and the protective ability was poorer. Furthermore, there were intraspecific variations in seed weight and nutrient composition depending on cultivation location, where *P. koraiensis* grown in the Baekdu Mountain in China had a higher 1000 seed weight, lower percent of shelling and lower oil quality than when grown in Lesser Khingan in China. The global distributions of the main edible pine nut tree species are shown in Table 9.1.

Table 9.1 Global distribution of the main species of edible pine nut trees (*Pinus* spp.)

Continent	Species	Distribution	Reference
Eurasia	*P. koraiensis* Sieb. et Zucc.	Far eastern Russia; northern Japan; north-central Korean Peninsula; Baekdu Mountain and Lesser Khingan in northeast China	Zheng (1983), Ma et al. (1992) and Lim (2012)
	P. armandii Franch.	Northwestern, south-central and southwestern China; 2000–3000 m elevation in north-central Taiwan; southern Japan	
	P. sylvestris (Loud.) Mayr	Russia (Europe and Siberia); northern Kazakhstan; northern Mongolia	
	P. pumila (Pall.) Regel	Japan; North Korea; northern Mongolia; east of the Ennis River in Siberia; Greater and Lesser Khingan in China	
	P. parviflora Sieb. et Zucc.	Japan (1300–1800 m elevation)	
	P. roxburghii Sarg.	India; Pakistan; Nepal	
	P. pinea L.	Low-elevation areas of Mediterranean, such as Italy, Spain, Portugal, France, Tunisia and Turkey	
	P. cembra L.	Alps and Carpathian Mountains of central European, including Poland, Switzerland, France, Italy, Austria, Germany, Ukraine and Romania, distributed at elevations of 1200–2300 m	
North America	*P. edulis* Engelm.	Semi-desert regions of USA, Canada and Mexico, mainly distributed at elevations of 1600–2400 m, and rarely occurring below 1400 m or over 3000 m	Mutke (2012)
	P. monophylla Torr. & Frém.	USA and northwestern Mexico, at elevations of 1200–2300 m, and rarely occurring below 950 m or over 2900 m	
	P. cembroides Zucc.	Western North America, from west Texas to southern Mexico, in areas of low precipitation (380–640 mm $yr.^{-1}$) at elevations of 1600–2400 m	
	P. sabineana Douglas ex D. Don	California, USA at about 1200 m elevation	
	P. coulteri D. Don	Southern California, USA and northern Baja California, Mexico at elevations of 200–2300 m	
	P. lambertiana Douglas	North American Pacific coast from Oregon to California and Baja California, Mexico	

9.2.2 Nutritional Value

Pine nuts are rich in fats, proteins and carbohydrates, and also contain trace elements (calcium, iron, zinc) and other micronutrients and vitamins and as such, are a highly nutritious, high value healthy foodstuff (Wolff et al. 2000). Pine seeds are generally rich in oil (fat) content; for example, *Pinus koraiensis* and *P. pinea* contain 67.89 and 44.90% fat, respectively. Pine nut oil is a high-grade edible oil and a raw material used in the pharmaceutical industry, since it contains high levels of unsaturated fatty acids, such as linoleic acid and pinolenic acid. Pine nuts are known to contain nutrients useful in the maintenance of brain health and have properties that prevent hypertension, lower blood lipids and glucose, and suppress allergic reactions. In addition to high oil content, pine nuts are rich in amino acids. A gram of protein of *P. koraiensis* or *P. pinea* pine nut contains 203 and 244 mg of essential human amino acids, respectively, and they are rich in glutamic acid that acts as a detoxifier and has positive effects on juvenile mental health and memory and language disorders caused by schizophrenia and cerebrovascular disorders. Micronutrients and trace elements are present in pine nuts, where content of calcium, zinc, iron, ascorbic acid (vitamin C), and riboflavin (vitamin B_2) per 100 g of *P. koraiensis* and *P. pinea* seed is 13.3 and 13.8 mg, 7.7 and 6.4 mg, 8.5 and 10.2 mg, 18.8 and 2.5 mg, and 0.41 and 0.28 mg, respectively. The fat content in pine nuts has been shown to be similar to that in walnuts and Brazilian *pine* and higher than that in hazelnuts (*Corylus* spp.), Siberian apricot (*Prunus sibirica*), yellowhorn (*Xanthoceras sorbifolia*) and African oil palm (*Elaeis guineensis*) (Fernandes et al. 2010; Nasri et al. 2005; Sathe et al. 2008; Savage 2001; Sharma et al. 2010; Venkatachalam and Sathe 2006; Zhang et al. 2015).

9.2.3 Market Value and Utilization

Nuts were one of the earliest human food sources and were an important symbol of the human civilization process; today, pine nuts are one of the most highly consumed nuts. Globally, commercial production of pre-processed pine seed is c. 55,000 mt, reaching about 65,000 mt in peak years, deriving from Russia (30,000 mt of *Pinus koraiensis* and *P. sylvestris*), China (20,000 mt of *P. koraiensis* and *P. armandii*), North Korea (3000 mt of *P. koraiensis*), Afghanistan and Pakistan (4000 mt of *P. roxburghii*), and Spain, Italy, Portugal and Turkey (2000 mt of *P. pinea*). When converted to processed pine nuts, the global production of only around 20,000 mt is substantially lower than the market demand. Previously, most studies of pine cultivation focused on forest species for timber, whereas recent studies have turned their focus to forests as sources of food, such as for pine seed production that mainly relies on natural, rather than commercially-planted forests. There has been a recent, gradual increase in plantation forestry for the production of pine seeds in China and Europe (Mutke et al. 2005).

Although pine nuts are one of the most expensive nuts on the market, prices are surprisingly stable, since the limited supply and lack of alternatives creates a market that differs from other agricultural products. Although there are inter-year variations in supply of pine nuts, fluctuations in price tend to be low. For example, the market price of pine nuts exported by the US in 2000 and 2002 did not reflect the 88% increase in production since prices fell by only 8% (Ren et al. 2006).

As pine seed production fails to meet market demand, pine nuts tend to be seasonally available, with periods of a lack of global stocks generally occurring a few months prior to harvest that inflates prices. For example, in the United States, the market price 2002 ranged between 20 and USD 35 kg^{-1} and the retail price of pine nut oil was USD 70–140 L^{-1}, or higher when used in the medicinal and food additive industries. Social consumer demand and purchasing power continues to drive demand for pine seeds.

In order to meet this growing demand, research is required to fully understand the economics of pine seed production with the goal of increasing and stabilizing productivity, appropriately managing existing wild pine nut resources, and improving selective breeding programs. In general, studies should focus on the use of superior natural resources for breeding and cultivation, pruning management of tree crowns, and water and fertilizer management.

9.3 Genetic Resources Conservation and Utilization

Previously, genetic improvement studies of pine trees were directed towards timber production. Some widely used pine trees have advantages of high output, such as *Pinus taeda* L. and *P. elliottii* Engelm. in the southeastern United States, *P. radiata* D. Don in Australia and New Zealand, *P. sylvestris* and *P. pinaster* Ait. in Europe. During the breeding process, metrics of multi-level genetic improvement are used to select genotypes with fast growth, good quality and strong adaptability for forestation. The directed, rapid-generation breeding using theoretical and technical systems are well established and have a proven track record in the development of commercial timber forestry around the world. The principal pine tree species in several countries have been bred for more than three generations to develop multiple targets, such as yield, quality and resistance improvement. For example, the genetic improvement of *P. taeda* in the United States, now in its fourth generation, has increased genetic gain from 10% in the first generation to 35% in the third generation, and the third generation of *P. radiata* has been completely improved in Australia and New Zealand, mainly through the use of a forward breeding strategy that increases genetic gain and maintains genetic diversity.

Modern forest tree breeding technology, which uses molecular and cell engineering at its core, has accelerated the process of selective breeding of new forest tree varieties with improved growth, quality and resistance properties. However, studies of collection and use of superior genetic resources of edible pine nut species and detailed research on their cultivation are relatively recent. Breeding of *Pinus*

koraiensis in its natural areas of distribution (northeast China, Far East Russia, Japan, North and South Korea) began in the 1960s and was essentially based on selection criteria for timber, such as growth and material improvement, crossbreeding, selection of seed families, management of flowering and fruiting, and asexual reproduction. However, selection of traits for pine trees for seed production is in its infancy, where forest seed resources and clones have been established in Northeast China. For example, *P. koraiensis* seed and superior tree progeny test nurseries have been developed to provide stratified support to seed collection from natural and managed forests and nearly 30 improved varieties with better seed quality and production have been put into commercial production (Zhang et al. 2015). Breeding of *P. armandii* was previously aimed at timber traits and recent preliminary research has focused on the variation and selection of fruit characters, grafting propagation, and construction of a seedling nursery, although the economic value of edible seed of *P. armandii* has not been confirmed. There are fewer studies on the edible properties of *P. pumila* and *P. sibirica*, probably because the seed is small and the distribution range is narrow. *Pinus koraiensis* has been the subject of selective breeding in Korea, where seedling genotype nurseries have led to varieties with high quality timber rather than edible seed traits. Although there are many edible pine nut species in the United States, and there is a long history of pine nut consumption, there is no dedicated edible pine nut tree breeding program. For instance, production of edible seed in *P. edulis* is combined with Christmas tree and ornamental tree cultivation.

There has been research on the breeding of *Pinus pinea* varieties for edible seeds in Spain and Portugal, where focus during the last few decades has been on genetic variation, provenance testing and fructification. In Russia, more attention has been directed towards pine nut production in *P. sibirica* (Mutke et al. 2005), where the current focus is on cultivation and selection of superior genotypes with improved seed yield. Since 1991, Russia has banned the logging of natural *P. sibirica* forests and determined that 70–90% of them are economically and ecologically important forests and has so designated one million hectares. The Altai Mountains in Russia is a center of *P. sibirica* cultivation, where nurseries contain 15 million selected genetically-superior seedlings and 4000 ha of forest are planted each year. The screening of high-yielding and large-fruited clones of *P. sibirica* resulted in a 24% increase that was achieved by high-branch grafting covering 70.5 ha, fruition to 150 kg per hectare. The research program of the former Soviet Union resulted in breeding varieties with higher branch fruition and yields of pine nuts and 180 trees were selected and grafted to create high-yielding clones.

In recent years, the breeding of edible pine nut tree has been paid increasing attention worldwide. However, some problems have emerged from previous studies. First of all, there was a lag in the breeding of pines with edible nuts. The commercial properties of edible pine nut trees are affected by yield, size and nutrient content. Therefore, it was necessary to increase the proportion of directional hybrid breeding and molecular-assisted breeding, and to develop genetic resources with special genetic traits and strong resistance. In addition, combining somatic embryogenesis technology was a very important new approach to meet the breeding and commer-

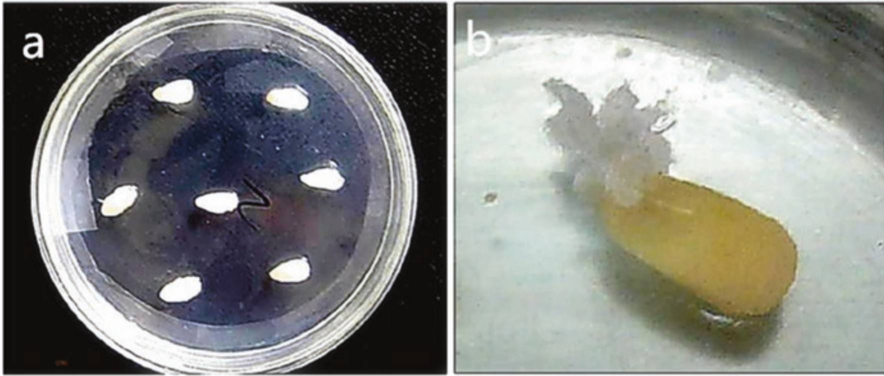

Fig. 9.1 The callus induction of *Pinus koraiensis*. (**a**) Immature embryos inoculated on induction medium for 1 day, (**b**) Callus induced after 30 days. (Source: Wang et al. 2009)

cialization of multi-variety forestry (MVF) (Find et al. 2014; Lelu-Walter et al. 2013). Embryonic callus was formed by callus culture in elite germplasm materials, then the elite germplasm materials were selected and preserved. Therefore, the induction of callus tissue, especially embryonal callus tissue, will be a leading issue in the study of conservation of edible pine nut tree germplasm resources. For example, Wang et al. (2009) used immature embryos of *Pinus koraiensis* to induce the callus, screened the suitable culture conditions and reported on the cryopreservation technology (Figs. 9.1, 9.2 and 9.3). Because many in vitro cultures of edible pine nut trees were still confined to the stage of experiment results, especially the regulatory mechanism of dedifferentiation and redifferentiation of isolated cells, the key gene regulation mechanism of somatic embryogenesis and the mechanism of variation in vitro culture still need further research (Klimaszewska et al. 2011). Similar to other pines, the technology of isolated pines to enhance the large-scale production process on the basis of successful laboratory culture needs to be further optimized.

9.4 Cultivation Challenges and Breeding Objectives

9.4.1 Problems Facing Pine Nut Production

Enhanced pine nut research is required to increase and stabilize production and is a key element of an effective research program to initiate tree improvement by breeding from appropriate stock. Zobel and Talbert (1984) clearly stated that *in most forest improvement, the largest, the cheapest and the quickest genetic gain is obtained through the selection of suitable seed production places*. If the individual edible pine nut trees that make up the base population are substandard, cultivated commercial varieties may show poor adaptability and growth leading to low yield and economic value.

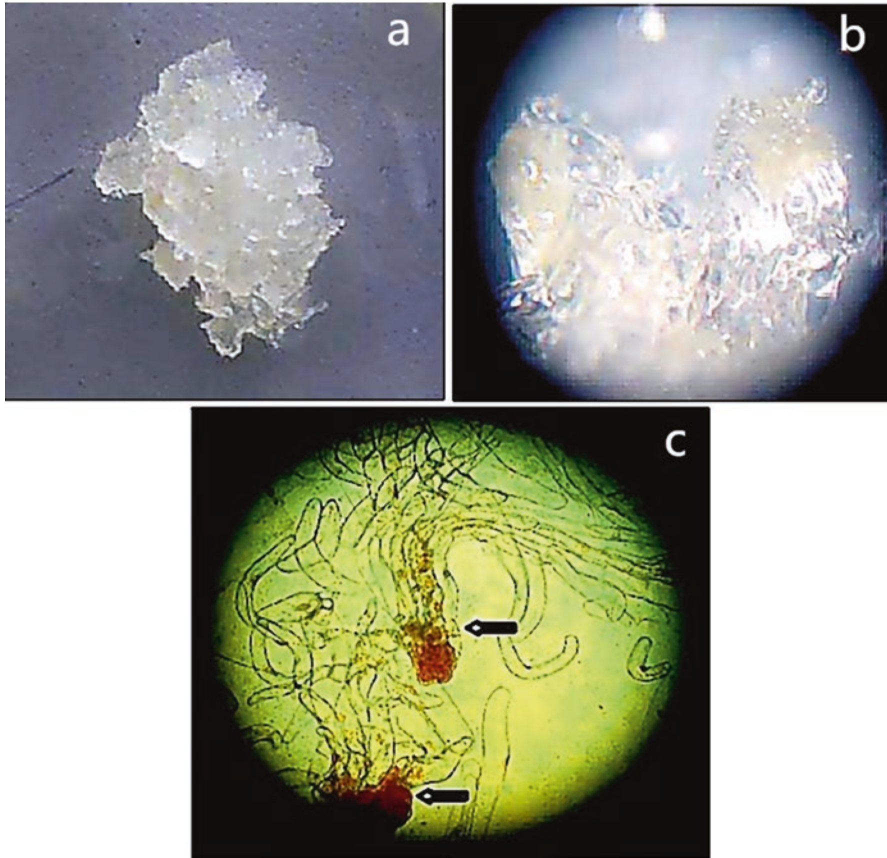

Fig. 9.2 *Pinus koraiensis* embryogenic callus. (**a**) Appearance, (**b**) Appearance under microscope, (**c**) Embryonal-suspensor mass (arrow). (Source: Wang et al. 2009)

9.4.2 Breeding Objectives

Forest tree breeding is a continuous and long-term process that includes selection, measurement and breeding of specimens to expand the population for allocation of commercial varieties and preservation of genetic resources. Therefore, breeding strategies should be based on a cycle that includes breeding, cultivation and selection of new varieties (Fig. 9.4).

The core activities in a breeding program are genotype selection and crossbreeding. These activities are carried out sequentially in each cycle so that elite genotypes are selected to form a breeding population from which individuals are selected for crossing to induce allelic recombination. The offspring produced by crossbreeding are planted to form a basic population ready for a subsequent breeding cycle and process of selection that increasingly produces genetic gain.

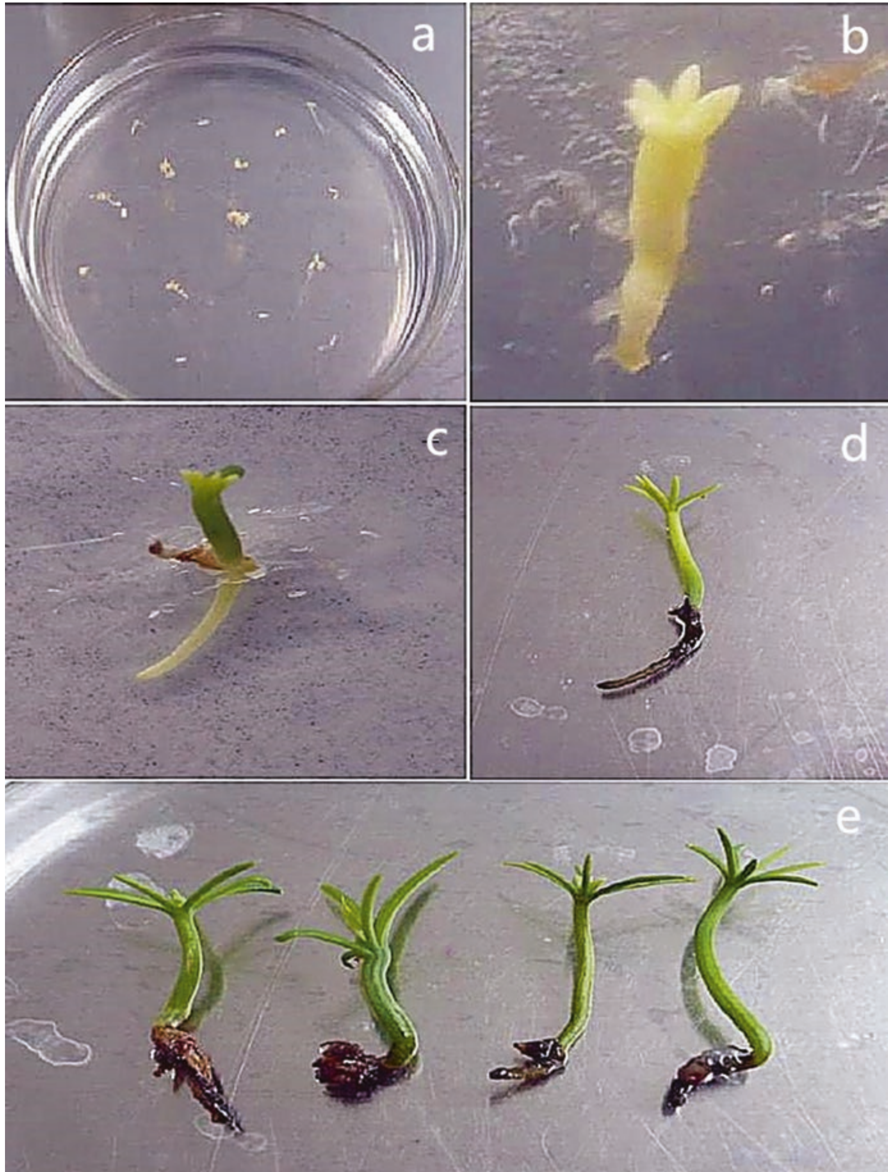

Fig. 9.3 Germination of somatic embryos on germination medium at different intervals. (**a**) 1, (**b**) 10, (**c**) 30, (**d**) 45, (**e**) 60 days. (Source: Wang et al. 2009)

Although there may be differences in the implementation of a program, such as population size and selection intensity, the steps of selection, hybridization, genetic determination and mass production of commercial varieties are the same.

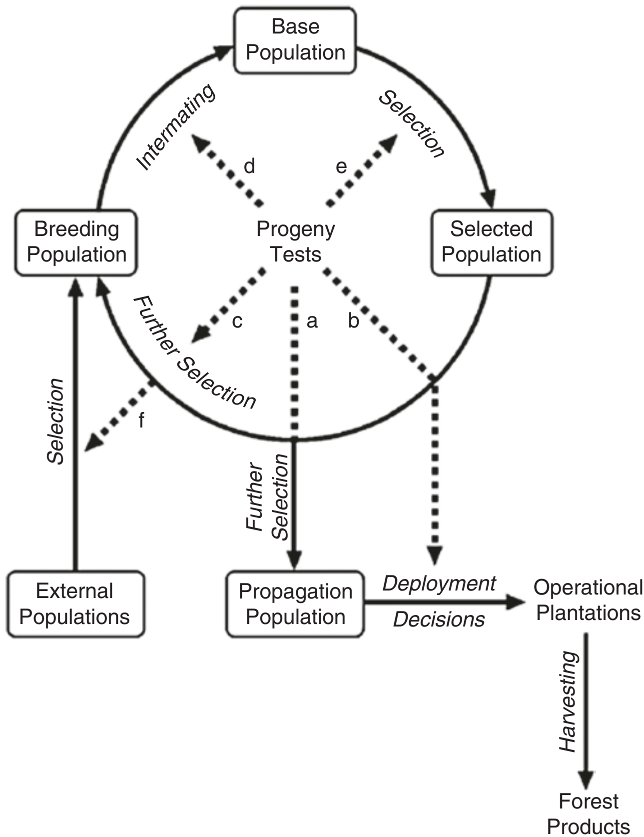

Fig. 9.4 Breeding cycle for improvements to forest tree species. (Source: White et al. 2007)

9.5 Conventional Breeding Approaches

9.5.1 Selective Breeding

9.5.1.1 Selection of Genetic Resource

Forest tree germplasm is the basic raw material to create new varieties and may be derived from propagative material of wild or cultivated, species and subspecies. The purpose of breeding improved in edible pine nut varieties is to cultivate those with high yield and high quality traits, where indicators in candidate genotypes include seed weight per plant, seed yield per 1000 grains and nutritional quality. The first step in the breeding process is to collect cuttings of trees with demonstrated high-quality traits and establish a cutting nursery for further cuttings or collection of seeds; the second step is to collect seeds to test for quality of generation and then select those offspring with high-quality traits.

In a comparison of multi-year data on seed yield of *Pinus koraiensis* from seed nurseries, managed forests, selected clones, the highest yields were from clones (Table 9.2). Therefore, it is necessary to combine seed yield and seed quality, insist on selecting the finest clones for a long period of time, carry out fine variety breeding and establish *P. koraiensis* nut orchards.

Zhu et al. (2006) studied seed traits of *Pinus armandii* and found annual variability in seed yield among provenances as well as yearly differences in seed yield of cones (Table 9.3). Relative seed stability was recorded among 95 clones and seed traits among 20 selected clones (Table 9.4). No correlation was recorded between seed yield and seed traits of clones; seed yield had no effect on seed quality. Therefore, in the selective breeding of *P. armandii*, seed yield as a selection index would not affect the quality of the seeds nor would it have great economic potential. We investigated the number of fruit and seed traits of 4316 individuals from 78 clones of *P. armandii* and determined that high yielding, high quality edible pine seed clones should be comprehensively evaluated using indicators of seed weight per plant, 1000 seed weight and seed production rate. This comprehensive selection index (CSI) method was used to screen out five high yielding, high quality edible pine nut clones.

Table 9.2 Comparison of seed yield of selected *Pinus koraiensis* clones

Type	Tree age (yrs)	Average seeds/plant	Maximum seeds/plant	Minimum seeds/plant	Age at first Fructification	Average seed yield (kg/ha)
Seed orchard	27	17.3	28.8	5.2	9	250
Fine clone	27	28.5	60	15	7	750
Artificial seed collecting forests	38	8.0	13.1	3	18	100

Table 9.3 Differences in *Pinus armandii* seed yield by Chinese provenance and year of fructification, 2001–2004

	2001		2002		2003		2004	
Provenance	Cones per plant	Number of plants	Cones per plant	Number of plants	Cones per plant	Number of plants	Cones per plant	Number of plants
Guizhou pingnbei	3.98	568	5.58	444	4.32	860	13.76	748
Yunnan chunxiong	4.56	1842	5.99	1406	6.68	1897	29.36	1719
Yunnan dongchuan	3.40	339	4.58	238	5.17	293	21.03	346
Sichuan huili	3.26	420	5.34	458	3.96	692	19.15	634
Yunnan baoshan	1.20	34	1.80	26	3.06	94	14.03	70
Mean	3.89	3203	5.64	2572	5.48	3936	23.15	3517

Table 9.4 Investigation on seed trait of different *Pinus armandii* clones

Strain code no.	1000 seed weight (g)	Number of seeds per fruit	Weight per fruit (g)	Satiation ratio (%)	Seed length (mm)	Seed width (mm)
8	409.45	172	70.29	15	14.1	9.2
19	327.15	82	26.83	28	12.9	8.73
21	342.25	105	35.82	9	13.6	8.9
22	327.45	117	38.31	7	12.7	9.1
23	272.9	94	25.65	5	11.9	8.8
24	307.65	145	44.71	4	13.4	8.4
25	336.85	87	29.42	25	13.6	9.0
32	399.85	141	56.38	23	14.7	9.0
34	386	128	49.40	10	14.4	8.9
35	198.7	176	52.67	8	12.1	8.6
36	305.25	178	54.23	8	12.8	8.3
38	309.45	127	39.20	6	12.1	8.9
40	391.9	111	43.50	15	14.1	9.1
41	446.1	110	49.07	10	15.3	9.3
42	537.5	119	63.78	4	13.6	9.0
45	323.75	122	39.50	15	12.8	8.8
48	319.55	104	33.23	9	12.6	8.5
49	347.5	137	47.68	13	14.2	9.0
84	275.51	53	14.51	22	11.8	8.5
98	351.85	132	46.33	15	12.8	9.8

9.5.1.2 Collection and Preservation of Tree Germplasm

Germplasm for the development of improved seed production should be collected from various regions and collections established for centralized management and according to use. For example, a temporary nursery is used to accommodate grafted cuttings for the construction of seed (nut) orchard, whereas a long-term collection is used to provide parents for crossbreeding and to cultivate subsequent generations as material for seed orchard construction. Here we describe the two types of collection areas.

A temporary nursery is a supply of tree scions for the construction by grafting of clones of asexually reproduced edible pine nut trees for seed orchards; this approach has been adopted by the *Pinus koraiensis* nursery in China that has existed for more than 10 years and is continuously updated as new varieties are selected. The quality of cuttings is a key factor that influences the survival rate of twig grafting, so grafted material tends to come from fresh local cuttings nurseries or healthy trees to reduce the effects of differences in growth trends and storage. Grafting survival rates using nursery material reach 75–96%, while that using healthy trees is only 50–70%.

Intensively managed, small-scale cuttings nurseries are preferred, since cuttings are of high quality and management conditions are good that lead to rapid growth of rootstock and early harvesting, and the efficient dense planting practice that produces large numbers of cuttings from a small area. For example, at the Maoer Mountain Experimental Forest Farm of the Northeast Forestry University in China, 50 selected clones of *P. koraiensis* were used to establish a cuttings nursery and each clone comprised 10–15 plants. The site was planted in the second year after grafting, and 5 years later, the seed orchard and breeding groups will be providing high-quality cuttings.

The basic methods of constructing a cuttings nursery, using *Pinus koraiensis* as an example, are as follows:

(a) Selection of cuttings: genotypes with high yield and high-quality fruit are selected following asexual reproduction and then cloned.
(b) Collection of cuttings: prior to sap flow, collect pest and disease free 1-year-old branches that measure 15–20 cm in length and exhibit strong growth with full head buds from the upper part of the crown of the fructification of cloned seed trees. Following removal, cuttings should be placed in a freezer plantlet vault.
(c) Grafted rootstock selection: select 4–5-year-old pest and disease free plantlets with strong growth, 20–30 cm in height that will be planted in a nursery site that has a gentle terrain, with a soil depth of 50–70 cm and a 20 cm-deep sandy loam topsoil containing 2–3% organic matter.
(d) Grafting time: Once rootstock sap begins to flow and the air temperature reaches at least 15 °C, grafting is done using the medullary heart-shaped layer splicing or splicing method.
(e) Nursery construction: the site should be gently sloping, with a free-draining soil depth of 50–70 cm and a 20 cm-deep neutral or acidic sandy loam topsoil containing 2–3% organic matter soil. The soil should be deeply cultivated and a base fertilizer applied prior to planting rootstocks 0.4 cm in diameter and less than 25 cm long at 1 x 1.5-m spacing, in April.
(f) Management: ensure the cuttings remain weed-free by removing weeds 3–5 times throughout the year and apply farmyard manure or topdressing compound fertilizer annually to the cuttings, before July.
(g) Monitoring: around September–October, when the shoot growth ceases and terminal buds form, survival rates of grafted seedlings should be assessed.

A long-term collection provides grafted cuttings for the establishment of primary seed orchards and improved first generation seed or nut orchards for a longer period of time, parent plants for crossbreeding full-sib families, and second-generation clonal seed orchards. The long-term collection is also known as a breeding orchard for first generation breeding. Since the breeding orchard undertakes long-term breeding tasks, its construction method differs from the temporary nursery to facilitate long-term preservation, ease of management and use.

9.5.2 Hybridization

Hybridization refers to the mating (crossbreeding) of two parents of different genetic composition and is an effective method and major approach for the genetic improvement of plants. Its effectiveness lies in the fact that through sexual hybridization, gene recombination results in greater genetic variation and greater vigor. Intra- and interspecific hybridizations are carried out to produce new hybrids of edible pine nuts, screen for more adaptable hybrids, and create conditions for the expansion of planting scope in the future. Studies have shown that the hybridization of species from two different habitats may result in adaptation to the two environments from which the parents derived, and solve the problems associated with simple introductions. For example, in 40 years, Russia has rarely succeeded in the direct introduction of *Pinus sibirica* to Europe, since its performance in the new environment usually declines. Crossbreeding is an important part of conventional forest plant breeding, and has only recently been used in edible pine nut trees.

The main purpose of hybrid seed production is to produce hybrid combinations with heterosis (stronger) traits. Pollination is often difficult in tall pine trees and the success rate is greatly affected by florescence that is influenced by climatic factors (Politov et al. 1999). Prior to using pollinating pine trees in a breeding program, the flowering characteristics of the tree species must be understood. Edible pine nut trees are monoecious, where female flowers (cones) are located in the upper part of the crown, some are born at the top of the crown (such as *Pinus koraiensis*), but most are located at the top of the trunk or on new branches of the main branch, whereas male cones are located in the middle and lower part of the crown. Long-term observation of the flowering period of the tree species is required to define the receptive period of the female cone and the period of pollen dispersal from the male cone. In general, a mating program is designed based on bagged female cone clones and collected pollen clones (Petrova et al. 2008).

Using *Pinus koraiensis* as an example for the determination and selection of new hybrid germplasm in edible pine nuts in China, we note there have been few studies on hybrid breeding. In 2014, the double-column hybridization of *P. koraiensis* was carried out in the *P. koraiensis* seed orchard in Amurensis and the Qingshan seed orchard in Linkou County in China (Fig. 9.5), where test material included parental selection after traits of tree growth, fructification and seed had been assessed in the previous year. After harvesting the hybrid cones in 2015, we analyzed the seed morphological traits, nutrients and pine nut amino acid content of the different hybridization combinations, and tested for variation among genetic parameters (Tables 9.5 and 9.6). The range in coefficient of variation (CV) of seed traits was 8.07–36.35% and the range in CV of 16 amino acid content of soluble protein from the pine nuts was 16.58–48.13%, the CV of essential amino acids was 29.37% (Mo et al. 2017).

Using the two-site, semi-biserial mating design of the *Pinus koraiensis* hybrid combination as test material, combining ability and heritability analysis showed that the heritability of seed traits, with the exception of hundred-grain weight and seed weight, were higher and genetically controlled (Table 9.6). The parents with

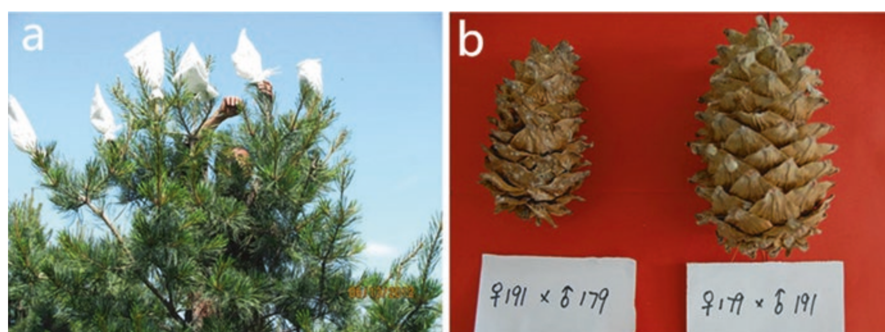

Fig. 9.5 The double-column hybridization of *Pinus koraiensis.* (**a**) Hybridization (bagging and pollination), (**b**) Cones (reciprocal cross)

Table 9.5 Analysis of variance of seed traits and nutritional components of *Pinus koraiensis* cultivated in two locations

	Weihe			Linkou			Total		
Trait	Mean	F-value	CV	Mean	F-value	CV	Mean	F-value	CV
Seed trait									
Cone weight (g)	115.22	**	30.00	92.31	**	34.19	101.69	**	34.10
Seed weight (g)	60.17	**	32.91	52.54	**	38.02	55.66	**	36.35
Seed length (mm)	16.41	**	9.06	15.14	**	10.50	15.71	**	10.65
Seed width (mm)	10.18	**	12.62	9.80	**	11.21	9.97	**	12.04
Length-width ratio	1.63	**	12.58	1.55	**	9.97	1.59	**	11.68
Kernel percent (%)	31.74	**	18.46	35.35	**	14.80	33.86	**	16.64
Hundred-grain weight (%)	51.94	**	19.31	48.11	**	25.22	49.97	**	22.83
Seed weight (%)	0.1738	**	25.78	0.1777	**	31.11	0.1759	**	28.79
Fat (%)	57.12	**	9.10	59.27	**	6.76	58.28	**	8.07
Nutritional Component									
Protein (%)	9.77	**	19.54	9.21	**	30.61	9.47	**	25.91
Polysaccharide (%)	9.66	**	32.40	10.27	**	29.56	9.99	**	30.89
Moisture (%)	4.21	**	20.61	3.71	**	25.58	3.94	**	23.99
Ash (%)	2.24	**	30.82	2.43	**	23.66	2.34	**	27.12

CV: coefficient of variation (%). ** significant at $P < 0.01$, * significant at $P < 0.05$.

Table 9.6 Estimation of heritability of various characters of *Pinus koraiensis* in Weihe and Linkou (%)

Location	Hundred-grain weight	Seed weight	Polysaccharide	Fat	Protein	Essential amino acids	Total amino acids
Weihe	55.78	61.99	75.23	70.27	76.76	50.27	69.45
Linkou	44.36	57.35	55.36	67.24	35.8	61.73	62.66

higher general combining ability (GCA) were hybrid combinations 042 and 79–9 and could be used as materials for the establishment of nut orchards; the hybrid combinations with higher specific combining ability (SCA) were 144 x 011, 79–9 x 10, 79–9 x 17, that could be used as a special serial number to provide materials for the establishment of nut orchards.

9.5.3 Clonal Breeding

Vegetative propagation or reproduction has the ability of maintaining parent characteristics and stability of reproduction that will enable the seed of forest trees to achieve higher genetic gain. Selection of edible pine nut clones focuses on variation in seed traits and biochemical composition, especially seed kernel size and type and content of oils, in individuals; therefore, selection of clonal propagated individuals with superior traits drives high quality in nuts. There are various methods of vegetative propagation; the most widely used are grafting, which is used to provide clonal grafted seedlings for establishing orchards, and cutting techniques that are used to provide afforestation seedlings for the construction of clonal forests. At present, the edible pine nut is mainly cultivated using grafted seedlings, because variation between individuals of the same genotype is small, leading to higher genetic gain than sexual reproduction.

In the 1980s, a large-scale, first-generation orchard was established using grafts to create seed orchards. Coniferous species such as *Pinus koraiensis* and *P. armandii* are more difficult to graft and cottage than broadleaf species and their survival rate is low. Following years of research, the survival rate of grafting is now greater than 80% and good results have been achieved in the large-scale establishment of the orchard.

9.5.3.1 Collection of Hardwood Scion

(a) The time to collect hardwood scion by cutting is before sap begins to flow and for *Pinus koraiensis*, this is usually from the middle to the end of March. Seed trees should be selected with good growth traits and strong fruiting ability. Practice has showed that graft survival rates are higher when scions are collected late, but before sap flow.
(b) Scion stripping site is selected from a robust, 1-year old peripheral branch of the upper part of the crown, usually the second to third branch up the trunk, and should measure c. 8 mm thick. In order to protect the seed tree for future scion strippings, only a small number of branches should be stripped at one time. The scions should be bundled separately from each plant for storage.
(c) Scions should be stored in a sealed container that is placed on a 50 cm ice block. The temperature in the cellar should be maintained above −6 °C.

(d) One day before grafting, the scion should be removed from the storage container, ensuring it is free from damage, pests and disease, and then cut 8–10 from the base of the terminal bud ready for grafting.

9.5.3.2 Grafting

In general, there are three types of pine nut tree grafting techniques: hard-branch, twig and bud that include techniques such as chipped scion or chipped rootstock. Methods of grafting vary and, using *Pinus armandii* as an example, three grafting methods are described below.

Hard-Branch Grafting Hard-branch grafting involves grafting to branches that are already lignified and have dormant terminal buds on the shoots. We used 3–4-year old *Pinus armandii* saplings that had an average height of 80 cm and DBH of 2–3 cm as rootstocks and collected 480 scions from 120 selected trees using between 5 and 10 strains of grafted trees. Hard-branch grafting has three procedures. The first is medullary cambium grafting which involves cutting branchlets 8–9 cm long from the branch of the scion and cutting the scion halfway (ideally 2/5) through the medullary at 1.5–2 cm from the tip of the terminal bud. Bark is cut from the top down to the main branch of the rootstock through the phloem and the xylem, exposing the layer, and the cut surface of the scion is aligned in all planes and attached to the cut surface of the rootstock. Second, side grafting involves cutting the scion to 8–10 cm in length obliquely from the base of the terminal bud to the medullary heart, and then longitudinally through the medullary heart to the base of the terminal bud. One-half of the scion is chipped-off and a strip of bark is removed from the entire length. At a point 10 cm above the base of the rootstock, a downwards incision is made from the cortex to the medullary heart to meet the longitudinal incision. The blade that is split into halves, one at the medullary heart and the other upper half is connected to the branch, with a 6–8 cm long incision. The medullary section of the scion has an inwards cut, exposing the inner layer, and the cut scion is inserted into the rootstock, ensuring alignment in both planes, and fastened with plastic tape from bottom to top. The third approach is cleft grafting, where the top 2–2.5 cm of the bud on the scion is cut into a 2–3 cm wedge shape on both sides. The main branch of the rootstock is cut at more than 10 cm from ground level to make a smooth, longitudinal 2–3 cm incision from the medullary heart. The cut scion is inserted into the incision, aligning in both planes, and tied with a plastic band from the bottom to the top that is subsequently loosened after 60 days. Normal scion growth indicates successful grafting.

Twig Grafting Scions used for twig grafting at the young-spike stage, which may be semi-lignified or not yet lignified, where annual shoots of semi-lignified (needle length 5–6 cm) or non-lignified (needle leaf sheath extends 1–2 cm) are used. It is thought that twig grafting is more effective than hard-branch grafting for species such as *Pinus elliottii*, *P. pumila* and *P. armandii*. Twig grafting adopts methods

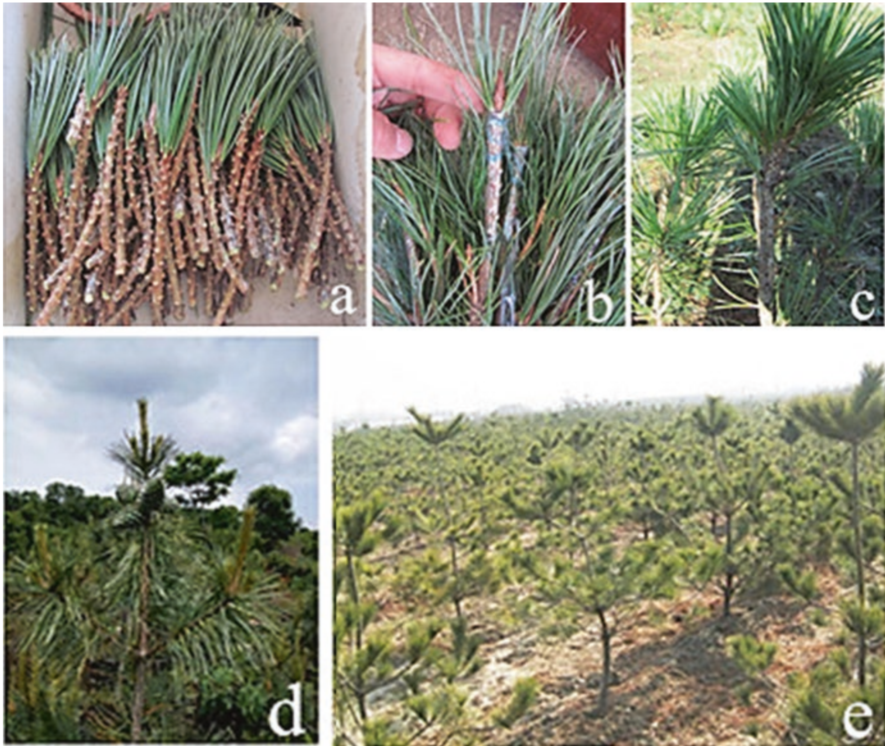

Fig. 9.6 Using *Pinus armandii* as an example, Twig grafting (notch grafting methods and here). (**a**) Scions, (**b**) Notch grafting methods, (**c**) Grafts take, (**d**) Produce cones and (**e**) Seed orchard establishment and into production

used in the medullary cambium cutting, abutting joint, picker abutting joint, cleft grafting and notch grafting methods. Here, we describe the cut-grafting method. Collect 4–5-year old *P. armandii* saplings with an average height of 2–3 m and DBH of 3–8 cm as rootstock, 260 twigs of *P. armandii* for scions, from which 3–4-cm length shoots should be cut. Next, a wedge shape of 1–2 cm in length is cut 2 cm from the base of the main bud and then a vertical cut of 2/3 s diameter of the section is made to a depth of 1–2 cm. Cut the wedge-shaped section of the scion lightly and straight down into the longitudinal incision of the rootstock, ensuring alignment in both planes, and then cover with rubber bands. We have recorded a 70% success rate using this technique (Fig. 9.6).

The average survival rate of twig grafts of *Pinus armandii* is 70%, which is 19.15% higher than the average survival rate of hard-branch grafts, which is 50.85%; the highest survival rate of medullary cambium is 83.8% (Table 9.7).

Bud Grafting There are two kinds of shoots, one is a winter bud and the other is an elongation bud. The first is a hard-branch bud and the latter is an elongation bud

Table 9.7 Different grafting methods for *Pinus armandii* and associated survival rates

	Grafting method	Grafting time (months)	Number of grafted plants	Number of successfully grafted plants	Fructification rate (%)
Hard-branch grafting	Medullary cambium abutting joint	02~03	80	67	83.8
	Lateral cleft grafting	02~03	80	54	67.5
	Medullary abutting joint	02~03	80	38	47.5
	Cambium abutting joint	02~03	80	39	48.8
	Cleft grafting	02~03	80	30	37.5
	Side grafting	02~03	80	16	20.0
Twig Grafting	Notch grafting methods	04	80	60	75.0
		05	80	52	65.0

formed in winter and spring. These types of bud are grafted onto the scion using the bud-grafting method. The elongation bud is a single bud shoot that has not yet formed an apical or lateral bud, or needle fascicles. There is no need to remove the needle fascicles when grafting, since the degree of lignification is low, rendering the grafting operation simple and highly efficient with a high survival rate. There are three approaches used in bud grafting. Horse-ear conjunction uses scion that are immersed in water and degreased for 1–3 days prior to grafting. A sturdy main bud or lateral bud is selected and the scion is cut to 3–6 cm in length and at the basal third, a 45–60° cut incision is made to create a horse-ear shaped section. Rootstock should be of a size similar to that of the scion and should be cut at 45–60° under the top shoots or lateral shoots and the graft should be sealed with binding tape. In side grafting, which is suitable for short shoots, spike diameters may differ. Since it is not necessary to cut off the rootstock, it may be reused many times, thus increasing propagation coefficient. At an appropriate height from the base of the rootstock, make a 4–5-cm incision at an angle of 30° from the cortex toward the medullary, then at the tip of the treated scion, make an incision (1–1.5 cm deep) at 30° and a 45–60° angle on the opposite side at the base of the scion to create a scalene triangle. Ensure alignment of the graft and apply binding. The third approach involves grafting needle buds from conifers with a cortex. Needles formed in the previous year may be grafted during the spring (March–April) while needles formed during the current year may be grafted in the summer (June–July). Selected needles should exhibit normal growth with healthy buds. The rootstock should be cut in cross-section, where the cortex is cut to form an ellipse with the needles (needle buds) that are embedded in the rootstock interface. Straps should be used to tightly secure the graft above and below the pine needle joints.

9.5.4 Seed Resources

The most effective way to increase production in the short term is to select high-yielding individuals and breed them asexually to create a seed orchard. It is believed that tree species such as *Pinus koraiensis* and *P. armandii* have high genetic variability in their fertility characteristics and some individuals have high yields, even after many years.

Creating orchard material is the core foundation for the establishment of a successful orchard, where selection of superior clones with high genetic gains is essential. In our experience first-generation seed orchard source material comprised superior tree clones that were not genetically determined according to their phenotypes; the one-generation reconstructed orchard was determined by offspring of the superior trees and selected from the original elite tree by backward selection and then panicles were cultivated to establish orchard clones. Second generation orchards were selected from superior forest trees to determine the highest quality individuals, and these were used to obtain hybrid combinations of seed to establish a full-sib progeny from which to cultivate clones. A third generation clonal seed orchard material is the product of the process from a phenotypic trait-preferred clone to create genetic assay-preferred clonality from which a hybrid-producing preferred clonal line is developed.

Superior orchard materials should demonstrate high genetic gain and comprise robust grafted seedlings with good fruiting traits. The seedlings represent excellent germplasm quality and seedling robustness since weak seedlings with superior germplasm are characterized by inconsistent growth that results in non-uniform, poor quality orchards. As the name suggests, the establishment of seed orchards is to produce seeds and the establishment of orchard clones must have good fruit-bearing traits, because reliable and consistent fruition has a direct impact on seed yield per unit area. In pine nuts, high volume growth stimulated by soil enrichment reduces flowering and seeding yield, therefore orchard establishment should have controlled fertilizer inputs.

Currently, seed gardens of edible pine nuts comprise second generation clones derived from first-generation clone seed orchard materials selected from superior individuals growing in high quality stands that are used to graft clone seedlings. Second generation parent material is selected from superior trees determined by progeny and hybridized to establish full-sib progeny from which superior individuals are selected and cultivated as cloned grafted seedlings. Seeds are collected from the superior trees to construct progeny forests for further cloning and cultivation of superior families and lines.

9.5.4.1 Seed Orchard Planting Scheme

The optimal number of clones (families) in a seed orchard is generally determined by the area and type of trees. Excessive quantities will lead to a decrease in the overall genetic gain, increase the workload of offspring determination and increase

costs. However, if the number is too small, genetic diversity will decrease, leading to an increase in rate of inbreeding and reduce seed quality. In addition, the pest and disease incidence in seed orchards is positively related to the level of genetic diversity. In general, 50–100 parents tend to be selected for 10–30 ha, 100–150 parents for 31–60 ha, and more than 150 parents for more than 60 ha. Since cloned pine nut orchards do not have problems of offspring traits, anamorphic coefficients may be reduced appropriately. Studies have shown that offspring of genetic diversity seed orchards do not decline significantly, but as there is a lack of consensus on rate of declines, genetic diversity must be taken into account when setting up an orchard (Kang and Lindgren 1998).

Clonal (family) configuration is one of the most important aspects of creating a seed orchard, because it not only directly affects genetic gain, but relates to inbreeding rate and seed composition that determine seed yield and quality. There are two traditional configuration methods: equivalent and truncated. In equivalent configuration, each parent is configured with the same number of plants that may increase the number of effective parents. For rapid generation seed orchards that are based on offspring measurements, equal allocation is not ideal because of the higher number of effective parents. Truncated configuration is the selection of parents with high-breeding values, but it may result in low fertility in selected materials. In order to overcome the disadvantages of these two configuration methods, Lindgren and Matheson (1986) introduced the concept of linear configuration, where selection of parents is based on breeding value and potential to obtain higher genetic gain to achieve an overarching balance of genetic parameters. Lindgren and Matheson proved that linear configuration approximates the best configuration when considering only outcrossing. If parental fertility is known, linear configuration may be corrected accordingly to optimize the effect of configuration.

The initial planting density of seed orchards affects the yield and quality of seeds; it is necessary to provide sufficient nutrient space to limit tree height to facilitate ease of seed harvest. It is important to maximize forest land use and create conditions for appropriate thinning and to reduce inbreeding, so it is recommended that the initial planting density of seed orchards should be two to three times that of mature forest tree density. Appropriate initial planting densities could be determined through research. Allocation of clones in the orchard should ensure heterogeneous distribution of parent types at equal spacings and standardized maintenance following thinning; a scientific research approach to orchard design will facilitate statistical analysis of data that will further inform robust, evidence-based orchard design and management.

9.5.4.2 Management

Production and operational activities of edible pine nuts are mainly concentrated in orchards. The forest tree seed orchard is also known as the *forest tree orchard garden* and agricultural technology is used in the intensive management of orchards that tend to be planted on steep mountainous slopes.

Many edible pine nut species, such as *Pinus koraiensis*, are managed as timber forests in the early growth stages, thereby constituting multi-function and multi-purpose tree species. Once an orchard is created, intensive production and management goals must be established to ensure the production of high-quality varieties, just as in horticulture, and soil nutrient status should be managed accordingly.

Management practices in tree seed orchards have only recently been developed. Managers use pruning techniques to manage and shape seed trees and to create forest tree orchards to facilitate fruit picking, improve production, and obtain high-quality and high-yield seeds. Edible pine nut trees are trees or shrubs, most of which are light-loving, and are characterized by apical dominance with cones mostly located in the upper part of the crown. To create an orchard for the purpose of harvesting seeds, tree height may be more than 10 m, otherwise the crown is large and disorderly, however, manual harvesting from coniferous trees is difficult and expensive. In order to overcome these difficulties, a number of strategies include maintaining a dwarf-tree body to limit the growth in height of a large number of flowering and strong adult female trees to about 6 m to allow convenient fruit picking; cultivating the tree to cup, open, round-head or ovary-conical shapes; update the fructification branches. In order to delay the expansion of the tree crown and update the fructification branches, the retracting method is used to retract the main shoots of the lateral branches that have grown in the current year. Retracting method is mainly to limit the expansion of new shoots to the expansion of the crown, while leaving a certain length of branches to promote its germination to produce new branches.

Pruning encourages pollination by increasing the amount and distribution of male and female flowers, reducing vegetative growth and promoting fruit growth. For example, there was a one-fold increase in the number of cones and an increase from 82 to 148 seeds in the cones of *Pinus koraiensis* where tree crowns had been removed compared with retained crowns. Generally, seeds from the mid-canopy have good plumpness and there are more seeds in cones growing on the windward side of trees than in the lee, due to increased wind-dispersed pollen and pollination. Studies of *P. armandii* clonal seed orchards have shown a significant difference in the quantity of fruit growing on all sides of trees, with southern orientations containing most fruit (average: 14.8), followed by westerly and northerly orientations (averages: 11.9 and 8.1, respectively). This is due to exposure to sunlight and the direction of the wind during pollen release. Cones are more likely to be distributed in the middle 3–7 branches of the tree, of which 75.07% will likely be pollinated, because upper branches develop later and do not coincide with phenology of pollination events, while the weaker lower branch growth results in smaller fruit. A study of flowering and seeding in *P. koraiensis* noted the number of cones was associated with position of the fructification branches and branch diameter. Spatial density of flowers for effective *P. koraiensis* pollination was found to be 66–200 flowers cm^{-2} to produce a fruit-set rate of 65–80% and when pollen density is 300–400 grains m^{-2}, the fructification rate is 85–90%. Therefore, it is recommended that management reduce the fructification layer, especially for the middle 4–6 branches; pruning is used to maintain stand density to increase cone yield.

As part of a structured management regime, soil fertility in managed orchard soils is maintained with phosphate and organic fertilizers; soil-poor orchard receive a large amount of organic fertilizer. Fertilization promotes tree growth and increases yield per unit area. In pine nut tree orchards, tree growth is directly proportional to the amount of flowering seed and the level of soil fertility. Analysis of soil nutrients in *Pinus armandii* seed orchards has found that when the Mn content was too high, toxins were produced that inhibited flower bud differentiation and reduced flowering. High B content is known to combine with toxic substances accumulated in the flower organs during reproductive growth, such as phytohormones, to form active substances that ameliorate toxicity and promote flowering. For the same clone, *P. armandii* planted in different seed orchards, the proportions of Fe, Mn, and B varies among soils in which *P. armandii* is planted and it has been found that there are beneficial effects of flowering and fruiting where Fe and Mn content is low and the B content is high. Excess Mn is controlled with the application of lime; however Ca in the lime inhibits absorption of B by the plant roots, so foliar spraying 75 g of boric acid plant^{-1} is used to increase B absorption. Different concentrations of N, P and K fertilizer have been applied to 25-year old *P. koraiensis* and it was found that the number of female cones was affected by N and P fertilizers, where N elicited the greater effect, but there was no effect of potash. The most effective treatment to increase the number of female cones (increase of up to 8.2) was application of NPK at 0.6, 0.3, and 0.05 kg plant^{-1}. The impact of N fertilizer was greatest on male cones, while K elicited the lowest effect and the most effective treatment with NPK was 0.6, 0.3 and 0.1 kg plant^{-1} that resulted in a maximum number of 1345.7 cones and was about twice the minimum (795.7) and a general increase of up to 6.7.

Tai (2000) stated that thinning was one of the most effective measures to promote flowering and fruition. It was found that thinning intensity had a significant effect on fructification number per unit area and yield per unit area in a *Pinus armandii* seed orchards, where 0.5 cutting intensity resulted in yields that were 175% greater than the lowest intensity. The optimal degree of canopy density for seed orchards or seed collecting forests is believed to be between 0.5 and 0.6; yields decline above these levels.

Climate is an important factor affecting cone yield, because it affects the formation of flower buds, flowering pollination rate, and growth and development of seeds and cones. The pollination period for female and male cones of *Pinus armandii* is from late April to early May and lasts for 7–10 days and 10–15 days, respectively; assisted, artificial pollination during this period may increase the seed-setting rate. Topography is known to affect growth and fructification of forest trees and a study of *P. koraiensis* found that individuals at lower elevations on shallow slopes grew better than that those at higher elevations on steeper slopes, and growth was better on shaded slopes than on sunny slopes due to greater water availability. However, fruiting was poorer in trees that grew on shady slopes. Therefore, it is recommended that *P. koraiensis* forests are planted on sunny and semi-sunny slopes.

Orchard pests are varied and the overall management policy is to prevent and control pests. Insecticide should be applied at the beginning of each year in early

spring, twice in succession, to target early development of pests that seriously harm flowers and fruits, such as the pine snout moth (*Dioryctria rubella*).

The flowering period of *Pinus koraiensis* is generally in mid-June, when the average temperature is stable at 17–18 °C. Observations of 8-year-old *P. koraiensis* orchards in the CaoHekou region of China found that the flowering rate of the female clones increased with tree age, where there was variation among lines, and the effect of auxiliary pollination was greater at early stages of establishment. Female cones grow at the top of the crown, with most located around the apical buds of the main trunk, whereas male cones tend to be concentrated in the canopy and below. Proportion of male:female cones per tree have been found to vary, for example, out of 195 ramets of 24 clones, 105 contained only female flowers (53.85%), 2 bore male flowers only (1.03%), and 54 plants were monoecious (27.69%). Dioecious clones should be removed to maintain genetic diversity. The annual flowering period of males and females is almost equal, where males flower for 3–6 days and the females for 3–5 days; the homologous flowering period is synchronous for 1–2 days and for 2–4 days across heterogolous lines.

Pinus koraiensis cones enter the mature stage and are ready for harvest when they change color from green to yellow-green, cone scales crack and gradually turn yellow, seed water content is about 75% and individual cones become detached. Analysis of seed ripening and maturing in *P. koraiensis* in a managed forest in CaoHekou showed a gain in seed weight between August 10 and September 10, of 54.9 g, and an additional 63.91 g by September 15 and increases of 13.68, 56.65 and 8.34% for 1000 seed weight, seed kernel weight and kernel rate, respectively. There were no significant increases after September 15 and there was no effect on seed size at different seed collection periods. In terms of seed collection efficiency, raw cone weight decreased by 27.84%, seed weight increased by 156.92% and seed production rate increased by 17.72% between August 10 and September 20. The most suitable season for *P. koraiensis* cone harvest is September 15–30. Where seed production is early, the combination of high raw cone weight and low rate of seed production of poor quality leads to greater seed costs and low economic efficiency.

9.5.5 Genetic Trait Variation and Correlation Analysis

Large-scale genetic improvement to pine nut tree species began in the 1950s; however, genetic improvements of pine cones for consumption were began later in the 1970s. Genetically-improved varieties are cultivated in a cost-effective way that maximizes the genetic gain per unit of time at the lowest cost; therefore, the evaluation and selection of genetic material directly affects genetic gains across generations and seed quality. The breeding and selection processes of superior clones of *Pinus koraiensis* and description of genetic variability of major economic traits have been reported by Zhang et al. (2015). A total of 60 clones were selected from four *P. koraiensis* seed orchards. Analysis of seed traits revealed variation and regularity between populations and clones that reflect interactions among trait indicators

and provide an effective basis for early selection of genotypes, shortens breeding cycles and increases the rate of generational breeding. Accurate assessment of the productivity level of a clonal orchard, estimation of genetic trait parameters and selection of multiple traits are used to screen for superior clones in Heilongjiang Province of China, and to provide material for local clonal breeding and creation of clonal seed orchards.

9.5.5.1 Genetic Trait Variation

Seed traits, seed nutrients, and fatty acid and amino acid content vary among *Pinus koraiensis* clones, whereas variation in seed morphological traits show a range of 8.40–17.81%. The CV of length is the smallest and that of kernel weight is the greatest. Oil content is the largest nutritional component and variation of the nutritional components exhibited a range of 10.79–68.88%. A total of 11 fatty acids, including 5 saturated fatty acids and 6 unsaturated fatty acids, were detected in the kernel oils; linoleic fatty acid content was the highest, reaching 42.02% and variation in fatty acid composition a range of 2.24–66.83%. Seventeen amino acids were detected in kernel protein, where the average mass fraction was 40.43%; average mass fraction of essential amino acids was 9.79%, which accounted for 24.21% of total amino acids, and variation in amino acid composition was 14.70–38.88%.

Seed trait variation and level of genetic diversity in *Pinus koraiensis* has been shown to be high with the average CV in phenotypic differentiation among populations was 29.82%, which was less than the intra-population variation (70.18%), indicating intra-population variation is the main trait source. With the exception of seed length, width, and aspect ratio, there were differences in seed traits among ($P < 0.05$) and within ($P < 0.01$) populations of *P. koraiensis* and generalized heritability was 61.54–94.81%, with a strong degree of genetic control, and a higher genetic gain (4.70–74.52%) obtained through clone selection (Tables 9.8, 9.9 and 9.10).

There is strong genetic variation among nutrient components of *Pinus koraiensis* seed kernels, where CVs of fatty acids range 2.24–66.83% and CVs of amino acid components range of 14.70–38.88%. There is high genetic nutrient improvement potential in seed kernels, since composition of fatty acids and amino acids indicated intra-population variation was the main source of variation (85.18 and 63.08%, respectively). All the clones from different source groups showed that traits were controlled by higher heritability ($h^2 > 80\%$) and all seed traits showed a similar trend in genetic gain (Tables 9.11, 9.12, 9.13 and 9.14).

9.5.5.2 Correlation Analysis of Traits

Four seed orchards were used as the independent variable (*X*) for seed traits and seed oil content was the dependent variable (*Y*). The dependent variable *Y* was tested to meet the normal distribution and a multivariate stepwise regression was

Table 9.8 Results of analysis of variance of *Pinus koraiensis* seed characters

Trait	Mean square (degree of freedom)			*F-value*		h^2 /%
	$MS_{t/s}$ (df = 3)	MS_s (df = 56)	MS_e (df = 180)	In seed orchard	Between seed orchards	
Kernel percent	194.520 (3)	33.496 (56)	5.964 (180)	5.81**	5.62**	82.79
Thousand Kernel weight	73,079 (3)	16,164 (56)	1486 (180)	4.52**	10.88**	77.88
Seed kernel quality	0.011 (3)	0.003 (56)	0.0001 (180)	4.29**	5.87**	76.69
Seed coat quality	0.043 (3)	0.008 (56)	0.001 (180)	5.10**	12.71**	80.39
Seed kernel quality / seed coat quality	0.120 (3)	0.018 (56)	0.005 (180)	6.73**	3.90**	85.14
Seed length	0.360 (3)	2.522 (56)	0.150 (180)	0.14	16.79**	–
Seed width	1.123 (3)	1.309 (56)	0.126 (180)	0.86	10.36**	–
Length-width ratio	0.023 (3)	0.018 (56)	0.005 (180)	1.28	3.66**	–
Oil content	1075.160 (3)	55.830 (56)	3.04 (120)	19.26**	18.35**	94.81
Protein content	98.460 (3)	12.730 (56)	0.084 (120)	7.73**	151.07**	87.06
Polysaccharide content	59.508 (3)	20.536 (56)	0.262 (120)	2.90*	78.30**	65.52
Moisture content	18.582 (3)	4.069 (56)	0.357 (120)	4.57**	11.41**	78.12
Ash content	90.838 (3)	5.781 (5)	0.827 (120)	15.71**	6.99**	93.63
Crude fiber content	39.686 (3)	4.593 (56)	0.127 (120)	8.64**	36.30**	88.43
Carbohydrate content	478.53 (3)	106.810 (56)	2.94 (120)	4.48**	36.34**	77.69

∗∗ difference at $P < 0.01$; ∗ difference at $P < 0.05$

performed. By discarding the independent variables with non-significant regression coefficients, the best regression equations were:

$$Y_{HG} = 45.12 + 18.421X_8 - 0.723X_{11} - 1.485X_{12} - 0.729X_{14}\ (R^2 = 0.990^{**})$$
$$Y_{LK} = 94.003 + 18.199X_8 - 0.117X_{12} - 0.297X_{14}\ (R^2 = 0.793^{**})$$
$$Y_{TL} = 65.851 + 17.221X_8 - 0.895X_{13} - 0.502X_{14} (R^2 = 0.871^{**})$$
$$Y_{WH} = 89.445 + 18.5031X_8 - 0.635X_9 - 0.793X_{10} - 1.354X_{11} - 1.009X_{12} - 0.857X_{14}\ (R^2 = 0.988^{**})$$

Multiple regression analysis showed that length-width ratio (X_8), moisture content (X_{11}), ash content (X_{12}) and carbohydrate content (X_{14}) were the main factors affecting oil content in the Hegang orchard; length-width ratio (X_8), ash content (X_{12}) and carbohydrate content (X_{14}) were the main factors affecting oil content in the Linkou orchard; length-width ratio (X_8), crude fiber content (X_{13}) and carbohydrate content (X_{14}) were the main factors affecting oil content in the Tieli orchard; and aspect ratio (X_8), protein content (X_9), polysaccharide content (X_{10}), moisture content (X_{11}), ash content (X_{12}) and carbohydrate content (X_{14}) were the main factors

Table 9.9 Index analysis and multiple comparisons of morphologies of *Pinus koraiensis* seeds from each seed orchard

Location	Kernel (%)	Thousand kernel weight (g)	Seed kernel quality (g/seed)	Seed coat quality	Seed kernel quality/Seed coat quality	Seed length (mm)	Seed width (mm)	Length-width ratio
Hegang	34.64	556.03	0.19	0.36	0.53	14.25	8.84	1.63
	(5.78)b	(10.13)a	(10.61)a	(11.00)a	(9.01)b	(8.30)	(12.19)	(12.90)
Linkou	32.64	546.39	0.18	0.38	0.47	14.29	9.02	1.60
	(9.14)c	(11.74)a	(16.30)b	(11.63)a	(13.09)c	(8.18)	(11.55)	(11.96)
Tieli	34.33	478.10	0.16	0.31	0.53	14.12	8.69	1.65
	(12.96)b	(17.43)c	(21.90)c	(18.62)c	(18.12)b	(8.41)	(12.67)	(12.62)
Weihe	37.00	519.24	0.19	0.32	0.60	14.30	8.83	1.64
	(11.23)a	(14.47)b	(17.56)a	(17.03)b	(20.71)a	(8.66)	(13.33)	(12.44)
Mean	34.65	524.94	0.18	0.34	0.53	14.24	8.84	1.63
	(11.09)	(14.54)	(17.81)	(15.94)	(17.80)	(8.40)	(12.51)	(12.47)

Location	Moisture content (%)	Ash content (%)	Crude fiber content (%)	Oil content (%)	Protein content (%)	Polysaccharide content (%)	Carbohydrate content (%)
Hegang	4.02	5.77	5.00	55.28	7.34	11.03	11.56
	(24.40)b	(24.5)b	(28.55)b	(10.25)c	(34.12)c	(15.47)c	(54.32)b
Linkou	4.75	3.22	3.42	63.44	9.60	10.76	4.82
	(30.77)a	(50.79)d	(35.81)d	(3.89)a	(17.27)a	(22.64)c	(80.66)d
Tieli	3.41	4.53	4.89	56.55	8.77	11.83	10.04
	(12.88)c	(34.65)c	(26.24)c	(6.76)b	(22.92)b	(17.89)b	(60.30)c
Weihe	4.72	6.44	5.64	51.77	6.28	13.32	11.84
	(35.9)a	(24.47)a	(17.81)a	(9.95)d	(29.22)d	(27.62)a	(61.35)a
Mean	4.22	4.99	4.82	56.76	7.99	11.74	9.56
	(32.01)	(39.52)	(31.22)	(10.79)	(29.82)	(23.49)	(68.88)

Numbers within parentheses are CVs. Different letters indicate significant difference at $P < 0.05$

affecting oil content in the Weihe orchard. Overall, length-width ratio and carbohydrate content were the main factors that affected oil content in the four orchards, of which length-width ratio had the greatest direct effect. Therefore, improvements to nutrient composition index and quality of germplasm resources via breeding selection processes have practical application value.

Table 9.10 Variance of genetic parameters in *Pinus koraiensis* seed characters

Seed character	Variance component (δ^2)			Variance component (%)			V_{st}/%
	$\delta^2_{t/s}$	δ^2_s	δ^2_e	$P_{t/s}$	P_s	P_e	
Kernel percent	2.684	6.883	5.964	17.28	44.32	38.40	28.05
Thousand kernel weight	949	3669	1486	15.54	60.11	24.35	20.54
Seed kernel quality	0.00014	0.0005	0.0004	12.68	47.79	39.52	20.97
Seed coat quality	0.00057	0.0019	0.0007	18.09	61.04	20.87	22.86
Seed kernel quality / seed coat quality	0.0017	0.0033	0.004	17.76	34.59	47.65	33.93
Oil content	26.652	17.594	3.043	56.36	37.21	6.43	60.24
Protein content	1.905	4.215	0.084	30.70	67.94	1.36	31.13
Polysaccharide content	0.866	6.758	0.262	10.98	85.69	3.33	11.36
Moisture content	0.323	1.238	0.357	16.83	64.57	18.61	20.67
Ash content	1.890	1.651	0.827	43.27	37.80	18.93	53.37
Crude fiber content	0.780	1.489	0.127	32.55	62.16	5.28	34.37
Carbohydrates	6.883	34.624	2.939	15.49	77.90	6.61	16.58
Mean				25.04	58.30	16.66	29.82

9.5.5.3 Clonal Selection Research

Clonal tests to calculate the repeatability of clones and repeatability refers to the continuous phenotypic spatio-temporal stability of genotypes (Xu 1988). Tests involved the determination of quantitative trait counts, prediction of future performance of individuals, estimation of generalized heritability and estimation of genetic gain.

In the tests, clonal repeatability of seed traits in the *Pinus koraiensis* seed orchard at Hegang was 48.19–98.66% (Table 9.15), whereas repeatability of oil, protein and polysaccharide content was high, above 90%. In Linkou, clone repeatability was 70.93–98.80% and, with the exception of length-width ratio and oil content, repeatability was above 90%. Repeatability at Tieli was 74.23–96.68% and, with the exception of length-width ratio, seed kernel weight, seed kernel weight/seed coat weight and clone repeatability was above 90%. Finally, clonal repeatability at Weihe was in the range of 49.24–98.84%, and repeatability of seed length, seed width and oil, protein and polysaccharide content was more than 90%.

Each seed orchard was evaluated for clonality, and one standard deviation of the mean value of the clones over the total trait average was selected. Clonal gains at Hegang, Linkou, Tieli and Weihe were 3.27–72.60, 6.19–60.90, 4.50–47.76 and 6.27–46.82%, respectively. This showed that the higher gains were obtained through asexual reproduction of selected superior clones. We found that seed kernel ratio, 1000 seed and seed kernel weight, oil, protein, polysaccharide, polyphenol and flavonoid content in the four seed orchards reached more than 5%.

Clonal repeatability of the fatty acid at Hegang, Linkou, Tieli and Weihe were 80.92–99.03, 85.34–98.26, 93.65–99.17 and 80.17–97.93%, respectively, and the average repetition rate of fatty acid composition index was high in the four orchards

Table 9.11 Analysis of fatty acid composition and oil content of *Pinus koraiensis* seeds from different seed orchards(%)

Location	C14:0	C16:0	C17:0	C18:0	C20:0	C16:1	C18:1	C20:1	C18:2	C18:3	C20:2	∑SFA	∑MUFA	∑PUFA	∑USFA	Fat content
Hegang	0.0349	6.8824	0.0636	3.8409	0.624	0.13	24.21	2.03	41.80	16.17	0.95	11.45	26.37	58.92	85.29	55.28
	(64.56) ab	(7.93) ab	(7.62)d	(9.4)c	(11.27) c	(23.08) b	(6.57)a	(6.89)c	(1.96)c	(4.02)a	(30.53) a	(6.36) d	(5.62)a	(2.29)c	(1.02)a	(10.25)c
Linkou	0.0447	7.0602	0.0862	4.153	0.7416	0.18	22.47	2.17	41.62	17.13	1.08	12.09	24.82	59.83	84.65	63.44
	(51.54) a	(5.33)a	(10.55) a	(8.08)b	(9.57)a	(16.67) a	(10.01) b	(8.29) b	(3.03) d	(12.32) a	(12.04) a	(5.11) c	(8.56)b	(2.36)b	(0.95)b	(3.89)a
Tieli	0.04	7.2698	0.0762	5.2313	0.7484	0.17	21.54	2.19	42.55	16.88	1.02	12.37	23.9	60.45	84.36	56.55
	(76.13) a	(6.90)a	(9.28)b	(10.67) a	(11.91) a	(29.41) a	(8.91)d	(7.76)a	(1.53)a	(4.74)a	(16.67) a	(6.85) a	(7.55)c	(1.92)a	(1.17)c	(6.76)b
Weihe	0.0347	7.2491	0.0713	4.2280	0.7149	0.17	22.42	2.20	42.13	16.37	1.11	12.3	24.79	59.61	84.40	51.77
	(74.46) ab	(7.41)a	(7.07)c	(9.68)b	(9.05)b	(17.65) a	(6.07)c	(8.64)a	(1.54) b	(3.85)a	(12.61) a	(6.54) b	(4.8)b	(1.38)b	(1.26)c	(9.95)d
Mean	0.0386	7.1154	0.0743	4.1133	0.7072	0.1627	22.66	2.1497	42.022	16.638	1.0422	12.05	24.97	59.7	84.674	56.76
	(66.83)	(7.24)	(24.05)	(10.22)	(12.58)	(25.15)	(9.01)	(8.47)	(2.24)	(7.61)	(19.26)	(6.9)	(7.59)	(2.2)	(1.18)	(10.79)

Saturated fatty acids (∑SFA): C14:0 + C16:0 + C17:0 + C18:0 + C20:0; monounsaturated fatty acid (∑MUFA): C16:1 + C18:1 + C20:1; polyunsaturated fatty acids (∑PUFA):C18:2 + C18:3 + C20:2; unsaturated fatty acids (∑USFA): ∑MUFA+∑PUFA. Numbers in parentheses are CVs. Different letters indicate difference at $P < 0.05$.

Note: Saturated fatty acids (∑SFA): C14:0 + C16:0 + C17:0 + C18:0 + C20:0
Monounsaturated fatty acid (∑MUFA): C16:1 + C18:1 + C20:1
Polyunsaturated fatty acids (∑PUFA):C18:2 + C18:3 + C20:2
Unsaturated fatty acids (∑USFA): ∑MUFA+∑PUFA
The number within parenthesis represents the variation coefficient
a, b, c and d indicate a statistically significant difference from each other at $p < 0.05$ level

Table 9.12 Analysis of protein and amino acid composition of *Pinus koraeinsis* seeds from different seed orchards(%)

Location	Asp	Ser	Glue	Glee	Ala	Cyst	Tyr	His	Argo	Pro
Hegang	3.1588(16.93)c	2.27(11.70)b	7.74(10.89)c	2.2854(18.08)b	1.932(16.16)b	0.7349(11.15)c	1.3229(11.16)c	0.8173(8.70)c	5.978(12.00)c	1.8025(17.5)c
Linkou	4.6118(5.92)a	3.014(8.14)a	10.603(2.24)a	2.5958(4.59)a	2.3623(6.39)a	1.0079(6.97)a	1.9083(8.64)a	1.0884(26.37)a	9.095(19.77)a	2.988(23.29)a
Tieli	3.4875(10.15)b	2.2015(12.52)c	7.943(9.08)b	1.8238(11.99)c	1.7321(13.37)c	0.8241(21.32)b	1.4272(16.58)b	0.9681(14.58)b	6.6545(9.98)b	2.034(42.6)b
Weihe	2.9733(16.37)d	2.1601(16.41)c	7.553(16.90)d	1.8022(17.93)d	1.6958(17.67)d	0.7381(13.89)c	1.2836(19.44)d	0.7504(15.29)d	5.726(17.94)d	1.4913(16.24)d
Mean	3.5578(21.89)	2.4114(18.93)	8.460(18.92)	2.1268(21.31)	1.9280(19.73)	0.8263(20.13)	1.4855(22.0)	0.9061(18.70)	6.864(23.15)	2.0788(38.88)

Location	Thru	Val	Met	Ile	Leo	Phi	Lys	TAA	EAA	Protein content mg/g
Hegang	1.0339(8.63)d	0.9755(9.36)d	0.6957(10.83)a	0.7482(10.16)d	2.3846(10.56)c	1.2020(9.77)c	1.3934(8.06)c	36.476(10.34)c	8.433(9.02)c	73.4(34.12)c
Linkou	1.4655 (10.07)a	1.6637(12.44)a	0.6830(21.73)a	1.3982(14.21)a	3.6753(5.22)a	1.7707(5.58)a	2.0247(19.51)a	51.956(8.48)a	12.681(9.04)a	96.0(17.27)a
Tieli	1.1903(14.52)b	1.1677(16.37)b	0.6207(19.06)a	1.0552(17.3)b	2.7472(12.94)b	1.3728(18.26)b	1.5727(9.76)b	38.823(7.91)b	9.727(10.2)b	87.7(22.92)b
Weihe	1.0632(16.58)c	1.0151(16.42)c	0.6663(15.84)a	0.8174(19.50)c	2.3241(16.53)d	1.1427(14.53)d	1.2845(15.13)d	34.477(16.40)d	8.313(15.67)d	62.8(29.22)d
Mean	1.1882(19.13)	1.2055(26.10)	0.6664(14.70)	1.0047(28.69)	2.7828(22.90)	1.3721(22.48)	1.5688(20.67)	40.433(20.13)	9.789(20.83)	79.9(29.82)

Numbers in parentheses are CVs (%). Different letters indicate significant difference at $p < 0.05$. Essential amino acids (EAA): Thru + Val + Met + Ile + Leo + Phi + Lys

TAA: Total content of amino acid content

EAA: Total essential amino acids content

Table 9.13 Variance in fatty acid composition and phenotypic differentiation between *Pinus koraiensis* pine seed clones

	Mean square (degree of freedom)			Variance component (δ^2)			Variance component (%)			
Fatty acid	$MS_{t/s}$(df = 3)	MS_s(df = 56)	MS_e (df = 120)	$\delta^2_{t/s}$	δ^2_s	δ^2_e	$P_{t/s}$	P_s	P_e	V_{st} (%)
C14:0	0.00206	0.00201	0.0000006	0.000001	0.00067	0.0000006	0.15	99.76	0.09	0.15
C16:0	1.4854	0.6644	0.0491	0.0182	0.2051	0.0491	6.68	75.30	18.02	8.15
C17:0	0.0041	0.0008	0.000003	0.0001	0.0003	0.000003	20.41	78.72	0.87	20.59
C18:0	1.5432	0.4783	0.00202	0.0237	0.1588	0.0020	12.85	86.06	1.09	12.99
C20:0	0.1479	0.0153	0.0010	0.0029	0.0048	0.0010	33.53	55.38	11.10	37.71
∑SFA	7.9137	1.6634	0.0581	0.1389	1.6634	0.0581	7.47	89.41	3.12	7.71
C16:1	0.0255	0.0038	0.0001	0.0005	0.0012	0.0001	27.47	67.03	5.49	29.07
C18:1	56.282	10.294	0.0029	1.0220	3.4303	0.0029	22.94	77.00	0.07	22.95
C20:1	0.2797	0.0901	0.0005	0.0042	0.0299	0.0005	12.16	86.48	1.36	12.33
∑MUFA	47.218	8.938	0.003	0.8507	2.9784	0.003	22.20	77.72	0.08	22.22
C18:2	7.4683	2.4378	0.0014	0.1118	0.8122	0.0014	12.08	87.77	0.15	12.10
C18:3	8.8762	4.6476	0.0024	0.0940	1.5484	0.0024	5.71	94.14	0.15	5.72
C20:2	0.2269	0.1154	0.0006	0.0025	0.1154	0.0006	2.11	97.41	0.48	2.12
∑PUFA	17.859	4.5681	0.0041	0.2954	1.5213	0.0041	16.22	83.55	0.23	16.26
∑USFA	8.447	2.7274	0.0065	0.1271	0.907	0.0065	12.21	87.17	0.62	12.29
Mean							14.28	82.86	2.86	14.82

MS Mean Square, *MS t/s* mean square among the seed orchards, *MSs* mean square within the seed orchard, *MSe* mean square of environmental, *df* degrees of freedom, *δ2* variance component, *δ2t/s* variance component among the seed orchards, *δ2s* variance component within the seed orchard, *δ2e* environmental variance component, *P* percent variance component, *Pt/s* percent of variance component among the seed orchards, *Ps* percent variance component within the seed orchard, *Pe* percent environmental variance component

Table 9.14 Variance in amino acid composition and CVs of phenotypic differentiation between *Pinus koraiensis* pine seeds clones

Amino acid	Mean square (degree of freedom)			Variance component (δ^2)			Variance component (%)			
	$MS_{t/s}$(df = 3)	MS_s(df = 56)	MS_e (df = 120)	$\delta^2_{t/s}$	δ^2_s	δ^2_e	$P_{t/s}$	P_s	P_e	V_{st} (%)
Asp	24.2498	0.6359	0.0018	0.5248	0.2113	0.0019	71.11	28.64	0.25	71.29
Thru	1.7448	0.0707	0.0004	0.0372	0.0234	0.0004	60.93	38.37	0.70	61.36
Ser	7.3552	0.2685	0.0018	0.1575	0.0889	0.0018	63.45	35.82	0.73	63.92
Glue	93.0101	3.1988	0.003	1.9958	1.0653	0.0030	65.13	34.77	0.10	65.20
Glee	6.6329	0.2980	0.00139	0.1408	0.0989	0.0014	58.41	41.02	0.58	58.75
Ala	4.2848	0.2282	0.0022	0.0901	0.0753	0.0022	53.75	44.92	1.33	54.46
Cyst	0.7368	0.0468	0.0010	0.0153	0.0153	0.0010	48.52	48.29	3.20	50.07
Val	4.5074	0.0744	0.0003	0.0985	0.0247	0.0003	79.77	20.00	0.23	79.95
Met	0.0484	0.0280	0.000044	0.0005	0.0093	0.000044	4.62	94.93	0.45	5.10
Ile	3.8734	0.0578	0.0001	0.0848	0.0192	0.0001	81.42	18.47	0.11	81.51
Leo	17.5038	0.359	0.0006	0.3810	0.1195	0.0006	76.03	23.85	0.13	76.12
Tyr	3.74	0.1398	0.0006	0.0800	0.0464	0.0006	63.00	36.52	0.48	63.30
Phi	3.6071	0.1102	0.0003	0.0777	0.0366	0.0003	67.79	31.95	0.26	67.97
Lys	4.7924	0.079	0.0002	0.1047	0.0263	0.0002	79.83	20.01	0.15	79.95
His	1.0380	0.0360	0.0001	0.0223	0.0120	0.0001	64.87	34.90	0.23	65.05
Argo	106.5909	2.3573	0.0001	2.3163	0.7858	0.0001	74.67	25.33	0.00	74.67
Pro	18.7393	1.0846	0.0001	0.3923	0.3615	0.0001	52.04	47.95	0.01	52.04
TAA	2797.72	61.77	0.0129	60.7989	20.5882	0.0129	74.69	25.29	0.02	74.70
EAA	185.759	3.338	0.001	4.0538	1.1123	0.0011	78.45	21.53	0.02	78.47
Mean							62.80	36.73	0.48	63.08

MS Mean Square, *MS t/s* mean square among the seed orchards, *MSs* mean square within the seed orchard, *MSe* mean square of environmental, *df* degrees of freedom, *δ2* variance component, *δ2t/s* variance component among the seed orchards, *δ2s* variance component within the seed orchard, *δ2e* environmental variance component, *P* percent variance component, *Pt/s* percent of variance component among the seed orchards, *Ps* percent variance component within the seed orchard, *Pe* percent environmental variance component

Table 9.15 Selection, evaluation, and estimation of repeatability in seed and fruit of *Pinus koraiensis*

Seed orchard	Trait	Kernel (%)	1000 seed weight (g)	Seed kernel weight (g grain^{-1})	Seed coat weight (g)	Seed weight / Seed coat weight	Length (mm)	Width (mm)	Length-width ratio	Oil content (%)	Protein content (%)	Polysaccharide content (%)
Hegang	Selection rate (%)	20.00	20.00	13.33	13.33	6.67	26.67	6.67	6.67	6.67	13.33	26.67
	Choice difference	2.27	66.14	0.03	0.05	0.08	0.47	0.78	0.10	15.31	5.33	2.24
	Reality gain (%)	6.54	11.89	14.21	14.60	15.59	3.27	8.82	6.09	27.69	72.60	20.30
	Repeatability (%)	65.64	83.39	80.62	81.55	66.56	79.72	52.83	48.19	94.55	98.66	98.24
Linkou	Selection rate (%)	6.67	6.67	20.00	6.67	6.67	20.00	20.00	6.67	6.67	13.33	6.67
	Choice difference	3.41	79.53	0.04	0.08	0.08	1.21	0.91	0.10	3.93	2.47	5.81
	Reality gain (%)	10.45	14.56	20.07	20.50	15.77	8.44	10.04	6.35	6.19	25.76	53.98
	Repeatability (%)	91.88	92.95	91.86	93.46	91.71	96.63	91.03	70.93	77.48	98.80	97.36
Tieli	Selection rate (%)	13.33	20.00	13.33	13.33	13.33	13.33	6.67	13.3	20.00	20.00	20.00
	Choice difference	3.07	188.6	0.07	0.14	0.08	2.85	1.97	0.07	7.51	1.55	1.69
	Reality gain (%)	8.93	39.44	41.63	45.68	14.65	20.19	22.64	4.50	13.29	17.69	14.31
	Repeatability (%)	90.94	94.55	87.94	97.05	88.86	98.17	92.58	74.23	94.25	98.68	96.99

(continued)

Table 9.15 (continued)

Seed orchard	Trait	Kernel (%)	1000 seed weight (g)	Seed kernel weight (g grain^{-1})	Seed coat weight (g)	Seed weight / Seed coat weight	Length (mm)	Width (mm)	Length-width ratio	Oil content (%)	Protein content (%)	Polysaccharide content (%)
Weihe	Selection rate (%)	6.67	20.00	6.67	20.00	6.67	13.33	6.67	20.0	6.67	20.00	20.00
	Choice difference	1.96	27.36	0.01	0.02	0.06	0.33	0.97	0.01	4.19	1.11	1.19
	Reality gain (%)	16.53	19.74	23.04	24.18	30.63	10.98	24.31	6.27	18.04	46.82	36.56
	Repeatability (%)	59.35	87.33	64.03	89.20	49.24	94.95	93.89	81.9	90.90	95.97	97.65

(shown in Table 9.16). Clonal gains at Hegang, Linkou, Tieli and Weihe were 1.25–45.18, 1.08–42.67, 1.42–55.78 and 6.27–26.47%, respectively, showing that the higher gains were obtained through asexual reproduction of selected superior clones. Heritability was estimated to be more than 80%, indicating that differences in fatty acid traits were mainly due to genetic effects. In the fatty acid composition, C16:1, C18:1, C20:1, C18:3 and C20:2 achieved gains of more than 5% and the greatest gains were for C20:2 (17.12–55.78%).

Clonal repeatabilities of the amino acid components at Hegang, Linkou, Tieli and Weihe were 90.68–98.97, 90.67–98.64, 92.06–98.94 and 91.86–98.97%, respectively, and clonal gains were 0.45–18.0, 6.56–28.61, 12.48–121.22 and 25.28–45.90%, respectively. Amino acid genetic heritability was estimated to be more than 90% with effects of selection >5% on Asp, Ser, Gly, Ala, Cys, Val, Met, Tyr, Pro, TAA (Table 9.17).

The objective of *Pinus koraiensis* improvement is to increase 1000 seed weight, seed kernel rate, content of oil, protein, unsaturated fatty acids and amino acids, and to reduce seed coat weight and content of ash, crude fiber, saturated fatty acids and non-essential amino acids. Analyses showed that 1000 seed weight, seed kernel rate, seed length, seed coat weight, seed kernel weight, oil content, protein content, ash content, crude fiber, ∑SFA, ∑MUFA, ∑PUFA, EAA, NEAA and TAA of amino acid composition were important indicators that varied among the orchards (Tables 9.15, 9.16 and 9.17). There were correlations between phenotypic traits and seed nutrient composition and the high clonal repeatability of the traits in the four orchards indicated they were under strong genetic control. Accounting for the results of the correlation analyses in the context of breeding objectives, we selected 1000 seed weight, seed kernel rate, seed length, seed coat weight, seed kernel weight, oil, protein and ash content, and crude fiber as integrated indictors of seed trait quality. Clonal repeatability for fatty acid composition was above 80% and strongly controlled by genetics, and ∑SFA, ∑MUFA, and ∑PUFA were selected as comprehensive indicators of seed trait quality, while clonal repeatability for amino acid composition was more than 90%, under strong genetic control (Tables 9.15 and 9.16) and EAA, NEAA and TAA were selected as comprehensive indicators of seed-trait quality.

The comprehensive selection index (CSI) method aims for greater improvement in certain important traits, but the economic importance of various traits should be considered. The largest comprehensive breeding value of a considered trait is the objective CSI upon which the constraints selection index is based, and artificially controls some traits by limiting or improving genetic function. In this multi-trait selection, the CSI method is considered ideal. Principal component analysis uses the idea of dimensionality reduction to summarize multiple indicators into a few comprehensive indicators that reflect information provided by original variables.

Analysis of the genetic parameters of the clones in the seed orchards indicated that the clonal repeatability of each trait was medium to high. Clones were selected by two methods of exponential selection and principal component analysis; three (HG8, HG21, HG14) were selected as superior clones from Hegang, based on the 20% clonal selection rate. The average values of 1000 seed weight, seed kernel

Table 9.16 Selection, evaluation, and estimation of repeatability in fatty acids composition of seed of *Pinus koraiensis*

Seed orchard	Trait	∑SFA	C16:1	C18:1	C20:1	∑MUFA	C18:2	C18:3	C20:2	∑PUFA	∑USFA
Hegang	Selection rate (%)	20.00	20.00	20.00	13.33	26.67	20.00	20.00	13.33	13.33	20.00
	Choice difference	0.79	0.05	1.85	0.18	1.67	1.17	0.88	0.43	2.45	1.07
	Reality gain (%)	6.96	37.57	7.65	8.76	6.33	2.81	5.46	45.18	4.16	1.25
	Clonal repeatability (%)	80.92	98.04	97.68	96.09	99.03	98.24	94.59	98.35	94.9	97.55
Linkou	Selection rate (%)	13.33	26.67	20.00	6.67	13.33	26.67	6.67	13.33	6.67	13.33
	Choice difference	1.28	0.03	2.75	0.48	2.79	0.99	7.31	0.24	3.61	0.92
	Reality gain (%)	10.59	16.83	12.23	22.08	11.26	2.37	42.67	22.06	6.04	1.08
	Clonal repeatability (%)	85.34	90.74	97.98	94.87	98.26	93.64	96.94	96.98	96.90	97.51
Tieli	Selection rate (%)	13.33	20.00	20.00	33.33	20.00	13.33	20.00	6.67	13.33	20.00
	Choice difference	1.34	0.07	2.98	0.21	2.87	1.12	1.13	0.57	1.97	1.20
	Reality gain (%)	10.80	39.00	13.81	9.81	12.02	2.63	6.69	55.78	3.26	1.42
	Clonal repeatability (%)	96.81	96.15	98.97	93.65	99.07	93.79	97.82	95.43	95.47	99.17
Weihe	Selection rate (%)	6.67	13.33	20.00	6.67	40.00	6.67	13.33	6.67	20.00	13.33
	Choice difference	2.57	0.05	1.93	0.24	1.07	1.20	1.03	0.19	1.23	1.19
	Reality gain (%)	20.89	26.47	8.62	10.88	4.31	2.84	6.28	17.12	2.06	1.41
	Clonal repeatability (%)	97.67	80.17	97.93	96.65	97.92	94.55	97.31	94.93	95.73	96.71

Saturated fatty acids (∑SFA): C14:0 + C16:0 + C17:0 + C18:0 + C20:0; monounsaturated fatty acid (∑MUFA): C16:1 + C18:1 + C20:1; polyunsaturated fatty acids (∑PUFA):C18:2 + C18:3 + C20:2; unsaturated fatty acids (∑USFA): ∑MUFA+∑PUFA

Table 9.17 Selection evaluation and estimation of repeat force in amino acid composition of seed of *Pinus koraiensis*

Seed orchard	Trait	Asp	Thr	Ser	Glu	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Pro	TAA	EAA
Hegang	Selection rate (%)	6.67	22.22	13.33	20.00	15.56	13.33	8.89	8.89	13.33	13.33	13.33	15.56	13.33	15.56	24.44	13.33	13.33	13.33	20.00
	Choice difference	0.16	0.04	0.13	0.35	0.20	0.24	0.07	0.05	0.06	0.02	0.10	0.07	0.05	0.01	0.02	0.29	0.32	2.16	0.31
	Reality gain (%)	5.05	3.50	5.80	4.58	8.54	12.25	9.71	5.12	7.91	2.24	4.08	5.24	4.19	0.45	2.07	4.88	18.00	5.91	3.72
	Repeatability (%)	96.06	98.03	92.86	98.41	97.21	95.17	94.81	97.31	96.86	91.39	93.22	90.68	98.75	96.20	95.99	95.79	98.13	97.01	98.97
Linkou	Selection rate (%)	31.11	22.22	6.67	46.67	22.22	17.78	15.56	11.11	13.33	13.33	20.00	24.44	26.67	4.44	15.56	26.67	6.67	13.33	20.00
	Choice difference	0.44	0.21	0.31	0.70	0.25	0.31	0.15	0.29	0.10	0.18	0.40	0.24	0.27	0.40	0.14	1.13	0.85	5.74	1.45
	Reality gain (%)	9.61	14.00	10.15	6.56	9.79	13.16	15.22	17.22	14.83	12.57	10.99	12.40	15.19	19.63	12.62	12.38	28.61	11.04	11.44
	Repeatability (%)	98.04	91.56	96.05	93.65	92.90	98.04	95.63	96.20	97.41	96.73	98.54	90.67	95.98	95.40	94.72	97.27	94.47	98.64	95.77
Tieli	Selection rate (%)	13.33	26.67	8.89	8.89	17.78	20.00	20.00	24.44	24.44	13.33	13.33	17.78	6.67	17.78	13.33	13.33	6.67	6.67	6.67
	Choice difference	0.62	0.21	0.59	1.03	0.35	0.32	0.28	0.25	0.15	0.30	0.59	0.37	0.66	0.20	0.24	1.21	2.47	4.85	1.76
	Reality gain (%)	17.70	17.82	26.59	12.93	19.29	18.27	33.41	21.02	23.69	28.62	21.55	25.91	48.13	12.90	25.07	18.21	121.2	12.48	18.08
	Repeatability (%)	96.93	96.84	98.94	98.34	96.67	98.88	92.06	98.47	98.82	95.36	98.47	98.15	98.75	98.29	93.16	96.19	96.78	95.26	97.08
Weihe	Selection rate (%)	15.56	20.00	13.33	13.33	13.33	13.33	13.33	13.33	13.33	13.33	13.33	13.33	13.33	13.33	13.33	13.33	13.33	13.33	13.33
	Choice difference	0.92	0.27	0.70	2.63	0.67	0.63	0.21	0.37	0.21	0.38	0.84	0.51	0.34	0.38	0.21	1.85	0.41	11.63	2.82
	Reality gain (%)	30.91	25.28	32.40	34.77	37.33	37.37	28.76	36.53	31.28	45.90	36.0	39.54	29.92	29.85	28.04	32.39	27.68	33.74	33.92
	Repeatability (%)	97.76	96.11	94.35	93.29	95.56	91.86	98.51	98.97	96.66	98.92	97.65	98.09	96.96	97.31	97.78	96.86	95.22	93.21	98.52

weight, seed length, oil, protein, ∑MUFA, ∑PUFA, EAA and TAA traits of these clones were higher by than the clonal averages of 7.87, 7.15, 2.73, 10.98, 27.35, 0.69, 1.89, 1.99 and 3.46%, respectively. At the Linkou seed orchard, three superior clones (LK20, LK19, LK18) were selected, and the seed kernel rate, 1000 seed weight, seed kernel weight, seed length, oil, protein, ∑PUFA and TAA traits were higher than the averages of 3.75, 11.88, 17.58, 6.84, 2.42, 4.35, 1.27 and 2.06%, respectively. Three superior clones were selected from the Tieli orchard (TL1104, TL1131, TL1383) and the seed kernel rate, 1000 seed weight, seed kernel weight, seed length, ∑MUFA, and EAA traits were higher than the averages of 4.54, 15.61, 21.50, 5.98, 5.3 and 4.75%, respectively. At the Weihe seed orchard, three superior clones (WH071, WH117, WH066) were selected and the seed kernel rate, 1000 seed weight, seed kernel weight, oil, protein, ∑PUFA, EAA and TAA traits were higher than the averages of 4.29, 9.85, 4.42, 8.14, 13.21, 0.74, 2.5 and 6.53%, respectively.

9.5.6 Analysis of Genetic Structure

Forest tree breeding research is slow, due to relatively slow growth and long reproduction cycles. The combination of conventional breeding with modern breeding techniques has resulted in a more efficient breeding strategy. Breeding is a continuous process and begins with the evaluation of the presence of important heritable economic or ecological adaptive traits of candidate plants. Traditionally, breeding programs are based on comprehensive evaluations of phenotypic characteristics to inform selection of individuals from a single gene population; however, the development of molecular genotyping technology has facilitated a genetic basis for breeding selection. Molecular markers are defined as heritable potential genotypes at different ages or in specific environments (Muranty et al. 2014) and as an indirect method of selection, genetic markers play an increasingly important role in shortening the breeding cycle and increasing selection intensity. The molecular markers currently used in forest trees include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite DNA (SSR) and single nucleotide polymorphisms (SNPs).

Molecular markers are used to detect genetic diversity of germplasm resources and to select suitable sample sizes and test materials; evaluate population size; construct core germplasm breeding material; and identify test materials (natural groups or parental sources).

South Korea established a 91-ha *Pinus koraiensis* clonal seed orchard using genetic markers to conduct in-depth research on the genetic structure and diversity of a *P. koraiensis* population established for breeding and gene conservation strategies that preserved genetic resources of *P. koraiensis* and *P. pumila* in forests measuring 55 and 2 ha, respectively. Thus, the 91 ha seed orchard was considered a genetically conserved forest. Analysis of isoenzymes in Russia showed natural

hybrids of *P. sibirica* and *P. pumila* in ecotone zones. In the 1980s, China conducted multi-point geographic provenance trials and offspring testing of the entire distribution areas of *P. koraiensis* and *P. armandii,* and established a number of *P. koraiensis* seed orchards with an expected genetic gain of 10% and a *P. armandii* seed orchard covering 133 ha. Studies have shown that both *P. koraiensis* and *P. armandii* have significant geographical provenance differences and GxE interactions, so China established several nature reserves in the natural distribution area of *P. koraiensis* to preserve in situ genetic resources. In addition, a gene bank was established for off-site preservation. Therefore, the development of high-generation pine tree breeding programs and genetic diversity studies is urgently needed to establish a scientific and rational strategy for the conservation of genetic resources. It is also important to strengthen and promote cooperation among countries in the region due to the transnational distribution of tree species of concern (Wang 2002). Here we describe genetic diversity analyses and genetic variation in the study of edible pine nut species.

9.5.6.1 Genetic Differentiation of Natural Populations

Xia et al. (2001) used RAPD technology to analyze the genetic diversity and differentiation among three populations of *Pinus koraiensis* distributed in northeast China. A total of 241 repeat sites were detected using 38 random primers, of which 139 were polymorphic (57.68%) (Table 9.18). In the Liangshui population, 227 loci were detected using 38 primers, including 121 polymorphic loci (53.30%); the Hulin province population comprised 216 total loci, including 114 polymorphic loci (52.78%); and of the 215 loci detected in the Heihe population, 108 were polymorphic (50.23%). The Liangshui population had the highest level of polymorphism, the Heihe population the lowest.

The Shannon and Nei indices of diversity indicated that genetic variation was mainly found within populations (Table 9.19) and genetic diversity of the Liangshui population was higher than that of the Heihe and Hulin populations. The average genetic similarity within a population was 0.927 and 0.845 between groups and may reflect evolutionary perturbations from the Quaternary Ice Age and recent human disturbance.

Table 9.18 Polymorphic loci within species and populations of *Pinus koraiensis*

Populations	No. of samples	No. of loci	No. of polymorphic loci	Polymorphic loci (%)
PLS	20	227	121	0.5330
PHL	20	216	114	0.5278
PHH	20	215	108	0.5023
Total	60	241	139	0.5768

PLS Liangshui population, *PHL* Hulin population, and, *PHH* Heihe population

Table 9.19 Genetic diversity and differentiation between populations of *Pinus koraiensis* using Nei's index and Shannon's phenotypic diversity index

Diversity index	Nei's index (H)	Diversity index	Shannon's diversity index
Within-group genetic diversity (H_s)	0.2469	Within-population genetic variation (H_{pop})	0.6078
Population total genetic diversity (H_t)	0.2964	Total population genetic variation (H_{sp})	0.9707
Intergroup gene diversity Dst	0.0494	Proportion of total genetic variation within populations (H_{pop} / H_{sp})	0.6559
Genetic differentiation coefficient, Gst (H_t – H_s / H_t)	0.1702	Proportion of total genetic variation between populations (H_{sp} − H_{pop}) / H_{sp}	0.3441

Petrova et al. (2014) used the isozyme method to analyze 11 ecotypes representing the latitudinal and longitudinal profiles within the species range, including samples from the geographic boundaries of the distribution of Siberian stone pine (*Pinus sibirica*). The genetic structure of the ecotypes was described on the basis of the variability for 26 isozyme loci encoding for 16 enzyme systems. The greatest genetic diversity was observed in the taiga ecotypes in the central part of the studied area, while the ecotypes along the species range boundaries were shown to be genetically depauperized. Approximately 8.1% of the observed genetic diversity is attributed to differences between the studied ecotypes. We detected high levels of genetic diversity for the Fest-2, Pgm-1, Sod-4 and a few other loci, as well as a correlation between allele frequencies and geographical locations of the populations. The results of multivariate analysis of allelic frequencies as well as cluster analysis allowed us to discriminate three major groups of ecotypes: northeastern, central and southwestern. In view of our results, we compare two hypotheses: one which attributes the spatial distribution of genetic variations to the selectivity for some of the polymorphic allozyme loci, and the other based in the history of the formation of the range of the Siberian stone pine.

9.5.6.2 Orchard Genetic Diversity

The genetic gain and genetic basis (genetic diversity) of seed orchards have always been the principal aspects of forest tree genetic improvement research. Genetic diversity of seed orchards is based on genetic gains that will ensure successful orchard establishment and maintain a diverse resource. In addition to genetic quality, seed quality relates to seed sowing and growing ability, 1000 seed weight, nutrient content and seed yield. A heterogeneous and abundant genetic base is required for genetic improvement programs of forest trees, therefore access to a sustained wild stock of forest trees is essential; however, threats from disease, pests and natural disasters place such stock at risk and may affect the sustainable development of the forestry industry.

Feng et al. (2010) studied the clonal, family and free pollination progeny of a seed orchard at Lushuihe Forestry Bureau, Jilin Province, China and analyzed genetic diversity and mating systems. Male parent analysis was performed on 100 offspring and results showed that the total reproductive fitness of the candidate parents was approximately 53% and of the 150 candidate parents, 48 (32%) provided active pollen for the offspring population and the remainder provided no effective pollen. Pollen dispersal was patchy and was reflected by mating ratios among the 53 open-pollinated progenies, which was highest at distances of 15–30 m (36%) and 30–45 m (30%); average mating distance was 32.6 m and the maximum mating distance was 67.88 m. The clonal populations in the orchards had a high level of genetic diversity (0.5333), where the average observational heterozygosity (Ho) of the population was 0.6481, the expected heterozygosity (He) was 0.6005. Compared with a clonal seed orchard, the diversity parameter of the offspring population was similarly high and did not decrease. The Fit value was −0.0517, indicating that the progeny population was close to the Hardy-Weinberg equilibrium, and was possibly related to random mating among orchard clones. Analysis of the mating system of seed orchards showed the multi-site outcrossing ratio was high (tm = 0.823) and inbreeding index was very low (tm-ts = 0.062), where single-site outcrossing rate ts = 0.785 and fixed index F = 0.375, indicating that inbreeding in the seed orchard was not significant.

Zhao et al. (2012) used SSR molecular markers to study the genetic diversity of *Pinus armandii* clonal seed orchards and progeny forests in two different years, in Pingba, Guizhou Province, China, and amplified 85 clones using 13 SSR primers. A total of 95 markers were obtained, of which 94 were polymorphic; there was an average of 1.717 effective alleles, average genetic diversity (h) was 0.4029, and the Shannon information index (I) was 0.5855, indicating that genetic diversity was high and variation exists within the provenance. Compared with the seed orchard parent groups, the genetic diversity index of the progeny population decreased in the early fructification period (1988) and genetic diversity of offspring in the late fructification period (2007) had decreased substantially, suggesting that measures need to be taken to increase seed yield and quality.

Edible pine nut trees are highly heterozygous, the probability of restructuring by its good genes or chromosome section reorganization of hybrid offspring is very small. This requires breeders to increase the probability of good offspring by increasing the number of F1 generations, which would greatly increase the selection workload. Marker-assisted selection is an important means to accelerate the process of breeding. The important means were mainly concentrated on the important traits, such as disease resistance, cone, etc. The complex traits developed were slow, such as production and abiotic stress aspects of molecular markers.

Since the application of marker-assisted selection began in the 1980s, it was mainly focused on simple traits controlled by a small number of genes, but was powerless on complex traits controlled by micro-effect polygenes (Bernardo 2008;

Heffner et al. 2010). Genomic selection is the detection of molecular markers in all genome-wide, but is not partial markers for single trait. Genomic selection is the main process through a test group to estimate each tag (usually was single nucleotide polymorphism) or effect value in different chromosome segment, and then reuse these effect values to calculate breeding population breeding. In the end, breeding efficiency was improved by individual selection of offspring (Meuwissen et al. 2001). Therefore, it is of great significance to carry out the systematic research of genomic selection technology and to apply it to the creation and breeding of edible pine nut tree materials.

9.6 Role of Biotechnology

Pine nut trees have a long growth cycle, high genetic heterozygosity, hybrid incompatibility and many important traits which are controlled by multiple genes. These factors limit genetic improvement by conventional breeding and make it difficult to meet the challenge of creating new varieties to serve the edible pine nut and timber markets. Nevertheless, recent developments in molecular biology and genetic engineering have identified genes linked to herbicide, insect and pathogen resistance which can be inserted into a plant genome to create needed genotypes without changing other traits directionally, and shortening the breeding cycle. Pine nut tree molecular breeding research is the key component to accelerate the process of genetic improvement.

9.6.1 Advances in Pine Nut Tree Molecular Breeding

Transgenic trees are very important for studying how different genes regulate specific traits. Candidate genes can be identified by molecular breeding. For pines, at present, there are two methods: particle bombardment and *Agrobacterium*-mediated transformation. Methods have been developed for transient and stable transformation using particle bombardment for *Pinus radiata* (Bishop-Hurley et al. 2001; Rey et al. 1996; Walter et al. 1994, 1998, 2002), *P. taeda* (Stomp et al. 1991) and *P. strobus* L. (Tian et al. 1997). In addition, stable transformation of embryogenic tissue of *P. nigra* has been achieved using particle bombardment (Terézia et al. 2005). *Agrobacterium*-mediated transformation is one of the common methods applied in conifers, which have been identified as a natural reservoir of *Agrobacterium* (Ellis et al. 1989; Morris et al. 1989; Stomp et al. 1990). These findings establish a solid foundation for *Agrobacterium*-mediated genetic transformation of conifers, including pines.

Using the *Agrobacterium*-mediated method, successful transfer of an exogenous gene into the *Pinus taeda* genome was made, the first successful transformation of conifers; however, transgenic plants were not obtained (Sederoff et al.

1986). Later, *Agrobacterium rhizogenes* (=*Rhizobium rhizogenes*) infection was induced in an aseptic seedling of European larch, achieving stable transformation; this represented the first transgenic plant produced by *Agrobacterium*-mediated transformation in a conifer. With this development in genetic engineering, additional pine genes were transformed. For example, a white pine (*P. strobus*) plant regeneration system was established using *A. tumefaciens* and the transgene expression was analyzed in pine T-DNA transformants carrying different numbers of copies of T-DNA insertions. The results showed that two or more T-DNA insertions in the same chromosome facilitate efficient gene silencing in transgenic pine cells expressing green fluorescent protein (Tang et al. 2007). Many studies have shown that *Agrobacterium*-mediated transformation has advantages over particle bombardment, such as a simpler integration pattern and a limited rearrangement in the introduced DNA. In addition, genetic material transformation can be used on seed and mature zygotic embryos (Tang and Newton 2005; Tang et al. 2007), and immature embryos (Terézia et al. 2005). However, embryogenic tissue as an experimental system for genetic transformation has more advantages because of its ability to produce high numbers of vigorous plantlets. This is an important feature because genetic transformation in conifers, including pines, frequently fails due to the low regeneration capability of transformed tissues (Terézia et al. 2005). For *P. nigra*, embryogenic tissue initiation, as well as successful plantlet regeneration, has been achieved (Salajova et al. 1999). Thus, we can study the genetic transformation of genes such as those related to flowering, on the basis of the abovementioned research methods, especially *Agrobacterium*-mediated transformation in pine nuts, which can quickly enhance needed gene expression, and also increase breeding resources.

Furthermore, with the development of molecular biology, we can further understand the molecular mechanism of gene expression using high-throughput sequencing technology, and additional genomes have been successfully sequenced in pines. High-throughput sequencing is now an efficient approach for detecting the expression of genes in non-model plants, thus providing useful and valuable information, despite the lack of the genome sequence. For example, defense-related genes triggered by nematode infestation were detected in both *Pinus pinaster* and *P. pinea*, which shown the genes' higher expression related to transcriptional regulation, terpenoid secondary metabolism (including some with nematicidal activity) and oxidative stress respectively. This is significant in molecular defense mechanisms and provides a better understanding of pine wilt disease (Santos et al. 2012). Similarly, after *P. monticola* was infected with WPBR (caused by the rust fungus, *Cronartium ribicola*), it was possible to carry out illumina deep sequencing of primary needles of *P. monticola*. The results shown that the DEGs up-regulated in resistant seedlings, included a set of putative signal receptor genes encoding disease resistance protein homologs, transcriptional factor (TF) genes of multiple families and so on. Many of the down-regulated DEGs were related to photosystems, the metabolic pathways of carbon fixation and flavonoid biosynthesis. This provides a basis for future studies of genetic resistance in no-model plants such as *P. monticola* (Liu et al. 2013).

For *P. tabuliformis* Carr., the genomics resource is relatively poor, which limits the discovery of valuable genes and breeding processes. However, with the help of high throughput sequencing technology, the study was able to infer a phylogenetic tree, evolutionary patterns and calculated rates of gene diversion, and these data will be applied in the breeding program of *P. tabuliformis* which will also lay the foundation for the comparative genomics in *P. tabuliformis* and related species (Niu et al. 2013). In conclusion, we can discovery many useful and valuable genes in edible pine nut trees by high-throughput sequencing technology, and additional molecular mechanisms of gene expression could also be understood.

Later studies also identified GA, gibberellin, important in the floral regulatory networks of angiosperm plants, metabolism genes (PtCPS gene, PtKAO genes, 1 PtGA20ox gene, 2 PtGA3ox genes and 12 PtGA2ox genes) in *Pinus tabuliformis*. During male and female cone development, the expression of most of the PtGA2ox genes, was higher than GA biosynthesis genes and the expression of PtKAO1 in cones peaked at a very early developmental stage (Niu et al. 2014). And, for further study, 21 high-confidence homologues involved in sRNA biogenesis (including DCL3 and AGO genes) and action in *P. tabuliformis* were identified by transcriptomic sequences. Meanwhile, miRNA and target genes were identified, which expressed in male and female cones and probably bind to AGO. It is important to investigate the roles of sRNA pathways in cone development in pines (Niu et al. 2015). Therefore, it can provide significant guidance for related genes such as flower genes for edible pine nut, and promote breeding development.

9.6.2 Advances in Edible Pine Nut Tree Molecular Breeding

Edible pine nuts have had limited development in molecular breeding which has mainly previously focused on molecular markers. For example, in *Pinus koraiensis*, the first genetic linkage map was constructed using an F1 progeny of 94 individuals by SRAP, SSR, ISSR markers. The map provides a basis and crucial information for future genomic studies of *P. koraiensis*, in particular for quantitative trait loci (QTLs) and mapping of economically-important breeding target traits (Chen et al. 2010). The influential factors of ISSR were researched previously, which was the basis for the study of gene isolation and identification among *P. koraiensis* populations. The complete chloroplast genome (cpDNA) sequence of *P. armandii* has been determined and contains 114 genes. A phylogenetic analysis revealed that the *P. armandii* chloroplast genome is closely related to that of *P. koraiensis*. These results will lay the foundation for other edible pine nut determining cpDNA (Li et al. 2016). In addition, pine cone extract can induce antimicrobial activity in mice infected with *Staphylococcus aureus*,

Escherichia coli and so on (Harada et al. 1988; Oh-Hara et al. 1990). Moreover, *P. roxburghii* essential oil has considerable anticancer activity and could be used as an anticancer agent. It is necessary to identify and purify the bioactive compounds for further investigation followed by in vivo studies (Sajid et al. 2018). The genetic transformation of edible pine nut also has corresponding research. For example, transferring the plasmid pAHC25 containing the bar gene into mature embryos of *P. roxburghii* by particle bombardment has been a success (Parasharami et al. 2006). One of the edible pine nuts, *P. lambertiana* (sugar pine), is an economically- and ecologically-important conifer with a 1600 km latitudinal range, extending from Canada into Mexico. Through deep sequencing, a variety of tissue types of *P. lambertiana* were used to generate more than 2.5 billion short reads, which is the first comprehensive survey in this species. The transcriptomic data were also used to address questions surrounding lineage-specific, dicer-like proteins that play a role in the control of transposable element proliferation and related genome expansion in conifers.

Given the importance of edible pine nuts, the yield and quality requirements of their seeds are particularly important. However, some studies have found that the unbalanced nature of the parents' flowering and fruiting and the phenomenon of empty seeds, directly affects the yield and quality of seeds. Gibberellin is considered as an important phytohormone that regulates the differentiation and stage transformation of bulbs. The gibberellin pathway is one of the important regulatory pathways in the flowering process of angiosperms, which can activate the expression of Flowering Locus T (FT), Suppressor of Overexpression of Constans1F (SOC1, LEAFY(YEY) and blossom ahead of time. In addition, the FLO-LFY gene and its homologue are one of the genes controlling the formation of flower meristem (Yue et al. 2005), which can make plants blossom early, while the TFL1 gene controls the inflorescence meristem. When the expression of the TFL1 gene and its homologue are inhibited, the flowering time of plants is also advanced. Besides, GID1 plays the role of a bridge in the process of gibberellin, accomplishing its function as a signal of gibberellin, by which GA binds DELLA protein to form GA-GID1-DELLA trimer, then degrades through the DELLA protein ubiquitin and removes the inhibitory effect of DELLA protein on plant growth in order to make plants produce the gibberellin effect (Sun 2010). Analysis of the structure and function of these proteins can help us build a basic model of signal transduction pathways (Fig. 9.7), so as to understand molecular mechanisms.

At present, there are studies that reveal the flower gene of pine (Marcelo and Adriana 2005; Guo et al. 2015). If we can clone and overexpress LFY and its homologous gene (LFY-like and NLY-like) through genetic engineering, edible pine nut trees will flower early. Thus, it could be possible to shorten the breeding cycle of the seed orchard, improve the economic benefit and produce offspring ahead of time.

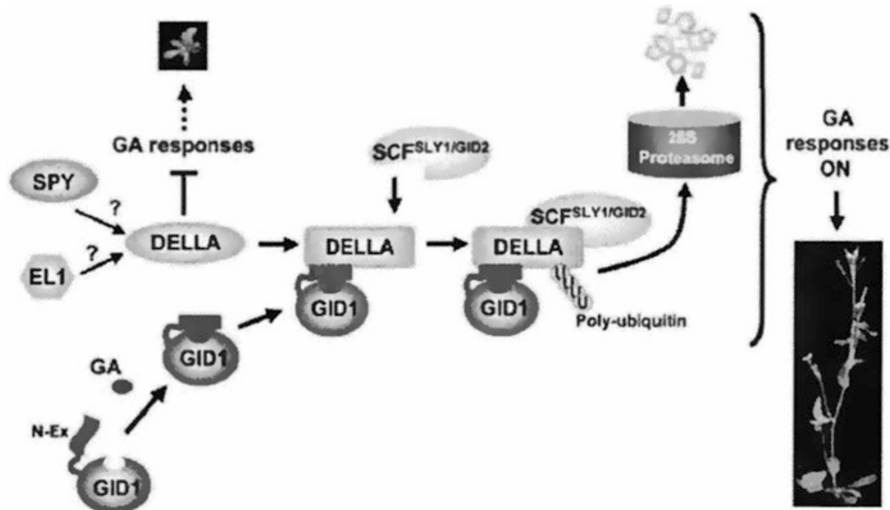

Fig. 9.7 The GA signaling pathway. GA Gibberellin, SPY Spindly, EL 1 Earlier flowering 1, GID 1 Gibberellin insensitive dwarf 1, SCF Skp 1 Cullin F-box, SLY 1 Sleepy 1, GID 2 Gibberellin insensitive dwarf 1, N-Ex N-terminal extension. (Source: Sun 2010)

9.7 Conclusions and Prospects

Edible pine nuts are an important nut resource rich in nutrients that are valuable in maintaining health as a dietary supplement. The edible pine nut trees are a prime candidate for resource collection, conservation, management and development as an economic crop. Multi-generation breeding of the species should be based on a three-stage recurrent selection based on selection and collection of breeding materials, hybridization and determination of clones, and breeding of improved varieties. The selection of cultivars with specific traits, such as high-seed yield, strong resistance, and high-nutritional quality, has developed globally and brought significant positive economic results, and promoted global food and nutrition security. Despite the collection of a wealth of breeding resources, current breeding of edible pine nut trees for human consumption has not yet formulated a long-term improvement plan. Although there are rich germplasm resources, they have not yet met market demand or provided food security. The slow process of conventional tree breeding has hampered the edible pine nut and timber markets; however, recent developments in molecular biology and genetic engineering have enabled genetic manipulation of plants to shorten the breeding cycle. Therefore, combining conventional and molecular breeding methods for edible pine nut improvement will help create new varieties of edible pine nuts and contribute to global food security in the twenty-first century.

Appendices

*Appendix I: Research Institutes Relevant to Edible Pine Nut Trees (*Pinus *spp.)*

Institution	Specialization and research activities	Contact information and website
School of Forestry, Northeast Forestry University, China	Clonal variations in nutritional components of *P. koraiensis* seeds collected from seed orchards	Prof. Dr. H G. Zhang Logan Campus, School of Forestry, Northeast Forestry University, Harbin, 26 Hexing Road, China Telephone: (86)0451–82,191,536 E-mail: hanguozhang1@sina.com
School of Forestry, Northeast Forestry University, China	Somatic embryogenesis and cryopreservation of *P. koraiensis*	Prof. Dr. H L. Shen Logan Campus, School of Forestry, Northeast Forestry University, Harbin, 26 Hexing Road, China Telephone: (86)13069875355 E-mail: shenhl-cf@nefu.edu.cn
Beijing Forestry University, China	Studies on fruiting quantity of *P. armandii* clones in the seed orchard and the seed quality	Prof. Dr. X H. Shen Logan Campus, School of Forestry, Beijing Forestry University, 35 east qinghua road, haidian district, Beijing, China E-mail: sxlkyhyc@163.com
Unidad de Anatomía, Fisiología y Genética Forestal, ETSI Montes, U.P.M., Ciudad Universitaria s/n	Variability of Mediterranean stone pine cone production: yield loss as response to climate change	Prof. Dr. L. Gil Telephone: (34)913367113 Fax: (34)915439557 E-mail: luis.gil@upm.es
Russian Academy of Sciences, Siberian Branch	Crossbreeding	Prof. Dr. N. Sergej Logan Campus, Siberian Branch, Filial of the Forest Institute, Academichesky pr., 2, Tomsk, Russia E-mail: gorosh@forest.tsc.ru
Grupo Silvicultura Mediterraénea	Breeding *P. pinea* uniform varieties	R. Calama Logan Campus, Apdo. 8111, Madrid 28,080, Spain E-mail: rcalama@inia.es Telephone: (34)913476868 Fax: (34)9–13,572,293
Korea Forest Research Institute, Chungju, Korea	Clonal variation in flowering abundance of *P. koraiensis*	Prof. Dr. I S. Kim Logan Campus, Forest Seed Research Center, Chungju, Korea P.O. Box 24, Suwon, Kyonggido, 441–350, Republic of Korea

Appendix II: Genetic Resources of Edible Pine Nut Tree

Country	Cultivar	Important traits
Heilongjiang province, China	*Pinus koreansis* – HG14	High oil content
Heilongjiang province, China	*P. koreansis* – LK20	High oil content
Heilongjiang province, China	*P. koreansis* – NB66	High yield
Heilongjiang province, China	*P. koreansis* – HG23	High yield
Heilongjiang province, China	*P. koreansis* – LK27	High protein content
Yunnan province, China	*P. armandii* – yema9	High yield
Yunnan province, China	*P. armandii* – CX42	High yield
Yunnan province, China	*P. armandii* – CX44	High yield
Yunnan province, China	*P. armandii* – CX56	High yield

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Chapter 10
Pistachio (*Pistacia* spp.) Breeding

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Abstract The genus *Pistacia* L. consists of 11 or more tree and shrub species belonging to the Anacardiaceae family. *Pistacia vera* L. is the only commercially-important species within this genus with nuts large enough to be consumed. The center of diversity of *P. vera* is northern Iran, southern Turkmenistan and parts of Afghanistan. Botanically, pistachio fruits are semidry drupes composed of a fleshy exocarp and mesocarp (pericarp or hull), and a hard endocarp (shell) containing the edible kernel. Pistachio is a wind pollinated dioecious tree with apetalous pistillate and staminate inflorescences on separate female and male trees. Currently pistachio cultivation is expanding in Iran, the USA, Turkey, Greece, Italy, Spain, China, Tunisia and many other countries. However, its commercial production has been affected by the undesired physiological characteristics of alternate bearing, shell indehiscence, blank nuts and susceptibility to abiotic stresses, including drought and salinity, and fungal foliar and root diseases. Genetic improvement of these characteristics should be a factor in future breeding attempts to produce superior pistachio cultivars. This chapter describes the advances in traditional and molecular breeding of pistachio cultivars. The traditional breeding and hybridization programs discussed are focused on new female and male cultivars, and rootstocks, introduced through pistachio genomics and breeding programs. The discussion of germplasm

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biodiversity and molecular breeding summarizes the current knowledge of intra- and inter-specific genetic diversity, cytogenetic evaluations, and marker-assisted selection in the genus *Pistacia.* The current knowledge of pistachio genetic engineering including micropropagation, regeneration systems, somaclonal variation and genetic stability, in vitro conservation and cryopreservation, and genetic transformation studies are also discussed.

Keywords Conventional breeding · Genetic diversity · Genetic engineering · Marker-assisted selection · Micropropagation · Molecular breeding · Pistachio

10.1 Introduction

The genus *Pistacia* L. belongs to the Anacardiaceae family with about 83 genera and some 860 named species (Christenhusz and Byng 2016), including mango (*Mangifera indica* L.), cashew (*Anacardium occidentale* L.), ambarella (*Spondias dulcis* Forst.), pepper tree (*Schinus* spp.), poison ivy (*Toxicodendron radicans* (L.) Kuntze), poison oak (*Toxicodendron diversilobum* (Torr. & A. Gray) Greene), and purple mombin (*Spondias purpurea* L.) (Hormaza and Wünsch 2007; Whitehouse 1957). The genus *Pistacia* consists of 11 or more (Zohary 1952) well-known tree and shrub species, among them *P. vera* is the only commercially-significant species producing large edible nuts. Based on archeological excavations and fossil evidence the *Pistacia* genus evolved about 80 million years ago (Hormaza et al. 1994a; Parfitt and Badenes 1997) and has been associated with humans since at least biblical times. The name pistachio is derived from the Persian word *pistak* in the ancient Persian language (Hormaza and Wünsch 2007). Pistachio cultivation slowly expanded west during the ancient Persian empire. The pistachio nut spread to Greece, Italy and Spain with the conquests of Alexander the Great. In 1929, the American botanist William E. Whitehouse collected the commercial pistachio varieties from Iran and introduced them to the US (Geisseler and Horwath 2016).

The natural population of wild *Pistacia vera* is centered in south-central Asia and appears to be the origin and center of diversity for this species (Fig. 10.1, textured area). The range extends from the Kopet Dagh mountain range of southern Turkmenistan, and northern Afghanistan to the Khorasan district and Sarakhs region in northeastern Iran, where significant undomesticated *P. vera* forests remain (Hormaza and Wunsch 2007; Parfitt et al. 2012; Whitehouse 1957; Zohary 1952, 1972). *Pistacia vera* is considered the ancestral species from which the other species are derived. *P. terebinthus* L. is the most recently evolved species. The chloroplast genome-based phylogeny of the genus *Pistacia* suggests *P. vera* var. Sarakhs is the common ancestor of currently cultivated pistachio varieties (Talebi et al. 2016).

Pistachio is a dioecious tree species and has pinnately compound leaves, with 3–5 leaflets. The female (pistillate) and male (staminate) inflorescences are panicles (Fig. 10.2). The floral rachis formed in the leaf axils of the previous year's shoot growth is comprised of 100–300 individual flowers. Both pistillate and staminate flowers are apetalous, lack nectaries and are wind pollinated (Crane and Iwakiri

Fig. 10.1 Countries with potential for pistachio production (gray color). Textured black area shows the natural population of wild *Pistacia vera* from the Kopet Dagh mountain range of southern Turkmenistan, and northern Afghanistan to the Khorasan district and Sarakhs region in northeastern Iran. (Source of blank map: http://onlinemaps.blogspot.com/2011_11_01_archive.html)

Fig. 10.2 Developmental stages of male and female inflorescences, and fruits of pistachio. (**a**) Male stage 1, beginning red, (**b**) Male stage 2, beginning open, (**c**) Male stage 3, full open, (**d**) Male stage 4, pollen, (**e**) Female stage 1, total brown, (**f**) Female stage 2, beginning green, (**g**) Female stage 3, extended green, (**h**) Female stage 4, opening, (**i**) Fruit set, (**j**) Rapid pericarp growth, (**k**) Fruit maturation, (**l**) Fruit ripening. (Photos: a–d by A. Sheikhi; e–l by L. Ferguson)

1981). The fertilized embryo or seed is the edible pistachio nut. Botanically, the pistachio fruit is a drupe with a hull, or pericarp, collectively the exocarp and mesocarp, and its hard-bony endocarp, the shell (endocarp) splits along its naturally preformed lateral suture when mature (Hormaza and Wünsch 2007).

10.2 Uses and Economic Importance

Within the *Pistacia* genus, *P. vera* is the only commercially-important species (Fig. 10.3). The other species which produce small inedible seeds are used as rootstocks but are also eaten fresh by local populations and processed into pickles, oil, chewing gum and soap (Kislev 1985). Among nut crops, pistachio ranks fifth, after cashews, walnuts, almonds and chestnut, in production. There are 639,296 ha of pistachio harvested with a production of 1,057,566 mt total in 2016 (FAOstat 2018). Currently the USA, Iran and Turkey are the major pistachio producing countries contributing over 84% of the world's pistachio production (Table 10.1) (Akbari et al. 2018a, FAOstat 2018).

The pistachio tree is referred to as *green gold* by Iranians due to its high economic value. Pistachio nuts are rich sources of essential nutrients for human health, proteins, vitamins, minerals, antioxidants and phenolic compounds (Akbari et al. 2018b; Aliakbarkhani et al. 2017; Ghrab et al. 2012; Tomaino et al. 2010). Pistachio nuts are also good sources of unsaturated lipids and shown to lower blood cholesterol, reduce cardiovascular diseases (Kris-Etherton et al. 2001) and blood glucose. The fruits, leaves, aerial parts and resins extracted from its stems, have multiple pharmacological and chemical uses as an antimicrobial, antioxidant and anti-inflammatory agent (Bozorgi et al. 2013; Tsokou et al. 2007).

10.3 Conventional Breeding and Hybridization

10.3.1 Current Challenges and Breeding Objectives

Given its rapid expansion in the western world, pistachios are still relatively unexamined genetically relative to other fruit trees. This suggests pistachios have excellent potential for genetic improvement to provide superior female and male cultivars and rootstock (Kallsen et al. 2009). Relying on a limited number of cultivars and rootstock, as in California, makes pistachio production vulnerable to new pests and diseases, such as mealybugs (*Ferrisia gilli* Gullan) (Ferguson and Haviland 2016) and pistachio bushy top syndrome (PBTS) (Stamler et al. 2015). Despite breeding efforts in different parts of the world (Chao et al. 1997; Kafkas and Kaska 1997; Mehlenbacher 2002; Parfitt et al. 1994; Vargas et al. 2001), most cultivars have undesirable characteristics such as a high percentage of unsplit and blank nuts, or

Fig. 10.3 Traditional and modern pistachio plantations. (**a**) and (**b**) Flood irrigated traditional orchards, (**c**) Nonbearing young modern orchard, (**d**) Fruit-bearing modern orchard, (**e**) Mechanical harvest in modern orchard, (**f**) Transportation of pistachios to processing plants. (Photos: a, b, f by A. Sheikhi; c, d, e by L. Ferguson)

extreme alternate bearing. Therefore, improvement of these physiological problems should be an important component of future breeding attempts (Hormaza and Wünsch 2007). Alternate bearing is a common challenge in almost all pistachio genotypes, during which the pistachio nut production fluctuates between an *on* year of heavy tree yields followed by an *off* year of little or no yields. In a region with concurrent alternate bearing, great economic losses occur during the off year which is very undesirable. The visible mechanism of alternate bearing is due to abscission of inflorescence buds (Crane and Nelson 1971).

Although the species used as pistachio rootstock are tolerant of saline and poor-quality soils, the best commercial production occurs in deep, well-drained, sandy

Table 10.1 The ten leading pistachio producing countries in the world

Ranking	Country	Production (mt)	Area harvested (ha)	Yield (kg.ha^{-1})
1	USA	406,646	96,720	4204
2	Iran	315,151	346,000	910
3	Turkey	170,000	60,814	2795
4	China	83,310	26,864	3101
5	Syria	56,833	55,406	1025
6	Greece	6338	3869	1638
7	Italy	3649	3848	948
8	Tunisia	3400	26,580	127
9	Afghanistan	2814	2311	1217
10	Spain	2418	6914	349

Source: FAOstat (2018)

loam soils with high lime content. In recent decades, soil salinization and alkalization, further exacerbated by drought and climate change, have negatively affected a large proportion of pistachio production in Iran (Taghizadeh-Mehrjardi et al. 2014). The California pistachio industry is having similar problems (Geisseler and Horwath 2016).

Pistachios evolved in high desert climates with extended hot summers and moderate winters. If grown in Mediterranean regions genotypes with lower chilling requirements are needed. Due to climate change and global warming, there is a serious concern in the pistachio producing areas due to lack of chilling hours (Benmoussa et al. 2017). Inadequate chilling causes unsteady low bud break out, delayed flowering, abnormal leaf form, decreased leaves and pollen amount, irregular sprouting of the buds, very low yield and late ripening (Akbari et al. 2018a). Moreover, insufficient chilling changes the bearing habit. The abnormal fruiting in the lateral and terminal axiles of the current shoots instead of on 1-year-old shoots, may happen in plants with unsatisfied chilling requirement (Crane and Iwakiri 1981). Insufficient heat accumulation increases the percentage of the unfilled, unsplit nuts (Rahemi and Pakkish 2009). Pistachios have specific chilling and heat requirements to break dormancy and bloom (Zhang and Taylor 2011). The chilling requirement is the number of hours at zero 0–7 °C (32–45 °F). Pistachio genotypes have different growing degree hours (GDH) requirements, with a range of 750–1400 h chilling and 8852–15,420 h heat. Persian cv. Kalle-Ghoochi requires lower chilling hours (750–950 h), and heat units (8852–9768 GDH). Ohadi and Ahmad-Aghaei cvs. require 1000–1250 h chilling and 10,656–13,320 GDH heat requirements. Akbari cv. requires 1200–1400 h chilling and 11,863–15,420 GDH heat units (Afshari et al. 2009; Rahemi and Pakkish 2009). Turkish cultivars have lower chilling requirements than Iranian cultivars. Uzun requires 400–600 h chilling and 10,800–11,200 GDH, Siirt needs 600–800 h chilling and 11,200–11,600 GDH, cv. Halebi needs 600–800 h chilling and 10,800–11,200 GDH and cv. Kirmizi needs 800–1000 h chilling and 10,400–10,800 GDH (Küden et al. 1994). For the Tunisian pistachio cv. Mateur, the

estimated chilling and heat requirements were about 600 h and 12,000 GDH, respectively (Salhi et al. 2013). The Kerman cv. grown in California, has an estimated chilling requirement of about 1000 h (Crane and Iwakiri 1981).

Flowering time is another pistachio breeding issue. Depending on the region, an early or later flowering genotype may be desirable. In the regions with late frosts and spring rains, late flowering is a desirable trait to avoid damaging bloom and the *Botrytis* and *Botryosphaeria* fungal infections (Chao et al. 2003).

Thus, the main breeding objectives and traits of interest of pistachio include:

Scion Breeding Objectives

(a) Increase in nut quality, size and appearance
(b) Reduction in percentage of blank or unfilled nuts
(c) Increasing the percentage of split nuts
(d) Increasing the green color of kernel at maturity
(e) Enhancing flavor and bioactive compounds
(f) High yield and productivity
(g) Precocity (early bearing)
(h) Low chilling requirement
(i) Abiotic stress (drought, salinity, cold, etc.) tolerance; pest and disease resistance
(j) Early season harvest (early fruit ripening)
(k) Reduced alternate bearing
(l) Introducing monoecious and hermaphrodite commercial cultivars
(m) Late flowering to avoid spring frost
(n) Shell-hinge strength (a successful pistachio cultivar will produce a nut that is split enough to open easily yet has enough shell-hinge strength to prevent it from falling apart in hulling or later in storage, transportation or the retail store).
(o) Minimum staining of the shell

Pollinizer Breeding Objectives

(a) Higher quantity of pollen
(b) Higher pollen viability and durability
(c) Extended bloom period
(d) Flowering precocity
(e) Large number of flower buds per cm of branch
(f) Overlap and uniformity in blooming with the female cultivar of interest
(g) Positive effects on nut quality

Rootstock Breeding Objectives

(a) Strong vigor and rapid growth
(b) Biotic stress (soil borne fungal disease, *Verticillium* spp., *Phytophthora* spp. and *Rhodococcus* spp. [causing pistachio bushy top syndrome], etc.) resistance.
(c) Resistance to nematodes
(d) Abiotic stress (drought, salinity, cold, heat tolerance, etc.) tolerance

(e) Compatibility with intended scion
(f) Higher micronutrient uptake efficiency
(g) Positive effect on scion performance (nut quality, scion vigor, alternate bearing, etc.)

10.3.2 Breeding and Cross-Pollination Techniques

Successful intra- and interspecific cross pollinations can be made between different cultivars and species of *Pistacia*. There is no fertilization barrier, except the difference in bloom time, to obtain F1 seedlings between *Pistacia* species such as *P. vera*, *P. eurycarpa* Yalt., *P. atlantica* Desf, *P. terebinthus*, etc. (Hormaza and Herrero 1998; Kafkas and Kaska 1997). Since pistachio is dioecious, and males and females may not necessarily bloom synchronically, pollen grains are collected and stored when female flowers are still closed. Staminate clusters are collected as soon as pollen shed is observed from the first open flowers (Fig. 10.2, male stage 3, full open and male stage 4, pollen) then they are dried on a paper sheet while preventing contamination from other pollen sources. Pollen grains are separated from the flowers using a 2 mm mesh, and then stored in a desiccator at 4 °C until used. Pistachio pollen germination and longevity varies considerably among male varieties (Parfitt et al. 2012). Pistachio pollen is only viable for less than 4 days at room temperature, about 3 weeks in a desiccator at 4 °C, and up to 8 months at −20 °C (Polito and Luza 1988). Parfitt et al. (2010) reported that initial pollen viability of 656 seedlings was 30% ± 23% SE with a value of 45% for cvs. Peters and 75% for Randy. After 4 weeks of storage, mean pollen germination for 622 seedlings was 2% ± 6% SE, 5% to 15% for Peters and 35% for Randy. Female flower buds should be bagged, before they are open (Fig. 10.2, before female stage 4, opening) in pollen and waterproof paper bags, sealed at the base with cotton batting fitted around the branch (Fig. 10.4a). Pollen should be introduced into the bag using a syringe when stigmas are receptive (Fig. 10.2, female stage 4, opening). Bags are removed 4 weeks after pollination when the stigmas are no longer receptive, and then replaced with breathable mesh bags to protect developing fruit from birds and animals (Fig. 10.4c). Nuts should be harvested in the fall when the hulls are easily separated (slipping) from the shells (Parfitt et al. 2012). Once the seeds are hulled and dried, they may be stored at 4 °C under desiccated conditions until they are planted in the next growing season. Before planting, seeds are water soaked for 12–24 h and stratified for 6 weeks at 4 °C.; thereafter, seeds are planted in forestry pots and transferred to the primary evaluation field when they are 40–60 cm tall. Pistachio has a long breeding cycle because of its long juvenile period (5–8 years), which can be shortened to 4–6 years by growing seedlings under ideal growing conditions without nutrient and water limitations (Parfitt et al. 2012). Superior pistachio genotypes with improved characteristics can be expected based on usable levels of heritability for most traits (Chao et al. 1997).

Fig. 10.4 Controlled cross-pollination in pistachio. University of California Davis, Wolfskill Experimental Orchards, Winters, California, 2018. (**a**) Female flower buds bagged in pollen and waterproof paper bags, (**b**) Pollen grains are poured into the bag using a syringe, (**c**) Breathable mesh bags to preserve the developing fruits, (**d**) Controlled pollination in pistachio germplasm. (Photos: A. Sheikhi)

10.3.3 Breeding and Hybridization Achievements

10.3.3.1 Female Cultivars

Most pistachio cultivars are chance seedlings collected by growers in primary pistachio-growing regions. In the past there was a major attempt at germplasm collection and evaluations in Iran, where there are more than 40 characterized cultivars of *Pistacia vera* (Ahmadi Afzadi et al. 2007) and hundreds of less documented varieties of this species cultivated by local growers, Among them cv. Akbari has almond-shaped large nuts but is highly susceptible to the common pistachio psylla (*Agonoscena pistaciae*), a minute insect pest. Ahmad-Aghaei is a high yielding cultivar with attractive white nuts and large size; this cultivar is highly alternate bearing. Ohadi (Fandoghi) is another Iranian cultivar (Fig. 10.5a), resistant to psylla with low alternate bearing tendency and medium yield. Cultivar Kalle-Ghoochi (Fig. 10.5b) bears large-sized nuts, is relatively high yielding but sensitive to spring frost.

Some pistachio cultivars have been released through breeding programs. In recent years there have been a few efforts to breed superior pistachio cultivars in Turkey (Kafkas and Kaska 1997), Spain (Vargas et al. 2001), Australia (Maggs 1990) and California (Chao et al. 1997; Parfitt et al. 1994). Breeding programs in Australia and California have been discontinued due to loss of funding (Parfitt et al. 2012). Cultivar Sirora was developed in Australia from a selection of Red Aleppo, by Don Maggs and Don Alexander at the CSIRO Merbein Laboratories. Sirora cv. seems to have a lower chilling requirement, higher yield and ripens 1 week earlier than cv. Kerman under Australia conditions. This cultivar has attractive green kernels, a character associated with superior taste and flavor, but has the drawback of being smaller than many of the other commercial cultivars (Maggs 1990). Kerman (Fig. 10.5c) was collected by W. E. Whitehouse in 1929, selected in 1936 and released for trial in 1957 by the USDA Plant Introduction Station in Chico, California. It has high yields and large nuts; however, it is not a perfect cultivar. It has a strong alternate-bearing tendency, a high percentage of blank and non-split nuts and minimal flavor. Kerman is considered a relatively late-ripening cultivar, which means that it can be exposed to the September flight of navel orangeworm moth. In regions of low heat unit accumulation, this cultivar may not mature until

Fig. 10.5 Pistachio female cultivars. (**a**) Fandoghi, (**b**) Kalle-Ghoochi, (**c**) Kerman. (Photos: a by A. Sheikhi; b, c by L. Ferguson)

after the first fall rains, increasing the risk of fruit fungal diseases. Damghan and Lassen cvs. were developed by Whitehouse from the same seed collection as Kerman from Iran. Damghan has very large nuts but very low yield under California conditions. Lassen has never been tested in replicated trials, but it is very similar to Kerman in nut characteristics and has good potential as a cultivar. Joley is another open-pollinated cultivar selected by J. Crane in 1980 at the University of California, Davis as seed from Damghan region in Iran. This cultivar was named in honor of the former director of the Chico USDA station; it matures 10 days earlier than Kerman. It is among the best tasting cultivars developed in California and its kernel is greener than that of Kerman. Smaller nut size, higher non-split percentage and low yield are drawbacks of this cultivar compared to Kerman. It does not appear to be a reliable cultivar for the pistachio industry; however, it can be used for breeding objectives (Parfitt et al. 2016).

The California Cultivar Development Program was begun in 1989 at the University of California, Davis. This program was a classical breeding and genetics program using controlled crosses (Parfitt et al. 1994). Three female cultivars, described below, were selected and released from this program. Golden Hills was released in 2005 from a cross made in 1990 between *Pistacia vera* seedling parents, female 2–35 × male ES#2. Golden Hills ripens 2 weeks earlier than Kerman so that growers can optimize the use of labor and harvest facilities, reduce navel orangeworm moth (*Amyelois transitella*) damage and associated aflatoxin contamination caused by the fungus *Aspergillus flavus* Link. Golden Hills also flowers 1–2 weeks before Kerman, and may have low chilling requirements and a higher percentage of split nuts, compared with Kerman (Parfitt et al. 2007). Lost Hills cv. was released in 2005 for distribution, and the cross that produced this cultivar was made in 1990 between the *P. vera* seedling parents, female 2–35 × male ES#6. This cultivar flowers from 1–2 weeks earlier than Kerman and is harvested 2 weeks before Kerman, permitting more efficient use of harvesting facilities when grown in combination with Kerman and avoiding the problems late harvest. Lost Hills also has a high percentage of split nuts and has a larger nut than Kerman. Lost Hills has loose kernels compared to Kerman. Loose kernels are not a desirable character for growers because more kernels may fall out during processing (Parfitt et al. 2008). Gumdrop, an open-pollinated progeny of female seedling B15-69, is a new female pistachio (*P. vera*) cultivar. B15-69 was developed as a part of California breeding program in the 1990s from a cross of female 2–35 × male ES#2, an early flowering male of unknown parentage. This cultivar produces gummy deposits on the fruit surface, hence the name. Gumdrop blooms about 5 days earlier than Golden Hills and 10 days earlier than Kerman. Gumdrop was selected because of its early ripening date; it can be harvested about 10–12 days earlier than Golden Hills and 24 days earlier than Kerman. Gumdrop, Golden Hills and Kerman cvs. comprise a harvest series, maturing over a month period. The early ripening of Gumdrop will permit pistachio growers to extend their harvest period and makes it less susceptible to insect damage from the navel orangeworm moth, a major pest of pistachio in California (Kallsen and Parfitt 2017b).

10.3.3.2 Male Cultivars

One of the well-known male cultivars is Peters which was found in the early 1900s by A. B. Peters at Fresno, California. It produces good quantity and quality of pollen that is shed over a relatively long period (about 2 weeks). However, under low-chill conditions of recent years it blooms later than cv. Kerman, resulting in poor pollination. Another less cultivated male cv. Chico was introduced from the USDA Plant Introduction Station in 1962. It was selected from seed introduced from Aleppo, Syria, probably of interspecific hybrid origin between *Pistacia vera* and *P. integerrima* Stewart. It produces a large quantity of pollen and blooms earlier than Kerman. However, it may have a negative effect on nut quality because of its interspecific origin (Parfitt et al. 2016). Randy, Famoso and Tejon, described below, are three male cultivars selected and released from the California Cultivar Development Program (Parfitt et al. 1994). Randy (*P. vera*) was released as a cross between *P. vera* seedling parents, female 2–35 × male ES#3 in California. Randy flowers 1 week earlier than Peters and is a superior pollinizer for early-season flowering cvs. like Golden Hills and Lost Hills. It is not recommended as a pollinizer for Kerman, except during seasons when Kerman and Peters bloom periods are asynchronous because of insufficient chilling hours (Parfitt et al. 2010). Famoso is a new male pistachio (*P. vera*) cultivar developed from a cross between female 2–35 × male ES#3 (an early flowering male of unknown parentage) parents in the University of California pistachio breeding program and released to replace cv. Peters in California cv. Kerman plantings. Famoso produces a large quantity of high initial pollen viability and a flowering period that is highly synchronized with the Kerman flowering period during both low-chill and normal years, whereas Peters often flowers much later and is unsynchronized with Kerman bloom period in years with low chilling accumulations. Famoso is a better pollinizer for Kerman (Kallsen and Parfitt 2017a). Another new male pistachio (*P. vera*) Tejon was selected as an open-pollinated offspring of female pistachio seedling selection B4-19, the cross- pollination having taken place in a pistachio breeding program trial plot near Bakersfield, California. It is believed that Tejon was the result of a pollination between the B4-19 female (a very early flowering selection) and Randy (the early flowering male variety). B4-19 is a female selection from a cross between the female 2–35 (*P. vera*) and an unknown *P. vera* male. Tejon produces many flowers and large quantities of durable pollens with a high germination percentage. Tejon is a very early flowering male cultivar, flowering 6–10 days before Randy. Tejon also has a synchronized bloom period with the female pistachio cv. Gumdrop. It seems that Tejon will provide the needed flowering overlap with Gumdrop and other early-flowering female cultivars during years with differing winter chilling accumulations. It is believed that use of low chill earlier flowering varieties will become increasingly common in the future as climate change continues to reduce average winter chilling hours (https://patents.justia.com/patent/PP28931).

10.3.3.3 Rootstocks

Although most *Pistacia* species can serve as rootstock, the most widely used around the world are *P. atlantica*, *P. integerrima* and *P. vera*. Iranian pistachio growers mostly rely on *P. vera* (cv. Badami-Riez Zarand) seedlings as rootstock because it is vigorous, and relatively tolerant to salinity, drought and *Phytophthora*. However, it is relatively sensitive to nematodes. Recently, high mortality rates of mature trees grown on this rootstock have been observed in the orchards of the Rafsanjan region (authors' personal observations), which could be due to changes in climate, and soil and water quality. Therefore, it is necessary to produce new rootstock tolerant to abiotic and biotic stresses. Recently, an organized breeding program was initiated at the Pistachio Research Center in Rafsanjan, Iran, from which promising rootstocks have been identified, such as: R-110 with increased tolerance to salinity; R-120 with increased tolerance to drought; H-142 with a high level of salinity, drought and *Phytophthora* tolerance; H-143 with tolerance to drought, nematodes and *Phytophthora* and H-144 with tolerance to drought, cold, nematodes and *Phytophthora*, which are under further evaluations.

During the early years of the California pistachio industry, *Pistacia atlantica* was used as the main rootstock to establish new orchards, until it showed susceptibility to *Verticillium* wilt, a fungal disease, and hundreds of thousands of trees on this rootstock declined or died. Genomic resources of *Verticillium* tolerance have been found among *P. integerrima* seedlings selected from an Iranian seedling tree planted at the USDA Plant Introduction Station in Chico, California. The selected *Verticillium* tolerant seedlings were quickly commercialized as Pioneer Gold I (PGI) and became the dominant rootstock in the rapidly expanding pistachio orchards of California. The most effective rootstock breeding effort has been conducted by Lee J. Ashworth, who introduced a new pistachio rootstock named University of California Berkeley 1, or UCB1. UCB1 is a hybrid of a specific *P. atlantica* female pollinized by a specific *P. integerrima* male (Fig. 10.6a). There has been an effort to produce the hybrid of a female *P. integerrima* and male *P. atlantica* named Pioneer Gold II (PGII) which is no longer commercially available under this name. However, the same cross named Platinum was produced by a commercial nursery (Ferguson and Haviland 2016). Currently, the California pistachio industry mainly relies on both seedling and clonal UCB1 rootstock (Fig. 10.7).

10.4 Molecular Breeding and Germplasm Biodiversity

Perennial woody plants, including fruit trees, typically require long breeding cycles, and the development of new commercial cultivars by plant breeders incurs considerable cost, energy, time and space to generate, maintain and evaluate breeding populations. For pistachio, as for other fruit trees, the main factor driving the length of the breeding cycle is the long juvenile phase. The juvenile phase for fruit crops has

Fig. 10.6 (**a**) Male (*Pistacia integerrima*, left) and female (*P. atlantica*, right) parents of University of California Berkeley 1 (UCB1) rootstock, Kearney Agricultural and Extension Center, Parlier, California, 2014, (**b**) *P. atlantica*, (**c**) *P. integerrima*, and (**d**) UCB1 seeds. (Photos: L. Ferguson)

Fig. 10.7 Pistachio rootstock production in California. (**a**) UCB1 seedlings ready for budding, (**b**) UCB1 seedlings 2 weeks after germination, (**c**) UCB1 seedlings 2 months after germination. (Photos: A. Sheikhi)

been variously reported to extend from at least 3 years in peach and almond to 15 or more years in avocado (Van Nocker and Gardiner 2014); this period is 5–8 years in pistachio (Hormaza and Wünsch 2007). Recently, it has become crucial to develop rapid-cycle breeding and efficient genotype selection pipelines to meet the fruit industry's rapidly changing demand for new superior cultivars. New advances in molecular plant breeding through the application of molecular biology or biotechnology, which includes marker-assisted selection (MAS), genome wide association studies (GWAS), genomic selection (GS) and genetic transformation, have the potential to reduce the juvenile phase and decrease the length of the breeding cycle in fruit crops (Iwata et al. 2016). However, novel technologies are just beginning to be applied towards the study of intra- and interspecific phylogeny, and marker-assisted breeding in pistachio.

10.4.1 Intraspecific Genetic Diversity

Several studies have been conducted concerning the genetic relationships of *Pistacia vera* cultivars. Identification of pistachio cultivars has been traditionally carried out using morphological traits (Zohary 1952). Since morphological markers may be influenced by environment, developmental stage and gene expression, later studies focused on the use of molecular markers for more reliable identification of pistachio cultivars. The evolution of molecular marker use in pistachio, like in many plants, began with isozymes, then restriction fragment length polymorphisms (RFLPs), non-coding chloroplast DNA, random amplified polymorphic DNA markers (RAPDs), amplified fragment length polymorphisms (AFLPs), inter simple sequence repeats (ISSRs), sequence-related amplified polymorphism (SRAPs), and progressed to sequence characterized amplified region (SCARs), simple sequence repeats (SSRs) and finally single nucleotide polymorphisms (SNPs). There has been some effort to characterize pistachio cultivars using isozyme markers (Aalami and Nayeb 1996; Barone et al. 1993, 1996; Dollo 1993; Loukas and Pontikis 1979; Rovira et al. 1994). However, their usefulness is affected by insufficient polymorphism among closely-related cultivars.

More recently, several types of DNA-based molecular markers in pistachio have been developed to fingerprint cultivars and to perform a more objective identification of genotypes (Hormaza and Wünsch 2007). Randomly amplified polymorphic DNA (RAPD) were first applied as DNA markers in pistachio by (Hormaza et al. 1994a). They used 33 RAPD primers to analyze 15 pistachio cultivars originating from Syria, Iran, Tunisia, Italy, Greece, Azerbaijan and the USA. UPGMA cluster analysis grouped the cultivars into two major clusters according to their geographical origin. The first major cluster was an Iranian-Caspian group, comprising cultivars originating from east of the Zagros Mountains. These are primarily of Iranian origin: cvs. Iran Large (Iran); Gazvin, Damghan and Rashti (selected in Israel from

seed collected in Iran); Kerman (selected in the USA. from seed collected near the city of Rafsanjan in Iran); O2-16 and O2-18 (selected in the USA. from seed collected in the Aspheron Peninsula in southern Azerbaijan close to northern Iran) and Peters (selected in the USA from a seedling tree of unknown origin growing in Fresno, California). The second major cluster was a Mediterranean group consisting of cultivars originating from the Mediterranean region: Bronte and Trabonella (Italy), Aegina (Greece), Sfax (Tunisia), Red Aleppo (Syria), and Ask and Nazareth (selected in Israel from seed collected in Syria). Kerman is related to Iranian-Caspian cultivars, which is consistent with its breeding history using Iranian materials imported through the USDA Plant Introduction Garden at Chico, California. Further studies by these authors confirmed the previous study (Hormaza et al. 1998); however, Caruso et al. (1997) could not separate the Mediterranean from the Iranian-Caspian genotypes using morphological descriptions and RAPD fingerprinting analysis.

Two separate populations of *Pistacia vera* in Turkmenistan have been characterized using RAPD markers. UPGMA cluster analysis divided 24 of the 27 accessions into two main groups (Kepele and Aghachli) according to their origins (Barazani et al. 2003). RAPD markers have also been used more recently to study the intraspecific diversity of *P. atlantica* (Kafkas 2005), *P. khinjuk* Stocks. (Karimi et al. 2012) and *P. vera* (Aliakbarkhani et al. 2015). RAPD markers have been widely used for genetic diversity, phylogeny and mapping studies due to ease of development and implementation and to cost- and time-effectiveness. However, drawbacks of this technique include dominant inheritance and low reproducibility between laboratories and technical replicates, which has motivated the development of several alternative molecular markers. Amplified fragment length polymorphism (AFLP) markers were also successfully applied for analysis of pistachio varieties from Afghanistan (Kafkas 2006), Syria (Basha et al. 2007) and Iran (Ahmadi Afzadi et al. 2007). Inter simple sequence repeats (ISSRs) can reveal diversity in the numerous microsatellite regions using single long oligonucleotide primers that may be anchored with one or two nucleotides on either end of a repeat region and extend into the flanking region (Esselman et al. 1999). ISSRs have been used for the study of genetic polymorphism of pistachio cultivars in Algeria (Kebour et al. 2012) and Iran (Noroozi et al. 2009; Tagizad et al. 2010). Sequence-related amplified polymorphism (SRAP) markers preferentially amplify open-reading frames and were used by Guenni et al. (2016) to study the genetic diversity of Tunisian pistachio. The authors screened 43 *P. vera* accessions using seven SRAP primer pairs. Non-coding regions of chloroplast DNA provide a practical opportunity to evaluate genetic diversity and to discriminate pistachio cultivars (Choulak et al. 2017). Genetic diversity of 15 pistachio varieties sampled in three areas of Tunisia were studied using sequence variation in the intergenic spacer between chloroplast genes trnL and trnF. The results revealed nine different haplotypes and three geographic groups (Choulak et al. 2017).

Microsatellites or simple sequence repeats (SSRs) are abundant and well-distributed markers throughout the nuclear genomes of eukaryotes. They are mostly

present in non-coding DNA, where they can accumulate mutations with less selective constraint than in coding DNA. SSRs as highly informative, reproducible, codominant, and multi-allele genetic markers, have been one of the most reliable molecular marker systems widely used for germplasm discrimination, genetic diversity evaluation and marker-assisted breeding (MAB) of fruit tree crops over the past 10 years (Bernard et al. 2018; Singh et al. 2010). Ahmad et al. (2003), developed and tested 25 SSR primer pairs for identification of pistachio cultivars from Iran, Turkey, Syria and the USA, using shell and kernel DNA to reveal the identity of the pistachios sold in the grocery store. Kolahi-Zonoozi et al. (2014) reported the development of 12 SSR markers isolated from a repeat sequence-enriched genomic library of *Pistacia vera*, and assessed genetic structure and diversity among 45 commercially-important Iranian cultivars. They found that the Momtaz cultivar collected from two different regions (Naseriyeh and Rafsanjan) clustered in two different groups. This might be an indication of homonyms (i.e. accessions with a same name, but genetically different). Similarly, SSR markers have also been applied to characterization of 61 genotypes of *P. atlantica* ssp. *atlantica* natural populations in northwestern Algeria (El Zerey-Belaskri et al. 2018), and to investigate the genetic diversity of 42 pistachio (*P. vera*) cultivars in Iran (Khadivi et al. 2018).

10.4.2 Interspecific Genetic Diversity

Interspecific genetic relations of *Pistacia* species have been studied using different morphological (Zohary 1972), isozyme (Dollo 1993) and DNA markers (Kafkas and Perl-Treves 2001; Karimi and Kafkas 2011; Karimi et al. 2009; Parfitt and Badenes 1997). The most complete taxonomic classification of *Pistacia* species, using morphological characteristics, was performed by Zohary (1952). He classified 11 *Pistacia* species into four sections: Lentiscella (*P. mexicana* H.B.K., *P. texana* Swingle); Eu-Lentiscus (*P. lentiscus* L, *P. weinmannifolia* Poisson, *P. saportae* Burnat.); Butmela (*P. atlantica*) and Eu-Terebinthus (*P. terebinthus*, *P. vera*, *P. palaestina* Bois., *P. khinjuk, P. chinensis* Bge.). The first classification of *Pistacia* species at the molecular level was reported by Parfitt and Badenes (1997). In an analysis of chloroplast genome RFLPs that classified the genus into two sections: Terebinthus, including deciduous species with imparipinnate leaves and large seeds and Lentiscus including evergreen species with a paripinnate leaflet arrangement and smaller seeds. In further studies, Kafkas and Perl-Treves (2001) and Kafkas (2006), using RAPD and AFLP markers, divided the genus into two main sections: the first section included species with single-trunked and large trees, and the second section included species with small trees or shrubs.

Pistacia vera var. Sarakhs, which is considered a wild relative of commercial pistachio cultivars, occurs in forests in the Sarakhs border region of Iran and Turkmenistan. Genetic relations of *P. vera* var. Sarakhs with other cultivars of *Pistacia* have been widely analyzed using AFLP (Ahmadi Afzadi et al. 2007),

SSR (Arabnezhad et al. 2011), SRAP (Talebi et al. 2012) and non-coding chloroplast DNA (Talebi et al. 2016) markers, suggesting that Iranian *P. vera* cultivars are evolved from *P. vera* var. Sarakhs. Hence, it appears that the wild Sarakhs variety plays an important role in the evolutionary history of the cultivated pistachio and may be an important source of new alleles for pistachio breeding programs.

10.4.3 Cytogenetic Evaluations

Chromosomal and karyotypic information are valuable to plant breeders and cytogeneticists in both plant taxonomy and phylogenetic analysis. The cytogenetics and chromosome numbers of *Pistacia* have been widely studied. The *Pistacia* genome size estimation was performed by Zerey-Belaskri et al. (2018) using flow cytometry on 97 samples. The results showed a very small genome size (2C = 1.21 ± 0.02 pg) for this species. Similarly, Siljak-Yakovlev et al. (2010) reported a small genome size for *P. lentiscus* (2C =1.19 ± 0.03 pg) and *P. terebinthus* (2C = 1.14 ± 0.02 pg). All species of *Pistacia* are diploid (El Zerey-Belaskri et al. 2018; Sola-Campoy et al. 2015) with the basic chromosome numbers of x = 12, 14 and 15 (Zohary 1952). Polyploid varieties have not been reported in *Pistacia* so far (Ghaffari et al. 2005). Chromosome numbers reported for different *Pistacia* species are as follows: *P. vera*, 2n = 30 (Ayaz and Namli 2009; Ghaffari et al. 2005; Harandi and Ghaffari 2001; Ozbek and Ayfer 1958); *P. atlantica*, 2n = 28 (Ghaffari and Fasihi-Harandi 2001; Ghaffari et al. 2005; Ozbek and Ayfer 1958), 2n = 30 (El Zerey-Belaskri et al. 2018); *P. khinjuk*, 2n = 24 (Ghaffari and Fasihi-Harandi 2001), 2n = 30 (Ozbek and Ayfer 1958); *P. terebinthus*, 2n = 30 (Ozbek and Ayfer 1958); *P. lentiscus*, 2n = 24 (Zohary 1952), 2n = 30 (Natarajan 1978); *P. chinensis*, 2n = 24 (Huang et al. 1986, 1989) and *P. integerrima*, 2n = 30 (Sandhu 1988).

The divergence and controversy in chromosome count in the genus *Pistacia* might be due to karyotyping errors. Such controversies in chromosome counting have been attributed to the small size of *Pistacia* chromosomes and to the fact that only a few cell divisions are visible in the root tip (Sola-Campoy et al. 2015). Ila et al. (2003) performed chromosome counts for *P. vera*, *P. terebinthus*, *P. atlantica* and *P. eurycarpa*. These authors have reported the same chromosome numbers of 2n = 30 for four *Pistacia* species, and basic chromosome number of x = 15 for all these species. This was the first study that reported chromosome numbers of *P. eurycarpa* and *P. atlantica* as 2n = 30. These results were confirmed by Al-Saghir (2010) and Al-Saghir et al. (2014) who reported that all pistachio species have the same basic chromosome number x = 15.

10.4.4 Marker-Assisted Selection

The most extensively studied trait in pistachio at the genetic and molecular level is the sex determination mechanism. *Pistacia* species are all dioecious; however, some cases of exceptional sex types have been reported. Ozbek and Ayfer (1958) found hermaphrodite (with male and female organs borne in the same flowers) trees of *P. vera*, or hybrids between *P. vera* and *P. terebinthus,* near the Antep province of Turkey. Crane (1974) described monoecious trees of *P. atlantica* and hybrids between *P. vera* and *P. atlantica.* A wild population of *P. atlantica* with several monoecious trees in the Yunt Mountains, Manisa Province, Turkey has been reported by Kafkas et al. (2000). Three monoecious phenotypes have been described by these authors: (i) fully monoecious trees with a mixture of male and female inflorescences, (ii) trees with several branches of staminate flowers, while the remaining branches bore pistillate inflorescences and (iii) trees with inflorescences of both sexes on several branches, and pistillate inflorescences on the remaining branches. Dioecy, where 50% of the progeny are male and 50% female, along with a very long juvenile period are main limiting factors of pistachio breeding programs. The dioecious character of *P. vera* is a breeding disadvantage, because nut characters of pollen parents cannot be evaluated directly. In addition, male plants represent half of the progeny in breeding populations, resulting in a considerable waste of time, labor, land and breeding resources (Kafkas et al. 2015; Khodaeiaminjan et al. 2017; Turkeli and Kafkas 2013).

Marker-assisted selection (MAS) of female progeny could be an important tool for pistachio scion and rootstock breeding programs. It has been difficult to identify sex in pistachio at seedling stage due to the lack of morphological markers. Thus, early detection of pistachio seedling gender by MAS is expected to help pistachio breeders with cost-effective management of germplasm collections and evaluation blocks. To date, several studies have attempted to develop of sex-linked DNA markers in pistachio species. Hormaza et al. (1994b) tested 700 decamer primers using bulked segregant analysis in order to identify sex-linked RAPD markers in *Pistacia vera* and found one sex-linked primer (OPO08945, CCTCCAGTGT) that produces a 945 bp band only in females. The low frequency of sex-linked bands suggests that a small genomic region is involved in sex determination in *Pistacia*. However, this RAPD marker was unable to distinguish between male and females in *P. vera* (Ehsanpour and Arab 2010) and in other *Pistacia* species (Hormaza et al. 1994b). Kafkas et al. (2001), screened 472 RAPD markers and found one male-specific (BC156, GCC TGG TTG C) and one female-specific (BC360, CTC TCC AGG C) in *P. eurycarpa*, and one female-specific (OPAK09, AGG TCG GCG T 39) marker in *P. atlantica*. Generating a sequence characterized amplified region (SCAR) marker from RAPD markers may yield markers that are more reliable and transferable among species. Yakubov et al. (2005) converted the OPO-08945 RAPD primer into a SCAR marker in combination with the Touchdown-PCR technique, which amplified a specific female 297-bp product. Esfandiyari et al. (2012) utilized RAPD and SCAR markers to identify a marker closely linked to sex loci in wild *Pistacia* species. According to the authors, the BC1200 (GCCTGATTGC) primer was found to amplify a female-specific band.

10.4.5 Application of New High-Throughput Sequencing Technologies in Pistachio Breeding

Accelerated breeding of superior pistachio cultivars using high-throughput sequencing technologies will require genome sequencing and assembly, saturated linkage maps and cost-efficient methods for genome-wide genotyping of hundreds or thousands of individuals. The past two decades have seen remarkable advances in genomic technologies and a rapid decrease in the cost and labor needed to sequence plant genomes. These advances have the potential to revolutionize the breeding of horticultural crops by reducing the length of the breeding cycle and by increasing selection accuracy and the efficiency of new cultivar development (He et al. 2014; Van Nocker and Gardiner 2014). Pistachio genome sequencing and assembly have been conducted by Kafkas et al. (2017) for the Siirt cv. using the Illumina sequencing platform. Assembly of the Kerman cv. is also in process by these researchers. A first genome survey of pistachio (*Pistacia vera*) was conducted by Motalebipour et al. (2016), using whole genome shotgun sequencing, and reported a genome size of 600 Mb for *P. vera*. The authors found 59,280 SSRs in the assembled genome, and a total of 206 polymorphic SSR loci were developed from which 41 were polymorphic in all 6 *Pistacia* species. The first genetic linkage map of pistachio was generated by Turkeli and Kafkas (2013) using ISSR, SRAP and AFLP markers. Restriction site-associated DNA sequencing (RAD-seq) has also been applied to identify sex-linked markers and to reveal the sex determination system in pistachio. Subjecting the genotypes to RAD sequencing in two lanes of a Hi-Seq 2000 sequencing platform generated 449 million reads, comprising approximately 37.7 Gb of sequence and yielding 33,757 single nucleotide polymorphisms (SNPs) between the parents. Among these SNPs, eight loci could distinguish sex with 100% accuracy in pistachio. Because females were heterozygous for all candidate SNPs, a ZZ/ZW sex determination system, in which females are the heterogametic sex, was reported for the first time in pistachio (Kafkas et al. 2015). Khodaeiaminjan et al. (2017) developed and analyzed sex-linked single nucleotide polymorphisms (SNPs) and expressed sequence tag-derived simple sequence repeats (EST-SSRs) to construct a linkage map of the sex chromosome in pistachio. The resulting consensus map showed a total length of 65.19 cM with the sex locus in the center of the chromosome at 31.86 cM.

10.5 In Vitro Culture Approaches

10.5.1 Micropropagation Without Integrating Callus Phase

Micropropagation has an important role in large-scale production of genetically improved and pure clones of fruit tree cultivars and rootstocks. The development of reliable protocols for micropropagation of fruit tree scions or rootstocks which are

recalcitrant to conventional vegetative propagation, would enable mass production demands to be satisfied. Hence, in vitro propagation has become indispensable for the mass propagation of genetically superior material released in fruit tree genetic improvement programs for the commercialization of new fruit crops cultivars (Akdemir et al. 2014; Arab et al. 2014a, b, 2016, 2017, 2018a, b; Benmahioul 2017; Benmahioul et al. 2012a; Garoosi et al. 2016; Jamshidi et al. 2016; Kılınç et al. 2015; Marín et al. 2016; Nezami-Alanagh et al. 2017; Ozden-Tokatli et al. 2005; Yıldırım 2012).

Propagation of pistachio is extremely difficult, and it is considered recalcitrant to conventional vegetative propagation, even when young plant materials are used in the in vitro culture process (Akdemir et al. 2014; Benmahioul 2017; Benmahioul et al. 2012a; Garoosi et al. 2016; Kılınç et al. 2015; Marín et al. 2016; Nezami-Alanagh et al. 2017; Ozden-Tokatli et al. 2005; Yıldırım 2012). Conventionally, pistachios have been propagated by different methods such as seeds and grafting onto seedling rootstocks to assist the development of pistachio plantations. Traditional propagation by germinating seeds is not appropriate for establishing commercial orchards because seedlings are not true to type. Also, grafting has not been successful to meet the demand of pistachio cultivars grafted onto suitable rootstocks (Marín et al. 2016). Therefore, the development of a reliable pistachio micropropagation system is the most commercially-feasible method for mass production of true-to-type plantlets within a short space of time (Akdemir et al. 2014; Benmahioul 2017; Kılınç et al. 2015; Marín et al. 2016; Nezami-Alanagh et al. 2017). Several micropropagation studies have been carried out on different pistachio species using various plant materials such as explants excised from in vitro grown juvenile seedlings obtained by germinating the seeds and mature plants, different media culture and different plant growth regulators (PGR). Some problems in the micropropagation process have been reported such as tissue browning in establishment and proliferation steps, shoot tips necrosis (STN), low shoot quality, lack of branching, vitrification and leaf yellowing (Abousalim 1990; Abousalim and Mantell 1994; Akdemir et al. 2014; Baninasab and Mobli 2002; Barghchi and Alderson 1985; Benmahioul 2017; Benmahioul et al. 2009, 2012a, b, 2015; Can et al. 2006; Dolcet-San Juan and Clavería 1995; Garoosi et al. 2016; Kılınç et al. 2015; Marín et al. 2016; Mobli and Baninasab 2009; Nezami et al. 2015; Nezami-Alanagh et al. 2017; Onay 2000a, b; Ozden-Tokatli et al. 2005; Picchioni and Davis 1990; Pontikis 1984; Tabiyeh et al. 2005, 2006; Tilkat and Onay 2009; Tilkat et al. 2012).

Since in vitro multiplication of pistachio is difficult, in vitro culture of pistachio has been initiated from germinated seeds and explants originating from in vitro seedlings subsequently used as a plant material for pistachio micropropagation process. In numerous studies different parts of pistachio seedlings obtained by germinating the seeds have been micropropagated on various media and plant growth regulator including Kerman cv. *Pistacia vera* and UCB-1 hybrid pistachio through shoot tips from juvenile 1-3-year-old seedling culture on modified Driver-Kuniyuki walnut (Driver and Kuniyuki 1984) medium (Parfitt and Almehdi 1994);

Pistacia rootstocks such as *P. khinjuk*, *P. atlantica*, *P. terebinthus* and *P. integerrima* via meristem and shoot culture on MS (Murashige and Skoog 1962) medium containing 4 mg l^{-1} 6-Benzylaminopurine (BAP) (Çetiner et al. 1996); *P. atlantica* rootstock using apical or axillary bud explants culture on a modified Quoirin and Lepoivre (1977) supplemented with BAP plus naphthaleneacetic acid (NAA) (Mederos et al. 1997); khinjuk pistachio (*P. khinjuk*) through seedling apical shoot-tip culture on MS medium with Gamborg vitamins 1 mg l^{-1} N6-benzyladenine (BA) (Tilkat et al. 2005); pistachio (*P. vera* cv. Kirmizi) through nodal explants culture on MS medium containing different cytokinins and silver nitrate ($AgNO_3$) (Ozden-Tokatli et al. 2005); *P. lentiscus* from axenic seedling-derived explants culture on MS medium supplemented 1 mg l^{-1} BA (Yıldırım 2012); pistachio (*P. vera*) nodal stem segments excised from 30-day-old aseptic seedling culture on MS containing Gamborg (B5) vitamins and supplemented with 4 mg l^{-1} (BA) (Benmahioul et al. 2012a, b); *P. vera* Siirt, *P. atlantica* and *P. khinjuk* via shoot tips and nodal buds culture on MS medium containing 4 mg L^{-1} BA and 0.1 mg L^{-1} GA3 in a temporary immersion bioreactor system (RITA) (Akdemir et al. 2014); genotypes of lentisk, *P. lentiscus*, through shoot tip explants (Kilinç et al. 2015) and *P. vera* via nodal explants on MS medium supplemented different cytokinins and various concentrations (Benmahioul et al. 2016). Also, a few studies have used adult material as an explant in micropropagation of pistachio such as *P. vera* cultivars: Mateur on MS medium (Abousalim and Mantell 1992, Dolcet-Sanjuan and Claveria 1995); Antep via nodal segments on MS medium (Onay 2000a, b); Siirt (Onay et al. 2004); male pistachio (*P. vera*) cv. Atli on MS medium (Fig. 10.8) (Akdemir et al. 2016; Tilkat et al. 2008); male pistachio (*P. vera*) cv. Atli and *Pistacia* rootstocks (*P. khinjuk*, *P. atlantica*) on MS medium in a temporary immersion bioreactor system RITA (Akdemir et al. 2014); and Ghazvini cv. on different modified media (Garoosi et al. 2016; Nezami-Alanagh et al. 2017).

Sometimes explants excised from in vitro grown juvenile seedlings were obtained by germinating the seeds and from mature plants, have been used. For example, in a study by Vatan Pur Azghandi et al. (2008) nodal explants of adult trees (15–20 years old) of six different pistachio rootstocks including *Pistacia vera* cv. Badami-Riez Zarand, *P. atlantica*, *P.atlantica* ssp. *mutica* F&M, *P. khinjuk*, *P. integerrima* and UCB1, and in vitro germinated seeds of these rootstocks, and then these explants were cultured on DKW medium containing with 2 mgl^{-1} BAP. In another study, to control shoot-tip necrosis, nodal segments of clonal UCB1 originating from in vitro culture (*P. integerrima* × *P. atlantica*) were cultured in modified MS medium containing Gamborg vitamins and 2 mg l^{-1} (BA) (Kermani et al. 2017; Nezami et al. 2015).

In conclusion, the efficacy of pistachio micropropagation is affected by many factors, including genotype, type of explant, culture medium, plant growth regulators, different combinations of growth regulators and agar. Therefore, different species have different nutrient and plant growth regulation requirements.

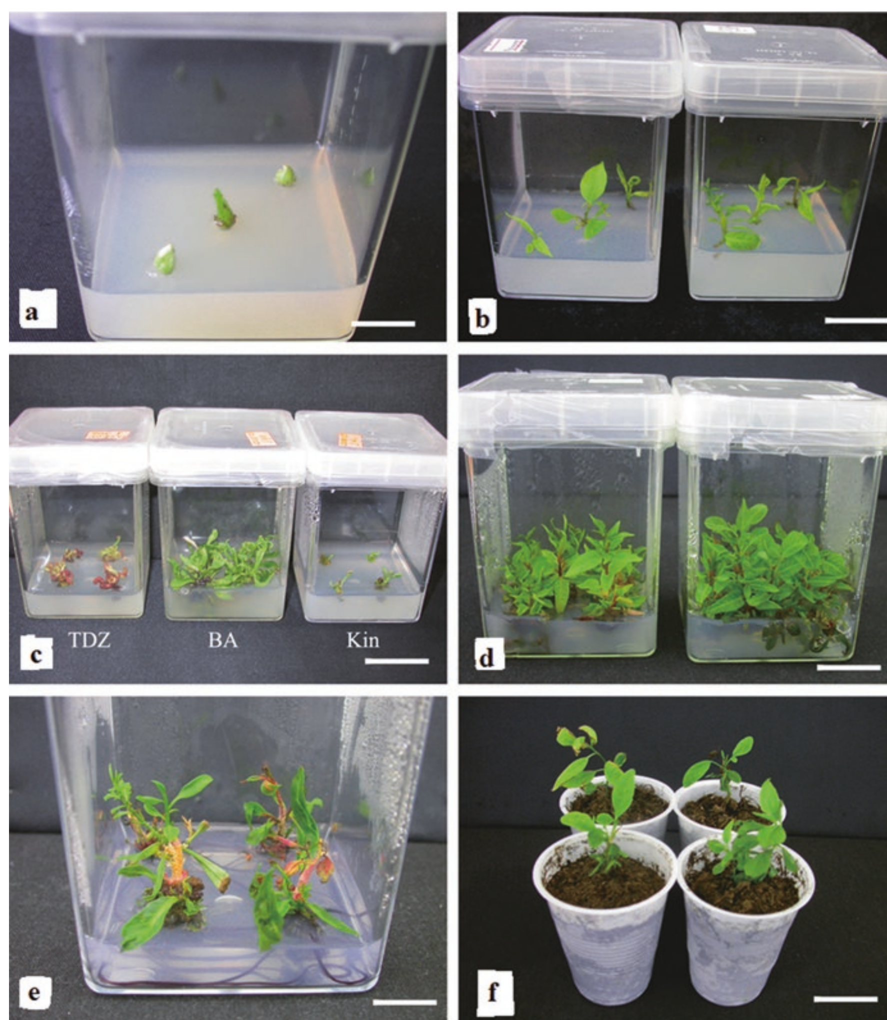

Fig. 10.8 Micropropagation of mature male pistachio (*Pistacia vera* cv. Atli). (**a**) Establishment of shoot tip cultures on MS medium, (**b**) Shoots cultured on MS medium containing BA 1 week after culturing, (**c**) Shoot multiplication on MS medium containing BA (middle), thidiazuron (TDZ; left), or kinetin (Kin; right), (**d**) Multiple shoot proliferation on MS medium, (**e**) in vitro rooting of microshoots 3 weeks after culturing on MS medium, (**f**) Plantlets derived from microshoots. (Source: Tilkat et al. 2008)

10.5.2 Micrografting

One of the major drawbacks of pistachio plantations is the lack of suitable clonal rootstocks which are well adapted to different environmental conditions. Although there are available some vigorous rootstocks for pistachio, i.e. the UCB1 hybrid

(*Pistacia atlantica* × *P. integerrima*) or *P. atlantica*, these are inadequate to meet the needs of the major pistachio production areas because they lack adaptation to cold winters and dry summers (Ferguson et al. 2005). Also, pistachio (*P. vera*) cultivars need to be grafted onto appropriate rootstocks, such as the terebinth tree (*P. terebinthus*) for their propagation. Therefore, developing of new grafting techniques is necessary (Marín et al. 2016).

In various previous in vitro culture studies of pistachio, grafting techniques for different purposes have been applied, including micrografting mature cultivar scions onto in vitro germinated seedlings of *Pistacia vera* (Abousalim and Mantell 1992); in vitro micrografting of mature pistachio (*P. vera* cv. Siirt) onto in vitro juvenile seedling rootstocks for rejuvenation purposes (Onay et al. 2004); in vitro micrografting of the shoot tip explants of pistachio (*P. vera* cv. Siirt) taken from in vivo grown trees on juvenile seedling originated from in vitro wild pistachio rootstocks (*P. atlantica* ssp. *mutica*, *P. terebinthus*, *P. atlantica*, *P. khinjuk*) (Can et al. 2006) and rejuvenation of mature lentisk (*P. lentiscus*) by micrografting (Onay et al. 2016). Recently, a novel method for propagation of recalcitrant pistachio cultivars has been developed. In this study a simple and reliable technique has been used to reduce large production of pistachio by grafting shoot tips originating from in vitro cultures onto 4- week-old terebinth (*P. terebinthus*) seedlings grown in pots (Marín et al. 2016).

10.5.3 Temporary Immersion System (TIS)

Commercial-scale mass propagation of pistachio plants and rootstock by in vitro culture in conventional agar-based semi-solid media is labor intensive and costly. Recently, in vitro culture studies in many plant species have focused on liquid cultures because its advantages include the reduction of cost and labor requirements, as well as an increase of in vitro multiplication rates. In order to commercialize the in vitro culture process in diverse plant species, different system have been used, including the application of shaking cultures, bioreactors and temporary immersion systems (TIS) (Akdemir et al. 2014). In one study, micropropagation of the male pistachio cv. Atlı and *Pistacia* rootstocks (*P. khinjuk*, *P. atlantica*) using juvenile and mature nodal bud explants have been done via a temporary immersion bioreactor system (RITA) and results revealed that this system could be used for large-scale propagation of pistachio (Akdemir et al. 2014).

10.5.4 Pistachio Media Culture Formulation Using Mathematical Modeling

Micropropagation is a reliable technique that plays a critical role in large-scale production of pistachio, and researchers have claimed considerable success with this technique (Nezami-Alanagh et al. 2017). However, the responses of pistachio

and its rootstocks to in vitro multiplication vary significantly. For instance, some pistachio and its rootstocks have exhibited shoot tip necrosis (Abousalim and Mantell 1994) and disorders to macro- and micronutrients of standard culture media (Abousalim 1990; Benmahioul et al. 2012a, b; Kılınç et al. 2015; Onay 2000a, b; Tilkat et al. 2005, 2006). According to the literature, MS Medium (Murashige and Skoog 1962), is known as a common medium in plant micropropagation, but this medium is not appropriate for micropropagation of pistachio and its rootstocks. Therefore, some pistachio researcher have recently focused on optimization of the culture medium and plant growth regulators (PGRs) for in vitro culture (Nezami-Alanagh et al. 2017). Optimization of MS media was employed by very different methods to overcome shoot-tip disorder (STN), calcium gluconate or Ca-enriched media e.g. DKW have been used in pistachio micropropagation (Abousalim and Mantell 1994). Recently, in *Pistacia atlantica and* in *P. vera* cv. Kirmizi, $AgNO_3$ up to 48 μM (8 mg l^{-1}) has been used to improve shoot regeneration and growth, and to prevent base callus formation (Ozden-Tokatli et al. 2005). Also, in several studies, the MS medium was adjusted by changing the level of plant growth regulator and vitamins (Akdemir et al. 2014; Dolcet-Sanjuan and Claveria 1995; Mederos et al. 1997).

Biological systems with high levels of interactions between chemical nutrients and the various nutrition necessities of the tissue culture process are difficult to understand and model. Because of the complexities of their systems, and their non-linear nature, high-throughput modeling systems can be used as powerful tools for culture medium optimization (Arab et al. 2016, 2017, 2018; Jamshidi et al. 2016; Nezami-Alanagh et al. 2017).

In recent decades, computer-aided techniques have emerged as promising methods to model high dimensional and non-linear processes (Arab et al. 2016, 2017, 2018; Jamshidi et al. 2016; Nezami-Alanagh et al. 2017). In order to achieve optimal protocols for micropropagation of *Pistacia vera*, artificial intelligence techniques, specifically artificial neural networks (ANNs) in combination with fuzzy logic (neurofuzzy logic) or with genetic algorithms (ANNs-GA), have been applied to predict and model, and to optimize the effect of the key ingredient combinations on growth parameters. The results of this study showed that artificial intelligence technology could be used as a powerful tool for media formulation and will improve large-scale multiplication of pistachio (Nezami-Alanagh et al. 2017).

10.5.5 Regeneration Systems

In vitro culture facilitates rapid multiplication of elite pistachio scion/or rootstock, and is a prerequisite for genetic improvement of pistachio via genetic engineering techniques. In vitro culture techniques such as organogenesis and somatic embryogenesis have been exploited in fruit crop genetic improvement programs through

genetic engineering, as well as large-scale micropropagation of selected superior clones (Benmahioul et al. 2009; Onay 2000a, b; Onay et al. 1995, 1996; Tilkat and Onay 2009; Tilkat et al. 2009).

Many factors have been affected direct (without callus formation) and indirect organogenesis and somatic embryogenesis of pistachio and its rootstock; namely, genotype, the type of explant tissue, physiological and chronological age of explants, light and photoperiod conditions, plant growth regulators, culture media and many others (Abousalim 1990; Barghchi 1982; Benmahioul et al. 2009; Kitto and Mcgranahan 1992; Onay 2000a, b; Onay et al. 1995, 1996; Tilkat and Onay 2009; Tilkat et al. 2009).

10.5.5.1 Organogenesis

Direct regeneration from explants is desirable, especially in modern breeding where increasing rapidity and reducing costs of regeneration are essential. There are some reports of adventitious regeneration from various tissues such as direct plant regeneration from mature leaves (Tilkat et al. 2009); as well as direct plant regeneration from in vitro-derived mature leaves (Tilkat and Onay 2009) (Fig. 10.9).

Organogenesis refers to plant regeneration by organ formation on explants or from cell masses. Within the last three decades, several researchers have focused on organogenesis to clone tissues of elite mature pistachio trees (Abousalim 1990; Barghchi 1982; Martinelli 1985; Onay 1996). Some protocols for clonal propagation of mature elite pistachio using organogenesis has been reported (Onay et al. 2000).

10.5.5.2 Somatic Embryogenesis

Somatic embryogenesis is the formation of embryos from cells other than gametes and is an alternative method of in vitro production of fruit tree crops which are recalcitrant to regeneration through organogenesis (Benmahioul et al. 2009; Onay 2000a, b; Onay et al. 1995, 1996).

Micropropagation by axillary budding is the ideal technique for the commercial propagation of hardwood species and is also considered the most suitable for ensuring the genetic fidelity of the regenerated plantlets (Benmahioul et al. 2009; Onay 2000a, b). Large-scale production of elite fruit cultivars is the first goal of optimization of somatic embryogenesis systems, followed by using somatic embryos as source material for genetic transformation to facilitate important accomplishments in the genetic engineering of fruit tree crops. In tree crops such as walnut and spruce, somatic embryogenesis has been widely used for the generation of transgenic plants. Over the past three decades, several studies have been conducted on somatic embryogenesis of pistachio, such as shoot organogenesis from cotyle-

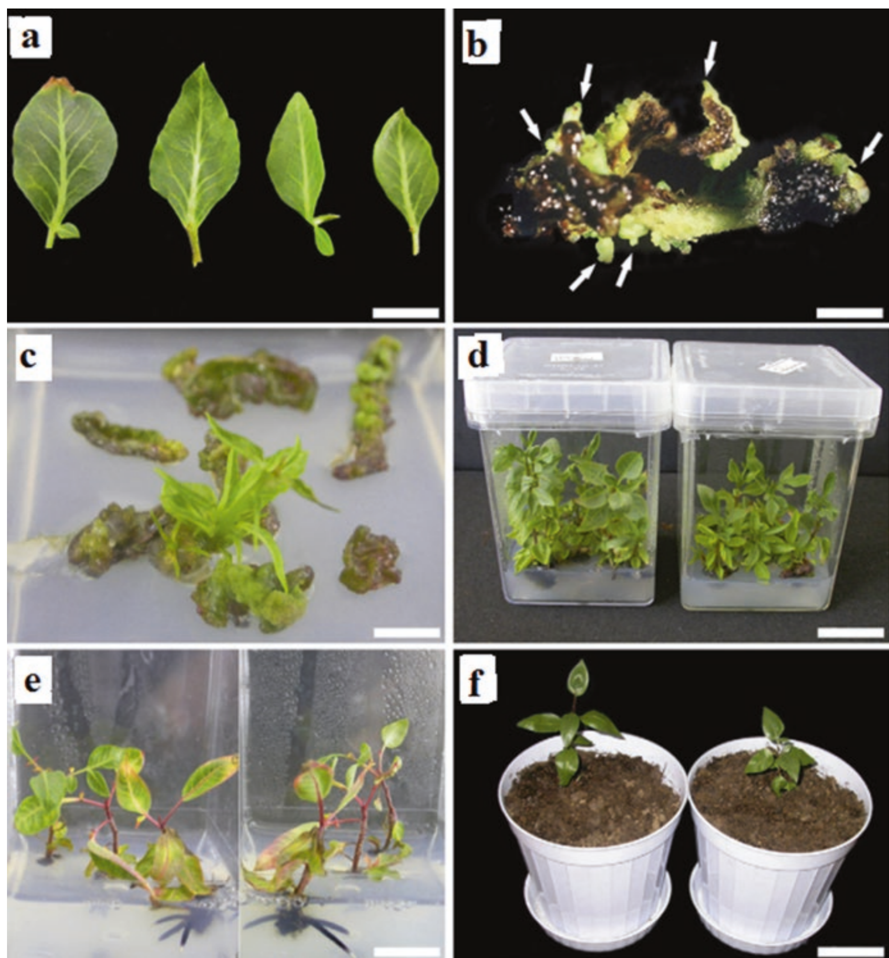

Fig. 10.9 Plant regeneration from in vitro-derived leaf explants of male Pistacia vera cv. Atli. (**a**) Regenerated leaves used for culture initiation, (**b**) Globular structures developed on the margin of the leaves after 4 weeks of culture, (**c**) Induction of shoot initials via direct organogenesis on MS medium, (**d**) Shoot proliferation on MS with 1 mg l^{-1} BAP, **e** Rooting micro shoots on modified MS medium, (**f**) Plantlets originated from micro shoots. (Source: Tilkat et al. 2009)

dons, embryonic axes (Kitto and McGranahan 1992); somatic embryogenesis from immature kernels (Onay et al. 1995); somatic embryogenesis from cotyledons, internodes and roots (Abousalim and Hafdi 1994); induction of embryogenic mass (EMS) from immature fruits of pistachio *Pistacia vera* (Onay et al. 1996); somatic embryogenesis in cultured leaf explants of pistachio (*P. vera*) (Onay 2000a, b), as well as embryo germination and proliferation of pistachio (*P. vera*) (Benmahioul et al. 2009).

10.5.6 Somaclonal Variation and Genetic Fidelity

The initiation of in vitro cultures from mature pistachio tissue is one of the main obstacles in pistachio tissue culture and several alternative approaches including shoot tip culture, organogenesis, and micrografting have been used to induce rejuvenation (Benmahioul et al. 2015; Tilkat et al. 2012). A natural problem of the regeneration process is the putative occurrence of somaclonal variations (Benmahioul et al. 2015). Regardless of the advantages of the in vitro culture, the occurrence of somaclonal variation in response to in vitro stresses in the regenerates is a serious limitation.

Therefore, understanding the causes and mechanisms involved in somaclonal variation, and screening of plantlets in order to verify the new regenerants as true to type is necessary to establish clonal fidelity of micropropagated plants and to confirm their suitability for commercial utilization (Akdemir et al. 2016; Benmahioul et al. 2015; Kılınç et al. 2015). Several strategies are available for analyzing the genetic conformity of in vitro-propagated progeny including phenotypic identification, cytological and protein analysis, as well as DNA analysis techniques (Akdemir et al. 2016; Kılınç et al. 2015). In recent years, DNA markers have served as an efficient tool for in vitro-propagated plants because they are stable, heritable, highly reproducible and reliable (Akdemir et al. 2016; Kılınç et al. 2015). In a study of DNA-based molecular markers, i.e. randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) were used to assess genetic fidelity of long-term micropropagated mature pistachio developed through direct shoot-bud regeneration using apical buds and in vitro-derived leaves. The results of this study revealed that the different marker system used in the study are reliable for the detection of variation in long-term micropropagated mature pistachio (Akdemir et al. 2016). In another study of genetic stability of selected genotypes of lentisk (*Pistacia lentiscus*) was developed using shoot-tip explants originating from in vitro seedlings and evaluated using IRAP markers. The results of this research confirmed that the clonal propagation of lentisk through shoot tips could be used for commercial utilization of the selected genotype (Kılınç et al. 2015).

10.5.7 In Vitro Conservation

In vitro culture techniques, i.e. organogenesis and somatic embryogenesis, together with in vitro conservation and cryopreservation procedures, can be used to avoid genetic erosion of pistachio. In vitro conservation and cryopreservation methods should be developed to safeguard pistachio germplasm (Akdemir et al. 2013; Benmahioul et al. 2015).

For the first time, slow-growth techniques related to temperature and nutrient reduction have been used for in vitro preservation of pistachio germplasm by Barghchi (1986); microshoots were stored for up to 18 months at 4 °C with the incorporation of abscisic acid (ABA, 0.25–4 mg l^{-1}) and mannitol (2.5–40 mg l^{-1}) to the culture medium. Then, somatic embryos and axillary buds excised from juvenile seedlings were encapsulated (Onay et al. 1996; Ozden Tokatli et al. 2010). In plant species like pistachio, the only available technique for safe long-term storage of germplasm is cryopreservation of vegetative tissue, cell cultures and seeds at ultra-low temperatures in liquid nitrogen (−196 °C) (Akdemir et al. 2013; Benmahioul et al. 2015).

10.6 Genetic Transformation and Gene Editing

Genetic improvement of fruit tree crops by conventional breeding is constrained by their long juvenile periods, and high degree of heterozygosity. Genetic transformation offers an attractive alternative to traditional breeding because it provides the potential to transfer specific traits into selected genotypes.

Tissue culture, in combination with genetic engineering techniques, has been successfully used to integrate specific traits through gene transfer. Transgenic plants have been produced from seedling-derived tissue, which is responsive to in vitro culture in fruit trees including apple, pear, citrus, walnut and *Prunus* spp. (plum, almond, apricot). To the best of our knowledge, so far, there has been no report of pistachio transformation. Although with molecular breeding, breeding periods have been reduced, but still not significantly enough. On the other hand, many studies have been done in the field of organogenesis and especially somatic embryogenesis which is the best resource for transformation in pistachio. Thus, the advent of next generation sequencing (NGS) technology and new genome-editing methods such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases have great potential for promoting the efficiency of pistachio breeding in future studies. This technology will accelerate breeding new pistachio cultivar and rootstock through reduction of the generation time and modification of traits of interest in a short time. Finally, insomuch as one of the most important barriers in pistachio breeding is long juvenility period, developing a FasTrack breeding system using these new transformation technologies can address this limitation by incorporating into pistachio early-flowering genes, i.e. the Poplar FT (*PtFT*) gene that induces pistachio trees to flower early and continuously.

10.7 Proteomics

Plants regularly experience numerous abiotic and biotic stresses throughout their life cycle. An enhanced understanding of the molecular bases involved in the plant responses to stress could facilitate the development of species with increased tolerance to environmental stresses. Genomics and proteomics, discovering novel genes and pathways involved in the plant's response to stress, are very important steps in crop improvement. Useful explanation of the molecular mechanisms engaged in the plant responses to stress, is inevitably tied to characterization of its components, which mainly include proteins. Proteomic analysis is a useful investigatory method that facilitates and expedites the comparison of a complex profile of proteins and generates a large body of information concerning the specific proteins involved in any biological pathways (Yousuf et al. 2016). Proteomic profiles of various plant species subjected to abiotic stress have been investigated (Aghaei and Komatsu 2013; Du et al. 2010; George and Haynes 2014; Jellouli et al. 2008; Li et al. 2013; Tanou et al. 2009, 2014; Vincent et al. 2007; Yuan et al. 2016). In pistachio, a recent high-throughput comparative proteomics study was carried out on two rootstocks subjected to salinity stress by Akbari (2018) and Katam et al. (2018), which appears to be the only proteomics study in *Pistacia vera* to date. In this study, which was performed on the roots of cv. UCB1 (as tolerant) and cv. Akbari (as susceptible), 153 proteins were upregulated, and 69 proteins were downregulated in the cv. UCB1; while in cv. Akbari, 340 upregulated and 18 downregulated proteins were identified. The identified proteins were functionally classified into signal transduction, stress responsive, cell wall and cytoskeleton metabolism proteins groups.

10.8 Conclusions and Prospects

Pistachio breeding began many years ago when local growers near its center of diversity started collecting and domesticating the best fruit-producing individuals in the wild populations. Most of the cultivars grown today, e.g. Kalle-Ghoochi, Akbari, and Ohadi, are the results of chance seedling selections. Recently, cvs. Golden Hills, Lost Hills and Gumdrop have been released through classical cross-breeding programs. The main traits of these cultivars are earlier blooming and ripening, which benefit growers by optimizing labor cost and harvesting equipment. However, most of these cultivars have undesired characteristics such as unsplit nuts, blank nuts, extreme alternate bearing, susceptibility to pests and diseases, etc. Therefore, improvement of these characteristics should be an important component of future breeding attempts to meet the pistachio industry's rapidly changing demand for new superior cultivars. Since pistachio is a highly heterozygous tree with a long juvenile period, developing new cultivars through traditional breeding is a long, expensive,

laborious and uncertain process. Modern molecular biology techniques are not yet widely used in pistachio breeding; one reason is that the genetics of pistachio is still not well understood, and there are inadequate molecular data in GenBank® for this fruit crop. As shown in this chapter, methodologies such as genetic engineering and marker-assisted selection in pistachio are still in their infancy. Although currently genomic data for pistachio are scarce, this will change soon due to the feasibility of next-generation sequencing at reasonable cost, and in the future the main limitation will probably switch to the availability of reliable phenotypic information. Recently the pistachio genome has been fully sequenced by researchers in California and Turkey and will provide genomic information to utilize in crop improvement programs. In the future, it is expected that the use of marker-assisted selection (MAS), genome wide association studies (GWAS), genomic selection (GS) and genetic transformation will improve the efficiency of breeding programs. Furthermore, QTLs for important traits should be identified. Progressive and fast development of genomic technologies, such as cisgenesis, intragenesis, functional genomics, somatic cell hybridization and CRISPR/Cas9 to edit and correct the genome, in addition to fast-track breeding system, will accelerate the process of rapid-cycle breeding to overcome the long juvenile period and high heterozygosity. We hope that in the future new pistachio cultivars with better characteristics than present ones will be created by new technologies.

More information on pistachio can be found in Joley (1979), Hormaza and Wunsch (2007), Parfitt et al. (2012), and in the *Pistachio Production Manual*, 5th edition (Ferguson and Haviland 2016).

Appendices

Appendix I: Research Institutes and Online Resources Concerned with Pistachio

Institute	Country	Website and contact information
Fruit & Nut Research and Information Center, University of California, Division of Agriculture and Natural Resources	USA	http://fruitsandnuts.ucdavis.edu/ Department of Plant Sciences Mail Stop 2, University of California One Shields Ave., Davis, CA 95616-8780 On Campus: 3043 Wickson Hall Phone Number: (530) 754-9708 Email: fruitsandnuts@ucdavis.edu
California Pistachio Research Board (CPRB)	USA	https://calpistachioresearch.org/ 4938 E. Yale Avenue, Suite 102, Fresno, CA 93727, USA Phone: 559.255.6480; 559.255.6485 Email: admin@acpistachios.org; bobk@acpistachios.org
Mechanical Harvesting of California Pistachios, University of California, Cooperative Extension	USA	http://ucanr.org/sites/mechpistachio
Pistachio Salinity Studies, University of California, Cooperative Extension	USA	http://ucanr.org/sites/psalinity
Administrative Committee for Pistachios (ACP)	USA	https://www.acpistachios.org/ Phone: (559) 255.6480 ext 103 E-mail: bobk@acpistachios.org
Pistachio Growers' Association	Australia	https://www.pgai.com.au/ 27 Ludgate Hill Rd, Aldgate SA 5154. Email: pgai@pgai.com.au
Australian Nut Industry Council	Australia	http://www.nutindustry.org.au/about-pistachios.html 42 Simpsons Road Currumbin Waters Q 4223 Phone: 0409 707 806 Email: exec@nutindustry.org.au

Appendix II: Genetic Resources of Pistachio

Cultivar	Uses	Taxon	Origin	Important traits	References
Kalle-Ghoochi	Scion, female	*Pistacia vera*	Iran	Filbert-shaped (round) nuts, large nut size, high productivity, early flowering, susceptible to spring-frost	Karimi (2010)
Ohadi (Fandoghi)	Scion, female	*P. vera*	Iran	Filbert-shaped (round) nuts, early ripening, low alternate bearing, resistant to pistachio psylla (*Agonoscena pistaciae*)	Karimi (2010)
Akbari	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, large nut size, high productivity, late flowering, late ripening, tolerant to spring frost, susceptible to psylla (*A. pistaciae*), uniformity in the fruit ripening	Karimi (2010)
Ahmad-Aghaei	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, large nut size, high fruit quality, high productivity, high alternate bearing	Karimi (2010)
Jandaghi	Scion, female	*P. vera*	Iran	Filbert-shaped (round) nuts, large nut size, late flowering, late ripening, high fruit quality, low alternate bearing	Karimi (2010)
Momtaz	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, early flowering, late ripening, high fruit drop at ripening	Karimi (2010)
Amiri	Scion, female	*P. vera*	Iran	Filbert-shaped (round) nuts, early flowering, low alternate bearing	
Shah-Pasand	Scion, female	*P. vera*	Iran	Almond shaped (oblong) nuts, late flowering, low vegetative growth, very low alternate bearing	Karimi (2010)

(continued)

Cultivar	Uses	Taxon	Origin	Important traits	References
Ebrahim-Abadi	Scion, female	*P. vera*	Iran	Filbert-shaped (round) nuts, large nut size, late ripening, resistant to pistachio psylla (*A. pistaciae*), low alternate bearing	Karimi (2010)
Ebrahimi	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, high non-split nuts, high vegetative growth, low alternate bearing	Karimi (2010)
Gholamrezaei	Scion, female	*P. vera*	Iran	Early flowering, late ripening, low alternate bearing	Karimi (2010)
Fandoghi-Riez	Scion, female	*P. vera*	Iran	Filbert-shaped (round) nuts, late ripening, high vegetative growth	Karimi (2010)
Fandoghi-48	Scion, female	*P. vera*	Iran	Filbert-shaped (round) nuts, large nut size, late ripening, low alternate bearing	Karimi (2010)
Fandoghi-Ghafouri	Scion, female	*P. vera*	Iran	Filbert-shaped (round) nuts, late ripening, high non-split and blank nuts	Karimi (2010)
Fandoghi-Zudras	Scion, female	*P. vera*	Iran	Filbert-shaped (round) nuts, large nut size, early ripening, low uniformity in fruit ripening	Karimi (2010)
Mousa-Abadi	Scion, female	*P. vera*	Iran	Early ripening, high vegetative growth, intermediate alternate bearing, low early-season hull-cracking	Karimi (2010)
Badami-Nishkalaghi	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, low alternate bearing, low early-season hull-cracking	Karimi (2010)
Badami-Raavar	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, late ripening, large nut size, high non-split and blank nuts	Karimi (2010)
Khanjari-Raavar	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, early flowering, early ripening	

(continued)

Cultivar	Uses	Taxon	Origin	Important traits	References
Khanjari-Damghaan	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, late flowering, late ripening, high fruit quality for fresh consumption	Karimi (2010)
Sefidpeste-Noogh	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, early flowering, late ripening, high alternate bearing, high fruit drop at ripening	Karimi (2010)
Rezaei-Zudras	Scion, female	*P. vera*	Iran	Filbert-shaped (round) nuts, large nut size, early flowering, early ripening, high non-split and blank nuts	Karimi (2010)
Harati	Scion, female	*P. vera*	Iran	Early flowering, late ripening, high non-split nuts, low blank nuts, low alternate bearing	Karimi (2010)
Hasan-zadeh	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, late flowering, late ripening, high productivity	Karimi (2010)
Sirizi	Scion, female	*P. vera*	Iran	Filbert-shaped (round) nuts, intermediate alternate bearing, high blank nuts	Karimi (2010)
Saifadini	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, high alternate bearing, high non-split and blank nuts	Karimi (2010)
Poost-Piazi	Scion, female	*P. vera*	Iran	Almond-shaped (oblong) nuts, high blank nuts, low vegetative growth	Karimi (2010)
Kerman	Scion, female	*P. vera*	USA	Large nut size, high productivity, high alternate bearing, high non-split and blank nuts	Parfitt et al. (2016)
Joley	Scion, female	*P. vera*	USA	Early flowering, green kernel, low productivity, small nuts	Parfitt et al. (2016)
Lassen	Scion, female	*P. vera*	USA	Similar nut quality to Kerman cv.	Parfitt et al. (2016)
Damghan	Scion, female	*P. vera*	USA	Large nut size, low productivity	Parfitt et al. (2016)

(continued)

Cultivar	Uses	Taxon	Origin	Important traits	References
Golden Hills	Scion, female	*P. vera*	USA	Early flowering, early ripening, high productivity, low non-split and blank nuts, balanced shell-hinge strength, uniform flowering, uniform fruit maturity	Parfitt et al. (2016)
Lost Hills	Scion, female	*P. vera*	USA	Early flowering, early ripening, large nut size, high productivity, low non-split nuts, balanced shell-hinge strength, uniform flowering, uniform fruit maturity, low shell-hinge strength	Parfitt et al. (2016)
Gumdrop	Scion, female	*P. vera*	USA	Earlier flowering than cv. Golden Hills, earlier ripening than Golden Hills	Parfitt et al. (2016)
Napoletana	Scion, female	*P. vera*	Italy	Predominant variety in Sicily	Parfitt et al. (2016)
Agostana	Scion, female	*P. vera*	Italy	Cultivated in Sicily, green kernel	Parfitt et al. (2016)
Girasola	Scion, female	*P. vera*	Italy	Cultivated in Sicily, green kernel	Parfitt et al. (2016)
Notaloro	Scion, female	*P. vera*	Italy	Cultivated in Sicily, green kernel	Parfitt et al. (2016)
Cappuccia	Scion, female	*P. vera*	Italy	Cultivated in Sicily, green kernel	Parfitt et al. (2016)
Femminello	Scion, female	*P. vera*	Italy	Cultivated in Sicily, green kernel	Parfitt et al. (2016)
Trabonella	Scion, female	*P. vera*	Italy	Green kernel, small nut size, high non-split nuts	Parfitt et al. (2016)
Bronte	Scion, female	*P. vera*	Italy	Green kernel, small nut size, high non-split nuts	Parfitt et al. (2016)
Sfax	Scion, female	*P. vera*	Tunisia	Large, tight nut clusters, nut size, productivity, percent split nuts inferior to Kerman	Parfitt et al. (2016)
Mateur	Scion, female	*P. vera*	Tunisia	Most common cultivar in Tunisia	Parfitt et al. (2016)
El Guettar	Scion, female	*P. vera*	Tunisia		Parfitt et al. (2016)
Aegina (Aegenes)	Scion, female	*P. vera*	Greece	Early flowering, susceptible to *Botryosphaeria dothidea*	Parfitt et al. (2016)

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Cultivar	Uses	Taxon	Origin	Important traits	References
Pontikis	Scion, female	*P. vera*	Greece	Oblong-ovate shaped nuts, large nut size, low non-split nuts	Parfitt et al. (2016)
Lamarka	Scion, female	*P. vera*	Cyprus	Main cultivar in Cyprus	Parfitt et al. (2016)
Sirora	Scion, female	*P. vera*	Australia	Main cultivar in Australia	Parfitt et al. (2016)
Rashti	Scion, female	*P. vera*	Iran	Late ripening, large nut size, low non-split nuts, good flavor	Parfitt et al. (2016)
Uzun	Scion, female	*P. vera*	Turkey	Early flowering, high alternate bearing	Karimi (2010)
Kirmizi	Scion, female	*P. vera*	Turkey	Early flowering, high alternate bearing	Karimi (2010)
Halebi	Scion, female	*P. vera*	Turkey	Early flowering, high alternate bearing	Karimi (2010)
Siirt	Scion, female	*P. vera*	Turkey	Intermediate flowering time, intermediate alternate bearing	Karimi (2010)
Beyazben	Scion, female	*P. vera*	Turkey	Early flowering, high alternate bearing	Karimi (2010)
Sultani	Scion, female	*P. vera*	Turkey	Early flowering, high alternate bearing	Karimi (2010)
Begirmi	Scion, female	*P. vera*	Turkey	High alternate bearing	Karimi (2010)
Bilgen	Scion, female	*P. vera*	Turkey	High alternate bearing	Karimi (2010)
Red Aleppo	Scion, female	*P. vera*	Syria	Splits and yields well but not as well as Kerman, nut size smaller than Kerman	Parfitt et al. (2016)
Achoury	Scion, female	*P. vera*	Syria	Almond-shaped (oblong) nuts, 90% split nuts, intermediate alternate bearing	Karimi (2010)
Red Oleimy	Scion, female	*P. vera*	Syria	Intermediate alternate bearing, 68% split nuts	Karimi (2010)
White Batoury	Scion, female	*P. vera*	Syria	Filbert-shaped (round) nuts, intermediate alternate bearing, low split nuts	Karimi (2010)
Peters	Scion, male	*P. vera*	USA	Produces high quantity of pollen, 2 weeks pollen shed, irregular flowering during low-chill years	Parfitt et al. (2016)
02–16	Scion, male	*P. vera*	Russia	Early flowering	Parfitt et al. (2016)

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Cultivar	Uses	Taxon	Origin	Important traits	References
02–18	Scion, male	*P. vera*	Russia	Late flowering, less durable pollen than Peters cv.	Parfitt et al. (2016)
Nazareth	Scion, male	*P. vera*	USA		
Chico	Scion, male	*P. vera*	USA	Early flowering, sheds pollen prior to and during the earliest part of the Kerman's bloom period, if xenia effects pistachio nut size, then this cultivar should not be used because it is suspected to be interspecific hybrid between *P. vera* and *P. integerrima*	Parfitt et al. (2016)
Randy	Scion, male	*P. vera*	USA	Early flowering, long bloom period, superior pollinizer for early-season flowering cultivars like cvs. Golden Hills and Lost Hills	Parfitt et al. (2016)
Famoso	Scion, male	*P. vera*	USA	Large quantity viable pollens, highly synchronized flowering with Kerman	Kallsen and Parfitt (2017b)
Tejon	Scion, male	*P. vera*	USA	Produces many flowers and large quantities of durable pollens with a high germination percentage, very early flowering, synchronized bloom period with the Gumdrop cv. (https://patents.justia.com/patent/PP28931)	
Gazvin	Scion, male	*P. vera*	Iran	Early flowering, poor pollen durability	Parfitt et al. (2016)
Adam	Scion, male	*P. vera*	Syria	Pollinizer for Ashoury	Karimi (2010)
Basem	Scion, male	*P. vera*	Syria	Pollinizer for Ashoury	Karimi (2010)
Caliphan	Scion, male	*P. vera*	Syria	Pollinizer for Ashoury	Karimi (2010)
Fady	Scion, male	*P. vera*	Syria	Pollinizer for Red Oleimy	Karimi (2010)
Ibrahim	Scion, male	*P. vera*	Syria	Pollinizer for Red Oleimy	Karimi (2010)

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Cultivar	Uses	Taxon	Origin	Important traits	References
Jamil	Scion, male	*P. vera*	Syria	Pollinizer for Red Oleimy	Karimi (2010)
Deeb	Scion, male	*P. vera*	Syria	Pollinizer for White Batoury	Karimi (2010)
Kamel	Scion, male	*P. vera*	Syria	Pollinizer for White Batoury	Karimi (2010)
Badami-Riez Zarand	Rootstock	*P. vera*	Iran	Vigorous, tolerant of *Phytophthora* spp., salinity tolerant	Karimi (2010)
Sarakhs	Rootstock	*P. vera*	Iran	Salinity tolerant, susceptible to *Phytophthora* spp.	Karimi 2010
Ghazvini (Kalle-Bozi)	Rootstock	*P. vera*	Iran	Salinity tolerant	Karimi (2010)
Baneh	Rootstock	*P. atlantica* Desf. ssp. *mutica* F&M	Irano-Turanian, Mediterranean	Resistant to root-knot nematode, less vigorous and difficult to bud than cv. Badami-Riez Zarand, scion-rootstock incompatibility, negative effects on yield	Karimi (2010)
Terebinthus	Rootstock	*P. terebinthus*	Mediterranean	Cold tolerant, efficient zinc and copper absorption, resistant to *Armillaria* root rot, susceptible to *Verticillium* wilt, difficult to bud, less vigorous and less uniform than other common rootstocks	Ferguson et al. (2016)
Atlantica	Rootstock	*P. atlantica*	Irano-Turanian, Mediterranean	Higher cold tolerance and less vigorous than Integerrima, susceptible to *Verticillium* wilt	Ferguson et al. (2016)
Integerrima	Rootstock	*P. integerrima*	Irano-Turanian, Sino-Japanian	*Verticillium* wilt tolerant, vigorous, buds easily, least cold tolerant of the commonly used rootstocks	Ferguson et al. (2016)
Khinjuk	Rootstock	*P. khinjuk*	Irano-Turanian	Drought tolerant, susceptible to *Phytophthora* spp., more vigorous than Baneh cv.	Ferguson et al. (2016)
UCBI	Rootstock	*P. atlantica* × *P. integerrima*	USA	Vigorous, positive effect on yield, moderately resistant to Verticillium wilt	Ferguson et al. (2016)

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Cultivar	Uses	Taxon	Origin	Important traits	References
Pioneer Gold I (PGI)	Rootstock	*P. integerrima* × *P. integerrima*	USA	Also referred to as Integerrima, has Integerrima's characteristics	Ferguson et al. (2016)
Pioneer Gold I (PGII)	Rootstock	*P. integerrima* × *P. atlantica*	USA	Vigorous, susceptible to *Verticillium* wilt, no longer commercially available	Ferguson et al. (2016)
Platinum	Rootstock	*P. integerrima* × *P. atlantica*	USA	Parents of this hybrid are genetically different from those used for UCBI	Ferguson et al. (2016)

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Chapter 11
Advances in Persian Walnut (*Juglans regia* L.) Breeding Strategies

Kourosh Vahdati, Mohammad M. Arab, Saadat Sarikhani, Mohammad Sadat-Hosseini, Charles A. Leslie, and Patrick J. Brown

Abstract Walnut (*Juglans regia* L.) is one of the oldest trees with harvestable products known to humans and has a history dating to 7000 BC in Persia. Walnut breeding programs aim to release productive scion cultivars with disease resistance and high-quality nuts, along with rootstocks resistant to biotic and abiotic stresses. Genetic improvement of walnut began with the selection of superior trees in their main centers of origin, primarily from the Persian plateau. The first selection and grafting of superior walnut genotypes began in France. The first organized walnut-breeding program employing targeted hybridization began in the USA in 1948, primarily using introduced French cultivars and selected local genotypes derived from seed imported from centers of origin (Iran, Afghanistan, China). Currently, both conventional hybridization with phenotypic evaluation and molecular breeding approaches are used in the USA programs as well as those in France, China, Iran, Spain and Italy. Recent advances in biotechnology and genomics show potential to accelerate cultivar development. In addition, the exploration, description, and preservation of biodiverse germplasm can provide a gene bank of desirable traits and enable biotechnologists to conduct breeding more accurately and rapidly in the future. Recent advancements have opened up new avenues to enhance the efficiency of walnut breeding to release new scions and rootstocks. These include next-generation sequencing (NGS) techniques, bioinformatics tools, high-throughput genotyping platforms and genomics-based approaches such as genome wide association studies (GWAS), marker-assisted selection (MAS), genomic selection (GS) and genome editing with the CRISPR-Cas9 system. In this chapter, we describe the

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background and development of conventional walnut breeding programs in the leading walnut producing countries of the USA, France, China, Iran and Turkey, and finally focus on the current use and status of molecular breeding and biotechnology in walnut breeding.

Keywords Bioinformatics · Biotechnology · Genetic diversity · Genomics · *Juglans regia* · Molecular · Rootstock · Cultivar · Selection · Marker

11.1 Introduction

Walnuts (*Juglans* spp.) are among the oldest tree foods known to humanity, with historical references dating back to 7000 BC in Persia (Dreher et al. 1996) (Fig. 11.1). *Juglans* is the most important genus in the Juglandaceae, containing 21 species that produce edible nuts (Karimi et al. 2010; Vischi et al. 2017). The genus *Juglans* is classified into four sections; *Rhysocaryon*, *Cardiocaryon*, *Trachycaryon* and *Dioscaryon* (McGranahan and Leslie 1991). Persian (or English) walnut is the only species in section *Dioscaryon* and the most widely cultivated *Juglans* species,

Fig. 11.1 Kourosh Vahdati stands next to one of the historic walnut trees in Rabor, Kerman, Iran estimated to be many hundreds of years old

grown throughout the temperate and semiarid regions worldwide for edible nuts (Amiri et al. 2010; Arzani et al. 2008; McGranahan and Leslie 1991; Molnar et al. 2011).

Persian walnut is a large deciduous tree with smooth bark and alternately arranged leaves. The male flowers develop as drooping catkins 5–10 cm long. Female flowers are borne terminally in clusters of 2–5. The characteristic of this species is having four-chambered nuts with hulls that dehisce and separate from the shells at maturity (Germain 1999; Ramos 1997). It is a diploid species (2n = 2x = 32) with an estimated genome size of 1315 Mbp (1.35 pg; monoploid genome size (1C DNA) = 657.80 Mbp). In comparison, the human genome (6153 Mbp) is approximately 4.7 times larger than walnut genome size (IHGSC 2004; Sarikhani Khorami et al. 2018).

Persian walnut, as its name suggests, originated in Persia (now Iran) and was distributed along the Silk Road eastward to Pakistan, Afghanistan, India, Uzbekistan and China, and westward to Turkey and Europe (Vahdati et al. 2014). Walnut has a long history of cultivation and widespread use from China to Western Europe. According to molecular phylogeographic studies and paleontology, its evolutionary history dates back to the Holocene in Eurasia (Pollegioni et al. 2017). Currently it is grown from 30 to 55° of latitude in the Northern Hemisphere in many countries of Asia, Europe and North America and from 30 to 40° in the Southern Hemisphere in Australia, New Zealand, South Africa, Chile and Argentina) (Fig. 11.2).

Walnut (*Juglans regia* L.) is an important nut and timber species and a valuable dietary source contributing to reduction of coronary heart disease (CHD) (Maguire et al. 2004; Zhang et al. 2009). Walnuts contain antioxidants that stimulate the immune

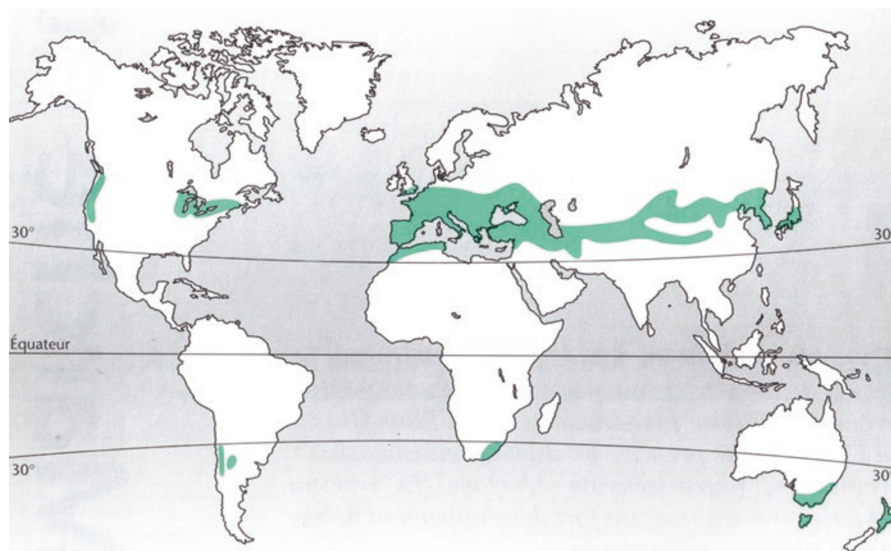

Fig. 11.2 Walnut distribution in the world; green areas on the map indicate walnut-producing areas. (Source: Germain 1999)

system and appear to have anti-cancer properties (Jahanbani et al. 2016a, b; Milind and Deepa 2011). Bioactive peptides in walnuts also appear to have value for prevention and treatment of hypertension (Huang et al. 2013; Jahanbani et al. 2018).

Due to their high content of unsaturated fatty acids (UFAs), antioxidants, proteins, tocopherols and other bioactive constituents, walnut kernels have been implicated in the control of heart disease (Pereira et al. 2008; Ros et al. 2004; Siqueira et al. 2015). Their high UFA content can also play an important role in decreasing total and LDL-cholesterol and increasing HDL-cholesterol. Dietary intervention studies have demonstrated that omega-3 fatty acid, found in abundance in walnut kernels, can help prevent some cognitive disorders including depression, dementia and Alzheimer's disease (Bourre 2005; Dogan and Akgul 2005). Due to its high nutrient value, walnut is a strategic product for human health and nutrition on the FAO list of priority crops (Gandev 2007).

With uses ranging from landscaping to timber production to human nutrition, walnut production plays a significant role in creating employment and generating income. According to FAO statistics (FAO 2016), the world's annual walnut production (shelled + in shell) is approximately 3.74 million mt with a commercial value of 2.77 (1.62 + 1.15) million USD. China, the USA and Iran, the largest walnut producers, account for more than 74.7% of world's production. The USA is also the largest walnut exporter (Table 11.1). The USA exports more than 50% of its walnut production, of which half is shelled walnut kernels, accounting for about 53.8% of the world's total walnut exports (FAO 2016).

Despite the high economic value and ancient cultivation of walnut, breeding has been limited, relative to other temperature-zone fruits, particularly in countries of the crop's origin. The first established walnut breeding programs dates back to the mid-twentieth century (Bernard et al. 2018). Prior to that, the genetic improvement of walnut involved selection of elite trees from natural populations. As in other tree crops, high yield and quality (large nut size, high kernel percentage, light kernel color) have been primary walnut breeding objectives.

Table 11.1 Leading nations for Persian walnut total production, area cultivated, export and import value in international trade (2016)

		World	Top three countries
Production (mt)		3,747,549	China, USA, Iran
Area harvested (ha)		1,186,399	China, Iran, USA
Export value (1000 USD)	(Shelled)	1,736,902	USA, Mexico[a], Chile
	(In shell)	1,218,144	USA, Mexico[a], France
Import value (1000 USD)	(Shelled)	1,401,865	Germany, Japan, Spain
	(In shell)	843,032	Italy, Turkey, Vietnam

Source: FAO (2016)

[a]Mexico data on walnut export is sometimes misleading and confused with data of pecan (Avanzato et al. (2014))

In this chapter, we discuss walnut cultivation, traditional breeding programs, germplasm biodiversity and conservation, molecular breeding, genetic engineering, mutation breeding, and hybridization.

11.2 Cultivation and Traditional Breeding

11.2.1 Current Cultivation Practices

Walnut cultivation is of great interest in suitable regions because walnut orchards require relatively low maintenance, are productive for at least 40 years, and nuts can be stored for up to 2 years under simple storage conditions (Adem 2009). Walnut trees require a temperate climate with 600–1000 chill hours (Aslani Aslamarz et al. 2009), can be seriously damaged by frost during late spring or early fall, and most cultivars are sensitive to both extremely high (>38 °C) and very low (< −20 °C) temperature. Walnut trees are very demanding in terms of soil texture and structure, requiring good drainage to allow water, air and nutrients to move to the roots. Due to their huge and spreading root systems, walnut trees prefer a deep and sandy loam soil. For commercial walnut production, soil pH of 7–7.5 is best and electrical conductivity (EC) should be lower than 2.5 mmhos.cm^{-1} (Germain 1999; Ramos 1997).

Most walnut trees in the past were grown from seed, but nowadays seedlings are mainly to provide rootstock. Typically, modern walnut orchards are established using one or two commercial scion cultivars, propagated by grafting or micropropagation. Tree spacing in walnut orchards ranges from 10 × l0 m to 6 × 3 m, giving approximately 100–550 trees per hectare, respectively (Adem 2009). Several walnut cultivars have been released to growers in the leading walnut producing countries, depending on the breeding objectives and the production areas. Chandler is the world's most common walnut cultivar, having a lateral bearing habit, strong yield and light kernel color (Tulecke and McGranahan 1994). Franquette, a late-leafing cultivar, is another common walnut cultivar in the world. It has long been the dominant walnut cultivar in France (Germain 1999); but due to its terminal bearing habit, it is being replaced by new lateral bearing cultivars such as Chandler and Fernor in modern orchards.

11.2.2 Walnut Production Challenges and Breeding Objectives

Leading challenges always dictate specific objectives in plant breeding, but high yield and crop quality are the main objectives for all crops. All targeted breeding traits also require high heritability, indicating the control of a high proportion of total phenotypic variance is dependent on genetic factors (Hansche et al. 1972; Ramos 1997). In initial walnut breeding work, nut size and kernel color were

primary targets. High kernel percentage, ease of kernel removal and reduced shell thickness were additional traits of interest. Lateral bearing, as a major component of yield, became one of the main traits pursued in walnut breeding programs beginning in the mid-twentieth century, leading to the introduction of the Chandler cv. Other commonly targeted traits are disease resistance (blight, anthracnose) and late leafing date. Some traits in walnut breeding depend on the production area and climatic conditions (Avanzato et al. 2014; Bernard et al. 2018; Ramos 1997). For example, due to late-spring frost, late leafing is an important trait in walnut breeding in France, Iran and Turkey. In addition, late leafing is helpful to avoid walnut blight (*Xanthomonas arboricola* pv. *juglandis*) which spreads by spring rains. Early harvest date is an important breeding trait in the US in order to widen the harvest window and avoid autumn rain (Akca and Ozongun 2004; Bernard et al. 2018; Ebrahimi et al. 2015; Germain 1990; Leslie and McGranahan 2014). Current and future breeding objectives are keeping in mind climate change and global warming, including rootstocks tolerant to abiotic stresses, especially drought, and with disease resistance.

11.2.3 The World's Walnut Breeding Programs

11.2.3.1 Cultivars

Although organized walnut breeding programs began during the last century, genetic improvement of walnut began much earlier by farmers selecting and propagating superior trees from their natural seedling populations. Crows have been the other early walnut breeders, selecting nuts from the highest parts of trees where nuts are larger because of more light and ventilation. Nuts hidden in the soil by crows as future food start to germinate after the cold season fulfills the chilling requirement. Walnut growers have used these seedlings to develop stronger trees. In Iran, walnut seedlings *planted* by crows are called Kalagh-kar, meaning *crow planted seedlings*, and were used extensively in the past to establish walnut orchards (Vahdati et al. 2014). Old literature reports that superior walnut genotypes, in terms of shell thickness, nut size and kernel color, were selected in the centers of walnut origin, such as Iran, China and other Silk Road countries, and used to establish new orchards. Abounasri Harvi (1515) in his book entitled *Agricultural Guidance* (*Ershad Al-Zerae* in Persian) reported that growers used thin-shelled walnut to establish orchards in the 1500s.

Development in walnut genetic improvement has occurred in 4 phases: 1700–1948, 1948–1979, 1979–2009 and 2009 to present. The first organized efforts at genetic improvement of walnut began in France with development of grafting methods that allowed selection and propagation of superior genotypes, leading to establishment of cultivars such as Franquette, Mayette, Crone, Grandjean and Parisienne. Walnut breeding in the USA, where Persian walnut is not native, began with the introductions of French cultivars and selection of superior local genotypes

grown from imported seeds from walnut centers of origin such as Iran and Afghanistan. The first breeding and selection efforts in the USA were by Felix Gillet (1835–1908), the father of the Northern California walnut industry and Josef Sexton (1842–1917), a walnut grower in Southern California. Their work, and additional seedling selections by other California walnut growers, led to the development and use of many terminal bearing cvs. including Eureka, Waterloo, Poe and Hartley. Discovery by California farmer George Payne of a highly productive seedling in his orchard fencerow led to widespread propagation and planting of this selection as the cultivar Payne. The precocity and abundant yield of this early-leafing selection was later determined to be due to its lateral bearing habit. Because cv. Payne was for many years the sole source of this trait in California, it became the most important parent in the University of California walnut-breeding program and almost all the University of California walnut releases have cv. Payne in their background (Ramos 1997; Tulecke and McGranahan 1994). The heritage of many current cultivars in the USA also includes Eureka (with a parent thought to be from Iran) and Pl159568 (originating from Afghanistan). In general, the objectives during this early phase of walnut genetic improvement were large nuts, high kernel percentage, thin shell and light kernel color (Table 11.2).

The walnut growers of the USA and France led the second phase of the world's walnut breeding. Work continued in the USA based on hybridization between French cultivars and genotypes originating from Silk Road countries including Iran and Afghanistan, but now strongly emphasizing incorporation of the lateral bearing trait. This breeding phase was led by Eugene F. Serr and Harold I. Forde, from 1948 to 1979. A total of 13 cultivars, namely Midland, Vina, Pioneer, Pedro, Gustine, Lompoc, Amigo, Chico, Tehama, Serr, Chandler, Howard and Sunland were released (Table 11.3). Chandler, the world's most prominent walnut cultivar, Serr and Howard were the most important of these. Chandler and Serr account for 75% and 12% of current California walnut acreage, respectively. Major breeding objectives were late leafing, lateral bearing, precocious production, moderate tree vigor, kernel quality and disease tolerance. Pedigrees of the Serr/Forde cultivars show that cv. Payne is a parent or ancestor of all of these (Fig. 11.3). More information about the California Walnut Improvement Program is available from the Walnut Research Reports Database at the University of California, Fruit and Nut Research Information Center website; http://ucanr.edu/sites/cawalnut/ (Ramos 1997; Tulecke and McGranahan 1994; Bernard et al. 2018).

The second phase of the French walnut-breeding program included 28 crosses (1900 intraspecific hybrids) between French and Californian cultivars (Fig. 11.4), led by Eric Germain at INRA (Bernard et al. 2018; Germain 1999; Ramos and Doyle 1984). In this phase, hybrids were evaluated. Lara, a natural seedling of Payne, served as a main parent in these crosses. The main objective was to obtain lateral bearing and late-leafing cultivars. Therefore, one of the main crosses was between Franquette (a late-leafing cultivar) and Lara (a lateral-bearing cultivar), led to release of Fernor in 1987 (Germain 1997, 1999).

The third and fourth walnut breeding phases cover the periods from 1979 to 2009, and 2009 until today. In addition to the USA and France, other walnut growing

Table 11.2 Walnut breeding programs and released cultivars and rootstocks in the world from past to present

Class	Country	1950–1700	1700–1948		1948–1979		1978–2009			2009 until now		
		Center of origin countries	France	United States	France	United States	France	United States	Other countries	France	United States	Other countries
Breeding for cultivars	Strategy	Selection	Germplasm evaluation and selection, introduction		Hybridization		Germplasm evaluation and selection, hybridization, molecular breeding			Germplasm evaluation and selection, hybridization, molecular breeding		
	Objectives	Large nut with thin shell, high kernel percentage, light kernel color			Lateral bearing, early production, kernel quality, disease tolerance, late leafing		Lateral bearing, No PFA, Precocity, Late leafing, Early harvest, Disease tolerance, kernel quality,			Early harvest, late-leafing, disease resistance, reduced water use, adapted to climatic conditions, winter hardiness		
	Released cultivars	-	Franquette, Crone, Marbot, Grandjean, Mayette, Parisienne	Eureka, Payne, Pl159568, Waterloo, Ashley, Adams, Olmo13-1048, Poe, Hartley	Lara	Midland, Vina, Pioneer, Gustine, Lompoc, Pedro, Amigo, Chico, Tehama, Serr, Chandler, Howard, Sunland	Fernor, Fernette, Ferjean	Cisco, Tulare, Robert Livermore, Sexton, Gillet, Forde	Jamal, Damavand, Baokexiang, Beijing 861, Jinglong 1,2, Lipin 1,2, Xilin 1, Xinzaofeng, Sebin, Bilecik, Maras 10, Yalova 1, Sutymez, Kaman 1	Feradam, Ferbel, Ferouette, Fertignac	Ivanhoe, Solano, Durham.	Dirilish, 15 Temmuz, Maras 12, Luguo series, Zanmei, Shuang zao, Jinbaoxiang, Rili, Persia, Caspian, Chaldoran, Alvand

Breeding for rootstock	Strategy	-	Selection	Inter-species hybridization	Germplasm evaluation and Selection, Inter-species hybridization			Germplasm evaluation and selection, interspecies hybridization		
	Objectives		Uniform orchards	Vigor and disease resistance	Disease resistance especially *Armillaria*, *Phytophthora*, Nematode and CLRV			Disease resistance, drought tolerance, dwarfness		
	Released rootstock		*J. regia* seedling	*J. regia* seedling, Paradox and Paradox series	*J. regia* seedling	Vlach, RX1, VX211	*J. regia* seedling	---	---	Jin RS-1, Jin RS-2, Jin RS3

- Cells with (---) indicate that there was no released cultivar or breeding program at that time

Table 11.3 Characteristics and phylogeny of some major walnut cultivars in the leading walnut producer countries

Cultivar	Parent	Origin	Leafing date	Tree vigor	Yield	Bearing habit	Nut weight (g)	Kernel weight (g)	Kernel percentage	Shell	Kernel color
Eureka	Superior genotype	USA	28-Mar	Vigorous	Fair	Terminal	15.2	7.40	49	Strength	Poor
Hartley	Franquette × Mayette	USA	3-Apr	Vigorous	Moderate	Terminal	14.3	6.50	45	Thin	Medium
Payne	Chance seedling	USA	18-Mar	Moderate	Moderate	Lateral	12.9	6.40	50	Strength	Light
Vina	Franquette × Payne	USA	26-Mar	Moderate	Strong	Lateral	12.6	6.20	49	Strength	Medium
Pedro	Conway Mayette × Payne	USA	5-Apr	Small	Strong	Lateral	13.5	6.50	48	Strength	Medium
Tehama	Waterloo × Payne	USA	31-Mar	Vigorous	Heavy	Lateral	14.1	6.80	48	Strength	Light
Serr	Payne × PI159568	USA	20-Mar	Vigorous	Variable	Moderate	14.4	8.10	56	Thin	Light
Chandler	Pedro × 56-224	USA	4-Apr	Moderate	Strong	Lateral	13.2	6.50	49	Thin	Extra light
Howard	Pedro × 56-224	USA	2-Apr	Moderate	Strong	Lateral	14.3	7.20	51	Strength	Light
Sunland	Lumpoc × PI159568	USA	20-Mar	Vigorous	Variable	Lateral	17.9	9.80	55	Strength	Medium
Cisco	Meylan × Pedro	USA	14-Apr	Moderate	Moderate	Terminal	14.2	6.20	44	Strength	Medium
Tulare	Tehama × Serr	USA	1-Apr	Vigorous	Strong	Lateral	14.1	7.60	53	Strength	Light
Robert Livermore	UC86-11 × Howard	USA	3-Apr	Moderate	Moderate	Lateral	12.9	6.40	50	Strength	Red skin
Sexton	Chandler × 85-8	USA	26-Mar	Small	Good	Lateral	15.6	8.30	53	Strength	Light
Gillet	Chico × 76-80	USA	25-Mar	Moderate	Strong	Lateral	15.2	7.70	51	Thin	Light
Forde	Chico × Lara	USA	30-Mar	Moderate	Good	Lateral	15.5	8.10	52	Strength	Light
Ivanhoe	UC 67-13 × Chico	USA	17-Mar	Moderate	Strong	Lateral	12.8	7.30	57	Thin	Extra light
Solano	UC67-13 × Chico	USA	28-Mar	Moderate	Strong	Lateral	14.6	7.90	54	Strength	Extra light
Durham	Chandler × PI159568	USA	30-Mar	Moderate	Strong	Lateral	15.1	8.30	55	Strength	Light

Franquette	Superior genotype	France	23-Apr	Vigorous	Fair	Terminal	11.0	5.50	50	Medium	Extra light
Lara	UC49-46 × Franquette	USA	13-Apr	Moderate	Strong	Lateral	11.5	5.50	48	Thin	Medium
Fernor	Franquette × Lara	France	21-Apr	Moderate	Strong	Lateral	11.0	4.90	44	Medium	Extra light
Fernette	Franquette × Lara	France	15-Apr	Moderate	Good	Lateral	14.9	7.50	50	Medium	Extra light
Ferjean	Grosvert × Lara	France	15-Apr	Moderate	Strong	Lateral	11.9	5.95	50	Thin	Extra light
Jamal	Superior genotype	Iran	Early Apr	Vigorous	Moderate	Moderate	11.42	5.80	50.77	Medium	Medium
Damavand	Superior genotype	Iran	Early Apr	Vigorous	Moderate	Moderate	13.09	6.27	47.07	Medium	Medium
Yalova 1	Superior genotype	Turkey	Early Apr	Vigorous	Low	Terminal	17.16	7.98	47.76	Medium	Dark
Yalova 3	Superior genotype	Turkey	Early Apr	Vigorous	Low	Terminal	13.42	7.28	53	Thin	Light
Sebin	Superior genotype	Turkey	8–16 Apr	Moderate	Strong	Moderate	12.68	6.34	61.24	Thin	Light
Bilecik	Superior genotype	Turkey	9–13 Apr	Vigorous	Moderate	Terminal	12.87	4.80	50.11	Medium	Medium
Maras 18	Superior genotype	Turkey	4–8 Apr	Vigorous	Moderate	Lateral	13–15	7–9	53–57	Medium	Light
Sütyemez 1	Superior genotype	Turkey	2–6 Apr	Vigorous	Moderate	Lateral	25–27	12–14	49–51	Medium	Light
Kaman 1	Superior genotype	Turkey	6–10 Apr	Vigorous	Strong	Lateral	13–14	7–8	52–57	Medium	Light

(continued)

Table 11.3 (continued)

Cultivar	Parent	Origin	Leafing date	Tree vigor	Yield	Bearing habit	Nut weight (g)	Kernel weight (g)	Kernel percentage	Shell	Kernel color
Zha 343	Superior genotype	China	Early Apr	Vigorous	Strong	Lateral	12.13	6.48	53	Thin	Light
Jinlong 1	Superior genotype	China	Early Apr	Vigorous	Moderate	Terminal	14.85	9.10	61	Thin	Light
Wen 185	Superior genotype	China	Early Apr	Moderate	Strong	Lateral	15.2	9.86	65	Thin	Light
Xiangling	Shangsong5 × Akesu9	China	Early Apr	Vigorous	Strong	Lateral	12.2	8.00	65	Thin	Light
Zhonglin 1	Jian92723 × Fenyangchuanzi	China	Early Apr	Vigorous	Strong	Lateral	14.0	6.29	56	Thin	Extra light
Liaoning 1	Dabopi10103 × Xinjiangzhipi11001	China	Early Apr	Vigorous	Strong	Lateral	9.4	7.29	65	Thin	Extra light
Jinboxiang1	Superior genotype	China	Early Apr	Moderate	Strong	Lateral	11.5	5.40	47	Thin	Light
Luguo 2	Shangsong6 × Luxiang	China	Late Mar	Vigorous	Strong	Lateral	14.5	10.01	60	Thin	Light

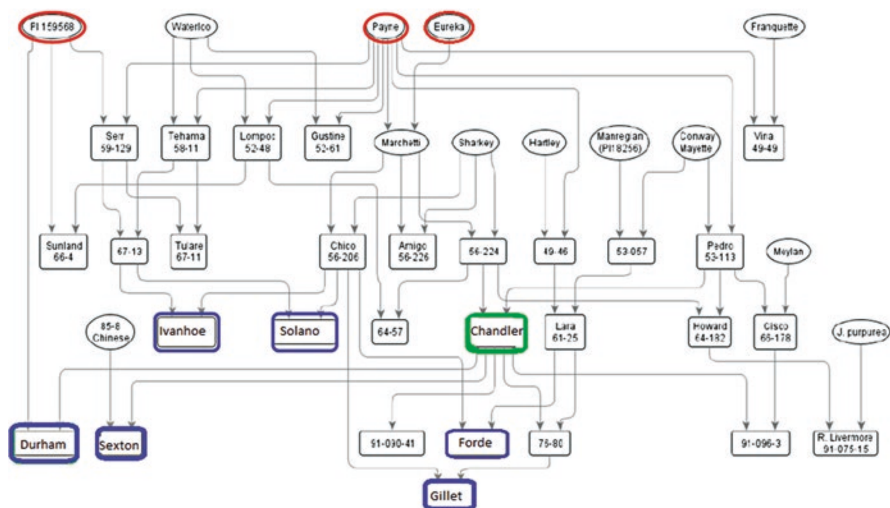

Fig. 11.3 The phylogeny of main California walnut cultivars; red, blue and green lines indicate main parents, new cultivars and the most common cultivar, respectively. (Source: Leslie 2016)

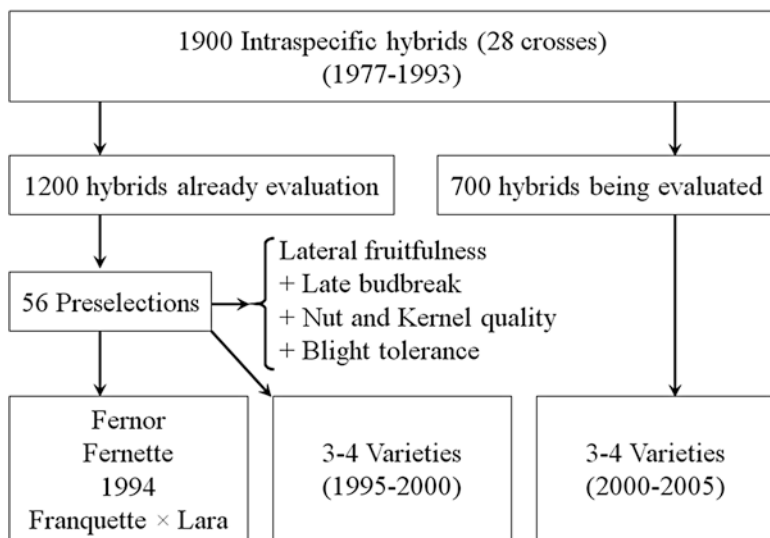

Fig. 11.4 The French walnut breeding program scheme in the second and third breeding phase. (Source: Germain 1997)

countries including China, Turkey and Iran started breeding programs in these phases. Walnut genetic improvement also started in some Asian (a primary center of diversity) and European (a secondary center of diversity) countries. Exploitation of genetic diversity and identification of superior genotypes were the main breeding strategies in these countries (Avanzato et al. 2014).

After the retirement of Harold Forde, the walnut breeding program in the USA continued under Gale McGranahan with the assistance of Charles Leslie (as the third phase). In addition to previous breeding objectives, by making 300 controlled crosses, late-leafing walnut cultivars with the additional trait of early harvest were released to growers. In addition, resistance to blight and blackline (CLRV) and absence of pistillate flower abscission (PFA) were other breeding objectives. Tulare, Robert Livermore, Sexton, Gillet and Forde were the main commercial cultivars obtained from this third phase of the walnut breeding program in the USA (Fig. 11.3; Table 11.2). Application of molecular markers along with traditional breeding was the most significant development in the third breeding phase (Leslie et al. 2009; McGranahan and Leslie 2004, 2005). The fourth walnut breeding phase continued under the leadership of Charles Leslie after the retirement of Gale McGranahan in 2009 in the USA. The main objectives are still early harvest dates, high yield and quality, and resistance to blight and blackline (CLRV). Ivanhoe (2010), Solano (2012) and Durham (2016) are three new cultivars with harvest dates earlier than cv. Chandler.

Modern biotechnology-based methods of plant breeding were also developed and introduced during this phase. A cooperative effort involving multiple laboratories, notably those of Abhaya Dandekar, Jan Dvorak and MingCheng Luo (UC Davis), and Malli Aradhya (USDA-ARS), with the participation of the UC Davis Genome Center, worked for several years to develop integrated physical, functional and genetic maps of the walnut genome (Leslie and McGranahan 2014). A major recent development has been the first sequencing of the Persian walnut genome by a group led by David Neale at UC Davis. This accumulating genetic information should accelerate the rate of breeding and varietal improvement in walnuts and help breeders select for desired traits (Martínez-García et al. 2016).

The third phase of the French breeding program continued under the leadership of E. Germain and F. Delort at INRA, Bordeaux. Additional hybrids, obtained from 28 crosses, were tested for late leafing and lateral bearing as the main objectives and more cultivars (e.g. Ferjean) were released. Germain (1997) described crosses carried out using the best previous hybrids with new sources from the Mediterranean (for lateral bearing) and Iran or Central Asia (for precocity). The French walnut breeding was then inactive for several years. In 2009, Fabrice Lheureux started a new phase of French walnut breeding (the fourth phase) and four cultivars (Feradam, Ferbel, Ferouette, Fertignac) were released (Bernard et al. 2018).

The walnut breeding program in Iran, initially led by Jamal Atefi at Horticulture Science Research Institute (HSRI), started to release new cultivars in 1983. Iran is one of the main centers of origin of walnut in the world and its traditional orchards contain great genetic diversity (Atefi 1993; Vahdati 2000). The main breeding strategy has been evaluation and selection from among this resource (Atefi 1990, 1993, 1997; Hassani et al. 2014). For this purpose, four collections of the selected superior genotypes are maintained in Karaj, Shahrood, Mashhad and Uremia. In 1994, seven genotypes from the Karaj collection (K72, Z63, Z30, Z60, Z67, Z53, B21) were selected and planted with eight French/Californian commercial cultivars (Chandler, Pedro, Hartley, Lara, Serr, Vina, Franquette, Ronde de Montignac) in Karaj. Among these genotypes, two promising genotypes, Jamal (Z63) and Damavad (Z30), were

released as the first Iranian walnut cultivars in 2009–2010 (Hassani et al. 2014). Currently, the fourth phase of the walnut-breeding program at HSRI continues based on germplasm evaluation and hybridization. Recently, four commercial walnut cultivars (Persia, Caspian, Chaldoran and Alvand) have been released which are characterized by high yield, lateral fruitfulness and late-leafing date.

In addition to HSRI, walnut breeding is in progress at the University of Tehran (Aburaihan Campus) under the management of Kourosh Vahdati. Germplasm evaluation in the walnut plantation areas (such as Kerman, Ilam, Fars, Qazvin, Alborz, Yazd, Kohgilooye and Boyer-Ahmad, Mazandaran etc.), hybridization, and molecular breeding are the main walnut breeding strategies used at the University of Tehran to release commercial cultivars and rootstocks. High yield, late leafing, lateral bearing, early harvest, dwarfing and drought stress tolerance are the most important objectives (Karimi et al. 2014; Vahdati and Mohseniazar 2016; Vahdati and Rezaee 2014; Vahdati et al. 2015).In China, walnut genetic improvement is based on germplasm evaluation and hybridization. The majority of germplasm evaluation is conducted in Xinjiang province, where there are extensive germplasm resources for walnut. About 80% of Chinese walnut cultivars are selected or originate from Xinjiang germplasm. Although the walnut breeding program in China began at the Liaoning Economic Forest Institute and China Academy of Forestry in the early 1960s, most of the current walnut cultivars were released after establishing uniform national selection criteria. Since the 1980s, based on these national standards, excellent progress has been made in selection of superior genotypes. Approximately 26 cultivars, including Baokexiang, Beijing 861, Jinglong 1, Jinglong 2, Lipin 1, Lipin 2, Lubo, Xifu 1, Xilin 1 and Xinzaofeng, were released (1979–2006) as a result of this work. In addition to selection, controlled crosses in China led to release of 16 cultivars, including Liaoning 1-8, Xiangling, Fenghui and Zhonglin 1-6. Most of the controlled crosses were between Chinese cultivars, especially Xinjiang walnuts (Baojun et al. 2010; Chen et al. 2014; Wu et al. 2010). The fourth phase of walnut breeding in China produced about 20 additional cultivars from controlled crosses. Chen et al. (2014) and Zhang et al. (2014) reported details about these cultivars.

Most Turkish walnut trees are early leafing and terminal bearing. Therefore, late leafing and lateral bearing are main breeding objectives. The walnut breeding program in Turkey is based on germplasm evaluation and selection of superior genotypes. In addition to Turkey being a center of origin, most walnut orchards in Turkey are seed propagated. Therefore, high genetic variation is retained in traditional orchards. Although many trees have been cut for timber, large seedling walnut populations remains.

The first walnut breeding program in Turkey began using identification of superior genotypes from 20 different sites in the Marmara region of northwest Turkey in 1971 (Ölez 1971). Subsequently, germplasm evaluation and selection of superior genotypes were continued by other researchers in different regions of Turkey (Akça and Polat 2007; Ertürk and Akça 2014). All Turkish walnut cultivars have been selected based on germplasm evaluation (Aslantaş 2006). Sebin, Bilecik, Acka 1, Maras 10, Bursa 95, Yalova 1 and Yalova 3 are some Turkish walnut cultivars released, based on selection from native walnut populations (Akça and Polat 2007). The leading cultivars are Yalova 1, Yalova 3, Yalova 4, Sebin and Bilecik. In addition

to germplasm evaluation, some controlled crosses between local and foreign cultivars were conducted in a new Turkish walnut breeding program since 2008. These controlled crosses produced about 1340 hybrids, which are under evaluation (Akça et al. 2016). Maras 18, Sutyemez 1 and Kaman 1 are new Turkish walnut cultivars released based on germplasm evaluation in 2009 and 2010. Also, Dirilis, 15 Temmuz, Maras 12 and Bayrak are Turkish walnut cultivars recently released (Ozcan et al. 2017). In addition to late leafing, lateral bearing and early harvest, the aim of the new Turkish walnut breeding program is to search for new promising rootstock candidates having tolerance to salt, lime, drought and disease stresses (Ertürk and Akça 2014).

In addition to these leading countries, walnut breeding is also conducted in other walnut growing countries including Spain, Germany (Bollersen 2017), Georgia, Italy, Hungary, Greece, Romania, Ukraine, Serbia and Azerbaijan. Walnut breeding in some of these countries dates back to the onset of the second phase of the world's walnut breeding period and most breeding objectives are the same in these countries. Some local cultivars were released based on genetic diversity evaluation and hybridization between local superior genotypes and French/Californian commercial cultivars. A brief summary of the released cultivars is contained in the book *Following Walnut Footprints* (Avanzato et al. 2014).

11.2.3.2 Rootstock

Rootstocks can play a crucial role in determining orchard efficiency. Combining the desirable attributes of two different plants by budding or grafting can produce different growth effects (Nimbolkar et al. 2016). Rootstocks are bred to grow in different soil types and conditions, and to provide the best anchorage, vigor, and resistance or tolerance to soil-borne pests and diseases and abiotic stresses. However, no individual rootstock is tolerant to all the factors impacting walnut production. The strengths and weaknesses of each rootstock needs to be considered in the context of a specific orchard location. Traditionally, the main walnut rootstocks were Persian walnut (*Juglas regia*) seedlings. Seedlings of Northern California black walnut (*J. hindsii*) were also traditionally used as rootstocks for Persian walnut scions in California.

The USA is a leading country in walnut rootstock breeding, having used Paradox cv., as walnut rootstocks for a long time. Paradox cv. rootstocks are hybrids of *Juglans hindsii* × *J. regia*. Other *Juglans* hybrids include Royal *(J. hindsii* × *J. nigra)* which is less vigorous than Paradox, perhaps due to their crop load, and not used as rootstock (Forde 1975). Because they display vigor and disease resistance superior to either parent, Paradox cv. hybrids are the most common rootstocks for *J. regia* in California. Currently, Paradox cv. rootstock accounts for 80% of walnut orchards (Baumgartner et al. 2013). Different types of Paradox have been introduced over the years (Tulecke and McGranahan 1994). Paradox is more vigorous, more resistant to some *Phytophthora* species, and more tolerant to soil salinity than *J. regia* (McGranahan and Catlin 1987). Despite its widespread use, the resistance of seedling Paradox root-

stock to *Armillaria* root disease, crown gall, and *Phytophthora* remains insufficient as evidenced by serious losses to the walnut industry from these root diseases (McGranahan and Leslie 1991). In addition, Paradox is susceptible to blackline disease caused by a hypersensitive response scions infected with cherry leaf roll virus. Therefore, walnut rootstock breeding in California was directed toward genetic tolerance to CLRV and resistance to soil-borne pathogens. In recent years, three clonal rootstock cvs. have become available (Vlach, RX1 and VX211). In addition, a few walnut growers use own-rooted English walnuts in areas where blackline disease (CLRV) is prevalent. Vlach cv. is vigorous but in trials has not demonstrated resistance to pathogens. RX1 cv., a *J. microcarpa* × *J. regia* hybrid with tolerance to *Phytophthora*, and VX211, a very vigorous *J. hindsii* × *J. regia* hybrid exhibiting nematode tolerance, have been released commercially (Leslie and McGranahan 2014).

Rootstock improvement work has also been proceeding in China. The Shanxi Academy of Forestry Sciences released the Jin RS-1 cv. rootstock series in 2011; Jin RS-2 and Jin RS-3, have been evaluated for cold, disease, and pest resistance, and seem to be ideal for northern Chinese areas subject to frost (Bernard et al. 2018; Wang et al. 2014). Walnut rootstock breeding is also proceeding, based on germplasm evaluation in other countries such as Turkey and Iran. Some of the main objectives in these countries are dwarfing, tolerance to salt and drought stress, and resistance to soil-borne disease such as *Agrobacterium*, *Phytophthora* and *Armillaria*.

Walnut rootstock breeding in Iran began at the University of Tehran (directed by Kourosh Vahdati) in 2003 with drought tolerance and dwarfing as the main objectives. Walnut germplasm from different regions of Iran were evaluated and superior genotypes were selected and planted in the Walnut Research Orchard at Aburaihan Campus, University of Tehran, Pakdasht, Tehran, Iran. Compatibility evaluation and molecular studies of these selected genotypes are ongoing.

11.3 Germplasm Biodiversity and Conservation

11.3.1 Genetic Resources and Biodiversity Conservation

Walnut, like other woody plants, typically has a long breeding cycle. Therefore, introduction of new cultivars may require many breeding cycles and dozens of years. Walnut breeders have been able to bypass long breeding cycles by using biotechnology alongside exploiting biodiversity. Recent advances in biotechnology and genomics have the potential to accelerate cultivar development greatly (van Nocker and Gardiner 2014), but exploitation of biodiversity also can be considered as a short cut to obtain commercial cultivars with desirable characteristics. Selection of superior genotypes provides a gene bank of desirable genes and enables biotechnologists to conduct breeding programs more confidently and rapidly.

The primary center of origin of walnut is the Persian plateau (including modern Iran and some central Asian countries). It is easy to find wild walnut populations in these countries. The existence of different types of walnut trees with a variety of

characteristics enables walnut breeders to implement any breeding program based on these populations. In addition, walnuts dispersed to other regions in the world, especially Europe and Eastern Asian countries using seed, so high genetic diversity exists in the secondary walnut diversity centers. Walnut genetic resources have played a key role in producing the current walnut cultivars. Many commercial walnut cultivars released in California originated from seedlings collected in Iran (cvs. Eureka and Olmo), Afghanistan (cv. Pl159568) and other countries of walnut origin such as China (Tulecke and McGranahan 1994).

Biodiversity conservation is a global concern. A large proportion of walnut genetic resources are threatened by destruction, from urbanization, the commercialization of traditional orchards and timber use. Currently, the only method of walnut germplasm conservation in center of origin countries is the identification of superior genotypes and their transfer to collections. Due to the existence of a huge genetic diversity in these countries (Iran is estimated to have more than 20 million walnut genotypes), this method is not efficient. On the other hand, classical methods of biodiversity conservation have certain limitations in terms of rapid production of plants and their long-term conservation. Therefore, it is necessary to conserve walnut biodiversity as international capital in the framework of an international project using new germplasm conservation techniques that make use of biotechnology. Biotechnological methods such as plant tissue culture, plant cell culture, anther culture, embryo culture etc. are quite applicable and useful techniques for ex situ conservation. It is axiomatic that modern biotechnology can help to counteract trends of genetic erosion in all agricultural sectors (Ogbu 2014; Pathak and Abido 2014).

11.3.2 Cultivar Characterization and Phylogeny

For a long time, walnut growers have used seeds of superior genotypes to establish new orchards, which has led to high genetic diversity in the walnut populations of the world. Following development of grafting techniques and walnut breeding programs in France and California, walnut cultivars were introduced. In addition to France and California, walnut cultivars were released in other countries based on exploitation of local genetic diversity and hybridization, although most are only locally cultivated. Several French/Californian cultivars are cultivated worldwide. The first of these was Franquette. Franquette, the main cultivar in French orchards, is late leafing with good nut characteristics but is terminal bearing and hence only moderate yield (Germain 1999; Tulecke and McGranahan 1994).

Nowadays cv. Chandler, a late-leafing and laterally-fruitful cultivar is the premier cultivar planted worldwide. Chandler is a cross between cvs. Pedro and UC 56-224 (Fig. 11.3) patented and released by the University of California, Davis in 1979. Chandler is highly lateral fruitful (therefore high yield) and quite vigorous with good nut characteristics (good shell thickness, strong seal, ease of kernel removal) and light kernel color. Table 11.3 presents important walnut cultivars in the leading walnut growing countries.

11.4 Molecular Breeding

Recent advancements in next-generation sequencing (NGS) techniques, bioinformatics tools, high-throughput genotyping platforms, and genomics-based approaches such as genome wide association studies (GWAS), marker-assisted selection (MAS), genomic selection (GS), and genome editing using CRISPR-Cas9 system have opened up new avenues to enhance the efficiency of fruit trees breeding, leading to release of new scions and rootstocks. Hindering walnut genetic improvement is the long juvenile phase and high degree of heterozygosity. In this section, we describe the potential of molecular breeding using novel genomic technologies in walnut genetic improvement to overcome conventional breeding barriers.

We first introduce molecular marker systems and whole nuclear and chloroplast genome sequence information that is available for walnut breeding. Next, we review QTL mapping, GWAS and GS studies conducted on walnut. We then review functional genomic studies including transcriptomics, proteomics and metabolomics done on walnut. Then we briefly review bioinformatics-assisted walnut breeding. Since fruit and nut tree crops, including walnut, have a long juvenile period, development of a new variety or rootstock may take 15–20 years via classical breeding and MAS and GS alone cannot accelerate the genetic improvement of walnut trees. Therefore, we note the future prospects of molecular breeding in walnut using novel technologies for rapid generation advancement.

11.4.1 Molecular Markers in Fruit and Nut Breeding

Many studies have described genetic variability in fruit crops. Additional and more direct indicators of genetic diversity are needed despite the considerable quantity of morphological descriptors, the large amount of walnut germplasm resources, and the prolific development of new scion and rootstock. Nowadays, genetic markers systems are widely used in germplasm characterization in order to assist phenotypic evaluation and accelerate breeding. Molecular markers have been widely used to analyze genetic relationships between wild and cultivated individuals and related species, interspecific hybrids identification, germplasm variability evaluation and for cultivar identification.

11.4.2 Molecular Marker Systems

Isozymes markers were once among the most widely used molecular markers in genetic studies. Isozymes have been used in different aspects of walnut genetic analysis including evaluation, interspecific hybrid identification, genetic diversity or relationship analysis and determination of genotype origin (Aletà et al. 1990;

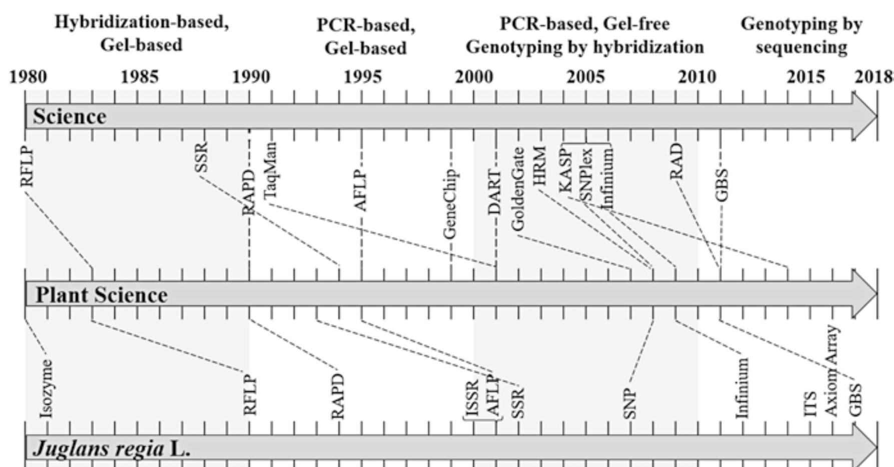

Fig. 11.5 Timeline for the first scientific documentation for the application of different commonly used molecular markers in science, plant science and Persian walnut genotypes. (Adopted from Obermeier and Friedt 2015)

Arulsekar et al. 1986; Busov et al. 2002; Cheng and Yang 1987; Fornari et al. 2001; Malvolti et al. 1993, 1994; McGranahan et al. 1986; Ninot and Aletà 2003; Solar et al. 1994; Vyas et al. 2003).

Isozymes were superseded by an emerging variety of more informative and robust DNA markers with higher rates of polymorphism, including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs). Some of these are still viable, cheap and quick methods to identify genetic variability, and RFLPs, RAPDs, AFLPs and SSRs consecutively contributed to the identification of walnut germplasm, evaluation of its genetic diversity, and assessment of uniformity and stability of cultivars (Fig. 11.5).

11.4.3 Overview of the Diversity and History of Fingerprinting Techniques in Walnut

11.4.3.1 Hybridization-Based Markers

Restriction Fragment Length Polymorphism (RFLP) The first DNA marker technology used in plants was RFLPs (Tanksley et al. 1989). Aly et al. (1992), for the first time, used a combination of isozyme and RFLP markers in *Juglans regia*, to determine the origin of somatic embryos derived from ovule tissues. RFLPs have been applied for different purposes in walnut, including genetic mapping (Fjellstrom and Parfitt 1994a, b) and genetic diversity assessment (Fjellstrom et al. 1994). Limitations of the RFLP technique include a requirement for high molecular weight

DNA, low levels of polymorphism and complexity of radioactivity or other staining techniques, which motivated the development of several alternative technologies (Bernard et al. 2018).

11.4.3.2 PCR-Based Markers for Walnut Genome Profiling

Random Amplified Polymorphic DNA (RAPD) RAPD markers are based on polymerase chain reaction (PCR) amplification of random genomic locations, and have been widely used for genetic diversity and mapping studies because they are easy to develop and are cost- and time-effective (Welsh and McClelland 1990; Williams et al. 1990). The first applications in walnut (Fjellstrom and Parfitt 1994a) identified RAPDs in a backcross population of [*Juglans hindsii* × *J. regia*] × *J. regia* and in the next step both RAPDs and RFLPs were used to construct a revised genetic map of walnut. In other studies, RAPD markers have been used for association mapping (Woeste et al. 1996a; Yang et al. 2002), assessment of genetic and morphological diversity (Erturk and Dalkilic 2011; Fatahi et al. 2010; Francesca et al. 2010; Nicese et al. 1998; Qianwen et al. 2010; Woeste et al. 1998), genetic mapping (Malvolti et al. 2001) and identification of interspecific hybrids (Emilia et al. 1995; Ross-Davis et al. 2008).

Sequence Characterized Amplified Region (SCAR) RAPDs markers are easy to develop and simple, but a lack of reproducibility makes them less reliable for genetic analysis in plant breeding. Therefore, there is an option to improve the reproducibility of RAPD markers by converting RAPD amplicons into sequence characterized amplified region (SCAR) markers. Recently, several studies have identified molecular markers linked to economically-important traits in walnut, including a RAPD marker correlated with precocity (Keqiang et al. 2002), a SCAR marker correlated with shell thickness (Li et al. 2007) and a SCAR marker correlated with precocity (Li et al. 2010).

Inter-Simple Sequence Repeat (ISSR) ISSRs are amplification reactions with a single long oligonucleotide primer anchored at both ends by a simple sequence repeat (Godwin et al. 1997). ISSR markers have been applied in several genetic studies in walnut, including genetic diversity studies (Christopoulos et al. 2010; Ji et al. 2014; Pollegioni et al. 2003; Potter et al. 2002), genetic mapping studies (Malvolti et al. 2001) and studies to determine the origin of a genotype (Malvolti et al. 2010). In total, RAPD and ISSR markers have been widely used for local *Juglans regia* germplasm characterization by Iranian, Romanian, Chinese, Greek and Turkish walnut researchers (Christopoulos et al. 2010; Erturk and Dalkilic 2011; Fatahi et al. 2010; Ji et al. 2014; Pop et al. 2010; Qianwen et al. 2010).

Amplified Fragment Length Polymorphism (AFLP) Another high-performing molecular marker based on the selective amplification of a subset of genomic restriction fragments is AFLP (Vos et al. 1995). Like RAPDs, informative frag-

ments from AFLP are generally sequenced to generate SCAR markers (Lecouls et al. 2004). Sütyemez (2006) first stressed the high potential of AFLPs for the study of genetic diversity in walnut. AFLP markers have been widely employed in walnut genetic studies for characterization of superior genotypes (Kafkas et al. 2005), assessment of genetic diversity (Ali et al. 2016; Bayazit et al. 2007; Qing Guo et al. 2010), evolutionary studies (Chen et al. 2008, 2009; Wang et al. 2010) and for fingerprinting walnut cultivars (He et al. 2010; Ma et al. 2011; Xu et al. 2012).

Simple Sequence Repeat (SSR) or Microsatellite Technique Since the late 1990s to early 2000s, microsatellites or simple sequence repeats (SSRs) have been a revolutionary tool for efficient genetic investigations, including genetic variability, population structure, genetic mapping and marker-assisted selection (MAS). SSRs have been the most widely used markers for marker-assisted breeding of fruit and nut tree crops over the past 10 years because they are highly informative, reproducible and codominant (Bernard et al. 2018; Singh et al. 2008; Wani et al. 2010).

The excellent properties of simple sequence repeats (SSRs) have made them the markers of choice for many uses in walnut genetic studies and breeding. In walnut breeding, SSRs are primarily used to evaluate genetic diversity of collected material before choosing the parental material that will help to maximize this diversity for subsequent optimal combinations. SSRs markers have been applied in numerous walnut genetic studies with different purposes as follows: fingerprint clones of *Juglans nigra* accessions with high-quality timber (Woeste et al. 2002), assessment of genetic diversity (Gunn et al. 2010; Victory et al. 2006; Wang et al. 2008 Pop et al. 2013), hybrid identification (Pollegioni et al. 2008) and identification of parentage among progenies (Pollegioni et al. 2009; Robichaud et al. 2006).

SSRs were originally developed from both coding and non-coding regions of plant genomes; in 2002–2018, hundreds of SSRs were developed in various walnut species. They were developed from several sources including a variety of DNA libraries (genomic, genomic-enriched for SSR, bacterial artificial chromosome and cDNA libraries), as well as public databases, including expressed sequence tags (ESTs) from NCBI. The advances made in next-generation sequencing (NGS) technology to sequence the genome or transcriptome especially RNA-seq have also provided a new scenario for detecting SSRs markers.

The development of SSR markers in walnuts was accomplished using enriched SSR libraries from genomic DNA in *Juglans nigra*, *J. cinerea* and *J. regia* (Dangl et al. 2005; Hoban et al. 2008; Topçu et al. 2015; Woeste et al. 2002), from expressed sequence tag (EST) collections from the NCBI database in *J. regia* and *J. hindsii* × *J. regia* (Qi et al. 2011; Yi et al. 2011; Zhang et al. 2010, 2013), from BAC-end sequences from the NCBI database in *J. regia* (Ikhsan et al. 2016; Wu et al. 2012b), from fast isolation by AFLP of sequences containing repeats (FIASCO) in *J. mandshurica* and *J. regia* (Chen et al. 2013; Najafi et al. 2014) and from next-generation sequencing of transcriptomes in *J. cathayensis*, *J. hopeiensis* and *J. regia* (Dang et al. 2015, 2016; Hu et al. 2015).

SSRs have been broadly used for landrace/cultivar identification and studies of genetic diversity in cultivated germplasm in walnut, as well as for studying evolution and relationships between cultivated and wild relatives (Aradhya et al. 2009; Bai et al. 2010; Dang et al. 2015; Dangl et al. 2005; Ebrahimi et al. 2011, 2017; Foroni et al. 2005, 2007; Gunn et al. 2010; Han et al. 2016; Hu et al. 2015; Karimi et al. 2010; Mahmoodi et al. 2013; Mohsenipoor et al. 2010; Najafi et al. 2014; Noor Shah et al. 2016; Pollegioni et al. 2011, 2017; Pop et al. 2013; Qi et al. 2011; Robichaud et al. 2006; Roor et al. 2017; Ruiz-Garcia et al. 2011; Victory et al. 2006; Vischi et al. 2017; Wang et al. 2008, 2015; Zhang et al. 2013).

11.4.4 High-Throughput SNP Assays in Walnut

Recent advances in next-generation sequencing technologies and the continuous decrease in cost have resulted in the enormous generation of sequence data sets, allowing the rapid discovery of single nucleotide polymorphism (SNP) markers. SNP markers are distributed throughout the genome and are broadly used in animal and human genetic analysis, but their application in plants, and particularly in walnut, is in the early stages. So far, the recent availability of genomic and transcriptomic databases has made possible the discovery of SNPs in silico, using bioinformatics tools.

A walnut genome sequence (cv. Chandler v1.0) has recently been released (Martínez-García et al. 2016; available at https://www.hardwoodgenomics.org/english-walnut-genome), 27 genomes of most important founders in the walnut improvement program at the UC Davis have been resequenced and, finally, a novel Axiom® Walnut700K SNP array has been designed by UC Davis for walnut variability, linkage mapping and association mapping analysis (Marrano et al. 2019; Neale et al. 2017). The availability of a high-density genotyping array in walnut opens new opportunities to apply GWAS and genomic selection in walnut-breeding programs and other walnut populations (Bernard et al. 2018; Marrano et al. 2019; Neale et al. 2017).

11.4.5 Nuclear Ribosomal Internal Transcribed Spacer (ITS)

The nuclear ribosomal internal transcribed spacer (ITS) region is an effective genetic marker for molecular identification of plants, because of its relatively high variability and facility of amplification. Sequencing of PCR amplicons from this region have been used for SNP discovery and characterization of English walnut (*Juglans regia*) cultivars. In one study, the first and second internal transcribed spacers (ITS1 and ITS2), as well as the intervening 5.8S coding region of the rRNA gene, were amplified and sequenced, and alignment of the ITS1-5.8S-ITS2 sequences from 18 walnut cultivars showed 244 SNPs and 1 short insertion-deletion

(indel) (Ciarmiello et al. 2011). Phylogenetic analysis of the ITS1-5.8 S-ITS2 region clustered the sequences into two groups that indicated these regions could be used to differentiate these walnut cultivars (Ciarmiello et al. 2011). In another study, a total of 32.6X walnut genome (cv. Chandler) equivalents of ABI SOLiD reads were mapped to 48,661 Chandler cv. bacterial artificial chromosome (BAC) end sequences (BESs) created by Sanger sequencing and 22,799 SNPs were discovered. Finally, 6000 SNPs were selected to construct an Infinium BeadChip that was used to genotype a walnut mapping population (You et al. 2012).

Recently, a new nuclear DNA marker from the sequence of the ubiquitin ligase gene (*UBE3*) region of nuclear DNA was developed for genetic diversity assessment of walnut genetic resources. The results showed that all walnut taxa (species/variety/cultivars) were distinguished using the ubiquitin ligase gene (*UBE3*) sequence (Suo et al. 2015). In another study sequences of 2 maternally-inherited mitochondrial DNA (mtDNA) markers (3–9 and nad5) 2 maternally inherited chloroplast DNA (cpDNA) intergenic spacers (trnL-F and trnS-G), 3 nuclear DNA sequences (15R-8, ITS and Jr5680) and 11 microsatellites (EST-SSRs) were obtained from 108 individuals of *Juglans hopeiensis*, *J. regia* and *J. mandshurica*. It was previously suggested that *J. hopeiensis* was simply a hybrid of *J. regia* and *J. mandshurica*, but results from this study showed that *J. hopeiensis* haplotypes are different from haplotypes found in both *J. regia* and *J. mandshurica* (Hu et al. 2017a).

11.4.6 Emerging Marker Technology: Genotyping by Sequencing (GBS)

Next-generation sequencing (NGS) has facilitated discovery of whole genome single nucleotide polymorphisms (SNPs) and development of high-throughput genotyping technology. New methods such as restriction site-associated DNA sequencing (RAD-seq) and genotyping-by-sequencing (GBS) have been established as powerful tools for reduced-representation sequencing of multiplexed samples that integrate genome-wide molecular-marker discovery and genotyping (Scheben et al. 2017).

In plant breeding programs, GBS was for simultaneous SNP discovery and genotyping in plants with and without reference genome sequences. The flexibility and low cost of the GBS method makes it a powerful tool for plant breeding (He et al. 2014; Scheben et al. 2017), that could be applied to various approaches for walnut genetic improvement, including genomic diversity studies, linkage maps, genome-wide association studies (GWAS), marker-assisted selection (MAS) and genomic selection (GS).

Whole chloroplast genomes, transcriptomes and genotyping-by-sequencing (GBS) are used in China to determine population genetics, phylogenomics, and hybrid speciation of *Juglans*. Reconstruction of the evolutionary history of *Juglans* through genomic and transcriptomic analysis revealed that climatic variation over the past years, associated with glacial advances and population isolation, have shaped Chinese walnut demography and evolution (Zhao et al. 2018).

11.4.7 Physical Mapping

A physical map indicates the physical distance (number of base pairs) between loci. The cloning of exogenous DNA into bacterial artificial chromosomes (BACs) is a new method for genome analysis (Choi and Wing 2000). To construct the physical map of the walnut genome, two bacterial artificial chromosome (BAC) libraries were built from genomic DNA isolated from in-vitro micropropagated shoots of Persian walnut (*Juglans regia* cv. Chandler) and fragmented with either HindIII or MboI restriction endonucleases. The average insert size for the HindIII and MboI libraries were around 135 kb and 120 kb, respectively. A total of 129,024 clones, 64,512 per BAC library, were arranged in 336 plates (Wu et al. 2012b). These BAC libraries represent around 27× genome equivalents assuming the walnut genome size is approximately 606 Mb. In this study BAC fingerprinting and BAC-end sequencing were performed using a fluorescence-based, high-throughput BAC DNA fingerprinting method (Luo et al. 2003). In total, 52,840 BAC clones from HindIII and MboI libraries of Persian walnut (*J. regia* cv. Chandler) were sequenced and the resulting 48,218 walnut BESs were deposited at GenBank (Wu et al. 2012b). The average GC content of the BES was 37.7%, which is slightly higher than papaya, poplar and *Arabidopsis* genomes with GC contents of 33–35%, but lower than that of the rice genome at 43%. Based on Blast2GO analysis, 1330 unique GO terms were assigned to 6396 BESs. Distribution of GO terms in the categories of biological process, molecular function and cellular component showed that the walnut genes cover a broad range of functional categories and biological processes. Through aligning of BES with ESTs and whole walnut genome shotgun sequences. Approximately 4000 SNPs were discovered and genetically mapped in a population of cvs. Chandler × Idaho, which ultimately led to anchoring of BAC contigs onto a linkage map.

The physical map of walnut enabled the discovery of two markers flanking the LB1 locus associated with lateral bearing. Recently, a 2-year project at the University of California, Davis was initiated to discover the causative mutation at the LB1 locus, develop a predictive SNP marker, and implement the marker in walnut genetic improvement. During the first phase of this project, over 700 progenies from a selfed cv. Chandler population were identified that carry recombination events in the LB1 region. These progenies are being phenotyped for bearing habit and genotyped with additional SNP markers in the LB1 region. Preliminary results from this study suggested three LB1 candidate genes (Dvorak et al. 2015).

11.4.8 Genetic Mapping and QTL Detection

Genetic mapping or linkage mapping in plants determines the relative positions of genetic markers and genes along chromosomes based on recombination frequencies. The procedure of constructing a genetic map is as follows: (1) grouping of

markers into linkage groups, (2) ordering of markers within these groups and (3) estimating genetic distances between the markers (Cheema and Dicks 2009; Paterson 1996). Distances on genetic maps are usually expressed as centimorgans (cM), and are related to recombination frequencies with possible correction for unobserved double recombinants and interference between crossovers. Applications of genetic maps in plant genetic improvement include: (1) identification of genomic regions or quantitative trait loci (QTLs) linked to agronomic traits of interest; (2) map-based cloning of major genes involved in important agronomic traits and the development of markers for MAS; (3) understanding chromosome evolution and phylogenetic relationships within and between species and (4) assisting with genome assembly (Luo et al. 2015; Semagn et al. 2006; You et al. 2012; Zhu et al. 2015).

Several genetic and QTL mapping studies have been conducted in walnut. Forty-two RFLP markers were applied for the first time by Fjellstrom and Parfitt (1994a) to construct a genetic linkage map from a progeny of 63 individuals from an interspecific backcross of [*Juglans hindsii* × *J. regia*] × *J. regia*. A few years later, 66 RAPD markers were used to investigate the same set of progeny and a new genetic map was constructed including the previously found RFLPs, along with the new RAPD markers (Woeste et al. 1996b). In another study, 120 RAPD and 4 isozyme markers were used for genetic map construction using 82 progenies from an intra-specific cross of walnut *J. regia* cv. Lara 480 and *J. regia* cv. Chandler 1036 (Malvolti et al. 2001).

11.4.9 Next-Generation Mapping

Integration of genetic linkage mapping and comparative genomics has been proposed as a powerful tool for map-based cloning and molecular marker development from functional genes involved in the trait(s) of interest. Establishment of segregating mapping populations by crossing two parents with phenotypic difference(s) in at least one trait of interest is required for QTL mapping. A 6 K Infinium SNP iSelect assay was used to genotype 425 F_1 progeny from a cross of cv. Chandler with cv. Idaho, and 1525 SNPs markers were mapped into 16 linkage groups (LGs) corresponding to the 16 walnut chromosomes. The LG lengths ranged from 37.7 cM (LG15) to 97.3 cM (LG7), and the total length of the genetic map was 1049.5 cM (Luo et al. 2015; You et al. 2012). This genetic map was used to construct a walnut bacterial artificial chromosome (BAC) clone-based physical map with 15,203 exonic BAC-end sequences (Luo et al. 2015). In another study, specific length amplified fragment sequencing (SLAF-seq) technique was used to generate large numbers of molecular markers to construct high-density genetic maps for walnut molecular breeding. In this research a F_1 population of 84 individuals was created from an intraspecific cross between cv. Yuan Lin (maternal line susceptible to anthracnose) and cv. Qing Lin (paternal line with resistance to anthracnose) and 2577 SLAF markers were used to construct genetic linkage maps. A total of 2395 of

these markers were assigned into 16 linkage groups (LGs) for the female map; likewise, 448 markers were used for the male map (Zhu et al. 2015). Finally, a QTL linked to walnut anthracnose resistance was identified on LG14. The 95% confidence interval for the QTL ranged from 165.51 to 176.33 cM on LG14, and 10 markers in this interval were considered to be linked markers to the anthracnose resistance trait. The phenotypic variance explained by each marker was 16.2–19.9% with LOD scores of 3.22–4.04. These results will assist molecular marker-assisted breeding and walnut-anthracnose-resistance gene identification (Zhu et al. 2015).

11.4.10 Comparative Mapping

Comparative mapping and comparative sequence analysis are valuable methods to identify similarities and differences between genomes, assist in the reconstruction of ancestral genomes, and consolidate genetic maps and to identify candidate genes underlying QTL (Luo et al. 2015).

A genetic map for walnut was constructed initially with walnut 6 K Infinium SNP assay, then used to construct a walnut bacterial artificial chromosome (BAC) clone-based physical map, and synteny was quantified with other plant genomes. These included three long-lived woody perennials, *Vitis vinifera*, *Populus trichocarpa* and *Malus domestica*, and three short-lived herbs, *Cucumis sativus*, *Medicago truncatula* and *Fragaria vesca*. The results of synteny analysis revealed that long-lived woody perennials were less diverged from the walnut genome than short-lived herbaceous annuals (Luo et al. 2015).

11.4.11 Walnut Genome Sequencing

11.4.11.1 Chloroplast Genome Sequencing

The advent of high-throughput sequencing technologies has facilitated a rapid improvement in the field of chloroplast genetics and genomics. Currently, more than 800 complete chloroplast genomes, including 300 from crop and tree genomes, have been sequenced, and the genomic information is available in the National Center for Biotechnology Information (NCBI) organelle genome database (Daniell et al. 2016). The genetic information gained from complete chloroplast genome sequences has improved our understanding of plant biology, diversity, phylogeny and evolution. In addition, considerable variation within and between plant species, in terms of both sequence and structural variation, have been revealed by chloroplast genome sequences (Daniell et al. 2016; Hu et al. 2016, 2017b). The information obtained from chloroplast genome sequences has been especially valuable for understanding plant adaptability to severe environmental conditions, assisting breeding of closely related species (Daniell et al. 2016).

The first complete chloroplast genome of walnut was sequenced using the Illumina MiSeq platform and was assembled and annotated using SPAdes and CpGAVAS software, respectively (Liu et al. 2012). The length of the chloroplast genome of walnut was 160,367 bp (GenBank accession number KT963008) with 36.11% GC content. A total of 137 functional genes included: (1) 86 protein-coding genes; (2) 3 pseudo genes (2 ycf15 and 1 infA); (3) 40 transfer RNA genes and 8 ribosomal RNA genes. Also the results of this study showed that there were 12 protein-coding genes, 14 transfer RNA and all 8 ribosomal RNA genes duplicated in the inverted repeat (IR) regions (Hu et al. 2016). Phylogenetic analysis of the walnut chloroplast genome with 11 chloroplast genomes from other species revealed that walnut was most closely related to the Fagaceae family and the genus *Populus* (Hu et al. 2016). Recently, the chloroplast genomes of 5 *Juglans,* including *J. regia* (common walnut), *J. sigillata* (iron walnut), *J. cathayensis* (Chinese walnut), *J. hopeiensis* (ma walnut), and *J. mandshurica* (Manchurian walnut), were sequenced to evaluate the structural patterns of their whole chloroplast genomes, to discover potential simple sequence repeats (SSRs) and divergence hotspots, and to determine their phylogenetic relationships (Hu et al. 2017b).

A combination of de novo and reference-based assembly strategies were used to reconstruct each species' chloroplast genome. Then, genome annotation and analysis were done using the online program Dual Organellar Genome Annotator (DOGMA, Wyman et al. 2004), and genomic sequences were analyzed using MISA software (http://pgrc.ipk-gatersleben.de/misa/) to identify potential simple sequence repeats (SSRs) (Hu et al. 2017b). Phylogenetic analysis powerfully supported division of the 5 walnut species into 2 previously documented sections including *Juglans*/*Dioscaryon* and *Cardiocaryon* (Hu et al. 2017a, b). In the other study, the complete chloroplast genomes and 2 nuclear DNA regions (the internal transcribed spacer and ubiquitin ligase gene) of 10 representative taxa of *Juglans* were used for phylogenetic analysis of the *Juglans* genus (Dong et al. 2017). The result of this study revealed that all 10 chloroplast genomes possessed 112 unique genes, including 78 protein coding, 30 transfer RNA and 4 ribosomal RNA genes. Also, based on 2 nuclear DNA regions, *Juglans* could be classified into 3 branches; *Juglans*, *Cardiocaryon* and *Rhysocaryon*.

11.4.11.2 Nuclear Genome Sequencing

The first complete high-quality draft genome of *Juglans regia,* from cv. Chandler, was obtained using the Illumina sequencing platform, resulting in 500 million reads and 120x genome coverage (Martínez-García et al. 2016). The nuclear genome of *J. regia* was 667 Mbp in length, with an N50 scaffold size of 464,955 bp (based on a genome size of 606 Mbp estimated by flow cytometry), 221,640 contigs and 37% GC content (Martínez-García et al. 2016). The genome assembly was performed using two different methods: SOAPdenovo2 (Luo et al. 2012) and MaSuRCA (Zimin et al. 2013). Extra scaffolding was done using RNA-seq transcripts from 19 different tissues that were assembled separately. The assembled genome was anno-

tated with MAKER-P and other genomic resources including expressed sequence tags (ESTs) and protein sequences from related species and the assembled *J. regia* transcriptome, which ultimately yielded 32,498 gene models. More than 1.2 million SNPs were discovered in the draft consensus sequence genome of *J. regia* (Martínez-García et al. 2016).

The availability of the first release of the walnut genome sequence (cv. Chandler v1.0) has helped foster genomic research in walnut breeding programs. However, short-read sequencing technologies were used for the first walnut genome sequence (cv. Chandler v1.0). To overcome the limitations of these technologies, the latest sequencing and optical mapping approaches have been used to improve the quality of the first genome assembly. In the first step, a second genome sequence of cv. Chandler was obtained using the Oxford Nanopore MinION sequencing platform, resulting in over 7 million reads and 35X genome coverage. In the next steps, chromosome-scale assembly and gene annotation will be done by optical mapping technology and Isoform Sequencing (Iso-Seq) to release walnut genome (cv. Chandler v2.0). The improved high quality walnut genome assembly (cv. Chandler v2.0) will provide a valuable genomic tool for genetic and genome-wide studies in walnut genetic improvement programs (Neale et al. 2017). Genome assemblies of tree crops are typically mosaics of the two distinct haplotypes found in a heterozygous diploid individual. To circumvent this problem, Zhu et al. (2019) generated a genome assembly from an interspecific hybrid of *Juglans microcarpa* and *J. regia* cv Serr, using BioNano and PacBio technologies. Due to the high divergence between *J. microcarpa* and *J. regia*, sequence reads for each species assembled separately, generating high quality genome assemblies from a perfectly phased gamete/haplotype from each parental species. This strategy can be efficiently applied to any heterozygous tree crop for which interspecific hybrids are already available or can be generated.

11.4.11.3 Walnut Genome Resequencing

To identify allelic variants at each genetic locus, the genetic variation in related *Juglans* species and in *J. regia* was discovered through sequencing and resequencing methodologies. A genome resequencing project was conducted at UC Davis to discover whole genome sequence variation in 27 accessions representing most important selections of their walnut improvement program. This genomic information will become an important resource for future genetic and population studies to identify alleles associated with phenotypes across walnut germplasm (Marrano et al. 2019).

SNP Discovery and SNP Genotyping Array Design After genome resequencing of the selected walnut accessions, BWA-MEN was used to align reads to v1.0 reference genome, and SNP calling, and yielded 17,800,528 SNPs, of which 609,658 SNPs covering 622 MB on 8079 scaffolds, were used for final array design (Marrano et al. 2019).

SNP Genotyping The novel Axiom® Walnut700K SNP array has been applied to genotype a total of 1284 trees in the walnut-breeding program at UC Davis and also a population of 95 walnut trees collected from different parts of Iran (Arab et al. 2019; Marrano et al. 2019). The results showed that a large majority of SNPs for both of California and Iran walnut trees (55.7 and 53%, respectively) fell in the class of PolyHigh Resolution (PHR) polymorphisms. Therefore, the Axiom® Walnut700K SNP array is a valid genomic tool for walnut genetic research including diversity and GWA studies worldwide (Marrano et al. 2019; Neale et al. 2017).

11.4.11.4 Phylogeographic Study in Walnut Using Whole Genome Sequencing and Resequencing

Demographic responses to climate change and diversification of the walnut genus *Juglans* were revealed by applying the pairwise sequentially Markovian coalescent approach to whole-genome sequences of 11 temperate *Juglans* species. Genome sequencing of 3 walnut species was done using the Illumina HiSeq 2500 sequencing platform to a depth of 90× for *J. mandshurica*, 57× for *J. regia* and 53× for *J. nigra,* and ALLPATHSLG (v.474117) was used for assembly. Then the genomes of 31 individuals from populations of 11 *Juglans* species (*Rhysocaryon*: *J. californica*, *J. hindsii*, *J. microcarpa*, *J. major*, *J. nigra*; *Cardiocaryon*: *J. cinerea*, *J. mandshurica*, *J. ailantifolia*, *J. cathayensis*; *Dioscaryon*: *J. regia,* J. *sigillata*) were resequenced to an average depth of 30–40X and >80% coverage using Illumina HiSeq 4000 paired-end sequencing libraries with insert sizes of 350 bp. Results indicated that the population histories of walnut species were not driven by extrinsic environmental changes alone, and possibly interactions with specialized pathogens have played a key role (Bai et al. 2018).

11.4.11.5 Walnut Rootstock Genome Sequencing

Recently the genomes of related *Juglans* species including*; J. sigillata*, *J. nigra*, *J. microcarpa*, *J. hindsii*, *J. cathayensis* and *Pterocarya* sp. were sequenced, assembled and annotated (Stevens et al. 2018). The main purpose of this genome-sequencing project was to resequence representative samples of *J. hindsii* and *J. microcarpa* genomes. In total, 34 *J. hindsii*, 13 *J. microcarpa* and 3 hybrid individuals were sequenced. Analysis of patterns of genomic variation in this study showed significant geographic structure (Stevens et al. 2018).

11.4.12 Genomics-Assisted Breeding in Walnut

Recent advancements in next-generation sequencing (NGS) techniques, bioinformatics tools, high-throughput genotyping platforms, and genomics-based approaches such as genome wide association studies (GWAS), marker-assisted

selection (MAS), genomic selection (GS), and genome editing using CRISPR-Cas9 system have opened up new avenues to enhance the efficiency of fruit and nut tree breeding to release new scion and rootstock.

The advent of next-generation sequencing technologies has accelerated the discovery of single nucleotide polymorphism (SNP) markers, facilitated genome-wide association studies (GWAS), and enabled marker-assisted selection (MAS), and genomic selection (GS) in fruit and nut crop breeding (Iwata et al. 2016; Laurens et al. 2018; Ru et al. 2015; van Nocker and Gardiner 2014).

Genome-wide association studies (GWAS) have become a vital methodology for detection of candidate genomic regions associated with simple and complex traits. Genomic selection goes a step further, selecting genotypes predicted to be superior based on their genomic estimated breeding values (Iwata et al. 2016; Laurens et al. 2018; Ru et al. 2015; van Nocker and Gardiner 2014).

Hampering conventional genetic improvement of fruit and nut tree crops is their long juvenile phase and high degree of heterozygosis. In addition, tree crops are affected by numerous biotic and abiotic stresses that complicate genetic improvement (Rikkerink et al. 2007). Selecting promising progeny during the juvenile period through genomics-based approaches, for example GWAS and GS, will speed the genetic improvement of trees through acceleration of the breeding cycle and increased selection intensity. Thus, genomic-based approaches have great potential for promoting the efficiency of fruit and nut tree genetic improvement (Ru et al. 2015).

11.4.12.1 Genetic and Association Mapping of Economically-Important Traits in Walnut

The University of California, Davis launched a walnut genome analysis in 2007 to develop new genomic tools in order to accelerate walnut breeding. The main objectives of the project included physical and functional mapping of the walnut genome and association mapping of horticulturally important traits in walnut. As a first step, genotyping of mapping populations from the cross (cvs. Chandler × Idaho) were done using 15 microsatellite markers. The result showed that 7 out of 265 F_1 individuals were half-sib origin. For association mapping, phenotypic data included lateral vs. terminal bearing, leafing and harvest dates, nut size, shell thickness, seal strength, kernel plumpness, percent kernel (kernel/nut ratio), kernel color and yield (Dvorak et al. 2008).

Evaluation of genetic structure and differentiation within the Persian walnut germplasm collection of 399 trees, from 204 diverse accessions at the USDA germplasm repository, and 62 elite germplasm frequently used in walnut breeding program at the UC Davis, was done using 14 polymorphic microsatellite loci. This analysis provides valuable information on the genetic diversity, which is key to the association genetic analysis, and helps with identification of diverse genotypes in this population for a resequencing panel to identify SNPs in the walnut genome (Dvorak et al. 2008). The results of this study showed that observed heterozygosity was consistently lower than the expected for all loci, which a range of 0.33–0.64,

with an average of 0.52, while the expected levels ranged from 0.41 to 0.85, for with an average of 0.69. Also, the analysis of molecular variance (AMOVA) revealed ~87% of the variation could be attributed to within populations, with only 13% accounting for variation among groups suggesting significant genetic differentiation within cultivated walnut (Dvorak et al. 2008).

Single nucleotide polymorphisms (SNPs) were discovered by comparing different sources of sequence information including; BAC-end sequences, SOLiD shotgun genome sequences and RNAseq data obtained from various walnut tissues. This comparison discovered ~6000 SNPs that were then processed by Illumina to generate an Infinium array. These SNPs were used to create the genetic map by analyzing 352 progenies from a cv. Chandler × cv. Idaho cross and this map has been used to align any phenotypic trait to linkage groups (Dvorak et al. 2011). Lateral bearing is one of the key determinants of yield and the most important breeding goal in walnut genetic improvement program. Therefore, the molecular markers discovered in a previous project by Dvorak et al. (2011) were used in developing a high-throughput genotyping platform (KASPTM genotyping) for lateral bearing (Martínez-García et al. 2014). In this project, an allele specific PCR (KASP) genotyping service was used because of its ability to genotype thousands of individuals using a small number of markers at low cost and high accuracy.

11.4.12.2 QTL Mapping, GWAS and GS in Walnut

Most economically- and horticulturally-important traits in tree crops, such as fruit and nut quality, are quantitative and controlled by multiple genes or QTLs. Due to the long juvenile phase of most tree species, generating segregating populations derived from biparental crosses is difficult and costly (Rikkerink et al. 2007). Therefore, genome wide association studies (GWAS) are more realistic for QTL detection in fruit trees compared to traditional QTL mapping in biparental populations.

High-throughput genotyping technologies are indispensable for genomics-assisted breeding, and various marker systems exist for molecular breeding in walnut. The availability of walnut genome sequences enables breeders to develop genome-wide markers for high-throughput genotyping and to construct high-density genetic maps (Bernard et al. 2018; Marrano et al. 2019).

Single nucleotide polymorphism (SNP) markers are cost-effective in terms of cost per marker and allow for higher-throughput genotyping and higher-density mapping, compared to SSR markers. To date, the walnut Axiom 700 k SNP array has been used to create linkage maps and to assist GWAS and GS (Marrano et al. 2019; Neale et al. 2017). So far, a few genome-wide association studies have been conducted on walnut. For example, assessment of water use efficiency (WUE) has been done on 260 individual clones of 64 cultivars located within the walnut improvement program in UC Davis. This panel has been genotyped with the Axiom® Walnut700K SNP array and then a two-step association genetics approach identified four loci associated with $\Delta^{13}C$, which were related to abiotic stress response

(Famula et al. 2019). The walnut breeding program population at UC Davis, genotyped with the novel Axiom® Walnut700K SNP array, was phenotyped for economically-important traits such as yield, harvest date, seal strength and kernel color, over the years, and this genotype-phenotype information is used for association analysis. Also, the seedlings of 95 trees from Iran, genotyped with the novel Axiom® Walnut700K SNP array, have been extensively phenotyped over 2 years for morpho-physiological and biochemical drought tolerance-related traits and will be used to carry out marker-trait association analysis for drought tolerance (Arab et al. 2019; Neale et al. 2017). Selecting the promising progeny during the juvenile period through GS will accelerate walnut genetic improvement via reduced breeding cycles and improved selection intensity. To the best of our knowledge, there has not been any scientific report on genomic selection research in walnut. Walnut breeding began at the UC Davis using the genomics-based approach. The genetic value of each individual in a breeding population is defined by breeding values (BVs) and can be used for choosing the best candidates to produce the next generation of offspring. Estimation of BVs for the four most important traits of interest: yield, harvest date, lateral bearing and leafing date have been done using 15 different families and phenotypic data collected for almost 16 years from walnut improvement program (WIP) at UC Davis. Linear mixed model (asreml-R or lmer) and Bayesian approaches (Rjags, MCMCglmm) have been used to estimate heritability and variance components for important traits such as lateral bearing, yield, harvest date and kernel color. Repeatability estimates for yield, harvest date, kernel color and lateral bearing, were 0.44, 0.47, 0.39 and 0.78, respectively, and average narrow-sense heritability estimates were 0.26, 0.38, 0.28 and 0.58, respectively, among different locations. Finally, a ranking of individuals, based on their breeding values (BVs) will help breeders with future selections. The results will guide future crossing designs in the walnut breeding program to implement genomic selection methods in walnut in the future (Martínez-García et al. 2017).

The walnut breeding population at UC Davis was genotyped with the novel Axiom® Walnut700K SNP array, enabling selection of superior genotypes based on their genomic estimated breeding values (GEBV).

11.4.12.3 High-Throughput Phenotyping in Walnut Breeding

Tree crop breeders need to phenotype large number of trees rapidly and accurately to identify the best progeny for traits of interest. So far, few studies have been done on walnut, but more attention will need to be paid to high-throughput phenotyping in walnut breeding programs. Two economically important traits of interest studied in walnut breeding programs are seal strength and kernel color. Recently, two accurate and consistent phenotyping methods including a texturometer and a virtual camera system were used for measuring seal strength and kernel color within the walnut improvement program in UC Davis (Neale et al. 2017). High-throughput phenotyping will be one of the most challenging future goals of fruit and nut tree genetic improvement programs.

Since fruit tree crops, especially walnut, have a long juvenile period, development of a new variety or rootstock may take 15–20 years via classical breeding. In summary, production of early-flowering walnut using continually flowering transgenic intermediates along with deployment of high throughput genotyping and phenotyping to select progeny will accelerate walnut genetic improvement.

11.4.12.4 Genomics-Assisted Walnut Rootstock Breeding

An active walnut rootstock-breeding program is ongoing in California and includes researchers from the USDA, UC Riverside and UC Davis. The main goals are to dissect the underlying genetic basis of resistance to crown gall, *Phytophthora*, root lesion nematode and *Armillaria* in walnut through genomic-based tools, and to use this information to release improved rootstock. This project focuses on identification and deployment of resistance QTLs for rapid screening to accelerate rootstock breeding. Several genomic and genetic resources such as transcriptome profiles, a 6 K Illumina Infinium SNP array, a SNP-based genetic map and a physical map have been developed by this working group.

Preliminary results show that resistance to both crown gall (CG) and *Phytophthora* spp. (PHY) is expressed in hybrids of wild *Juglans* spp. and in *J. regia*, including the commercially-released rootstock cv. RX1 (*J. microcarpa* × *J. regia*). Some mother trees of *J. microcarpa* showed significantly greater half-sib family mean resistance to PHY and CG than the population mean. Similarly, some mother trees of *J. cathayensis* showed significantly greater half-sib family mean resistance to lesion nematode (NEM). These mother trees were used for QTL mapping of resistance genes (Kluepfel et al. 2015).

Genotyping by sequencing (GBS), a series of genetic analyses that includes single SNP discovery and genotyping using NGS technology, have opened new possibilities in walnut breeding and genetic studies. GBS can simultaneously perform SNP discovery and genotyping with or without reference genome sequences. Therefore, GBS and the newly designed Axiom *J. regia* 700K SNP array can be applied to walnut breeding and genetics studies, including genotyping and genetic map construction, genome-wide association studies, genomic selection and population genetic studies (Fig. 11.6) (Arab et al. 2019; He et al. 2014; Marrano et al. 2019; Scheben et al. 2017).

The reference genome sequences of *Juglans regia*, *J. microcarpa* and *J. cathayensis* is facilitated application of GBS and discovery of genes controlling targeted traits. In a study, GBS information from 600 interspecific hybrid progeny was used to generate a genetic map for *J. microcarpa* × *J. regia*. Also, this group has identified QTLs for *Agrobacterium tumefaciens* (crown gall) and *Phytophthora* resistance. Detailed information about the rootstock program is available from the walnut rootstock website (http://www.rootstocks.net/).

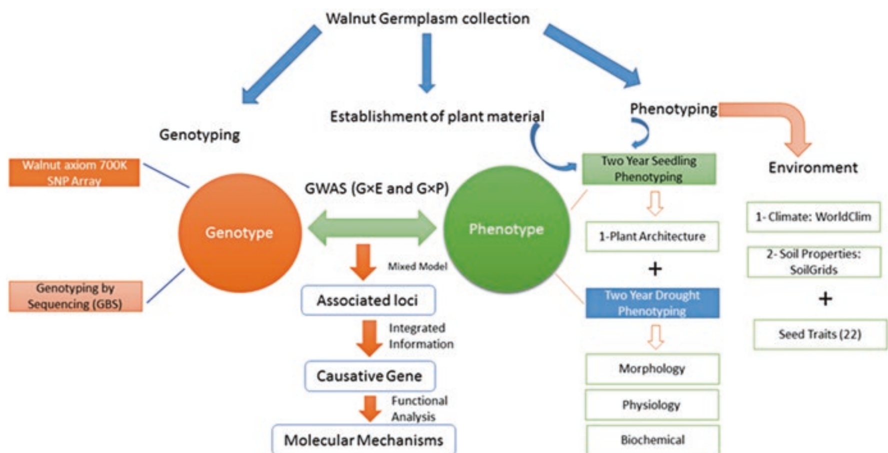

Fig. 11.6 Schematic representation of genomics-assisted breeding in walnut; an ongoing project by the University of Tehran and the University of California, Davis, on drought-resistant rootstock

11.4.13 Application of Functional Genomics in Genomics-Assisted Breeding

Functional genomics includes genome scale approaches to define gene function and gene networks at the transcript or protein level. Functional genomics is a most efficient tool and could be integrated into breeding at various stages (Dvorak et al. 2008). Functional genomics methods can be employed to produce useful molecular markers to increase the efficiency of variety development in fruit crop breeding program and to identify new genes of interest.

Functional mapping integrates mathematical and molecular genetics with developmental mechanisms underlying biological processes to shed light on the genetic basis of complex traits. In 2008, over 95% of more than 18,000 walnut sequences in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) were produced by the Dvorak (2008) Lab.

For functional mapping, 18 samples of walnut tissue were collected from cv. Chandler trees at UC Davis in 2008 and paired-end (85 bp × 85 bp) sequencing conducted on an Illumina Genome Analyzer. In the next step, to generate a Unigene set, these new sequences were combined with the 18,000 walnut ESTs and this set of genes was annotated using Blast2GO (http://blast2go.bioinfo.cipf.es/). A total of 21 cDNA libraries were constructed for the characterization of the walnut transcriptome using next-generation DNA sequencing. Over a billion reads were handled to obtain consensus sequences or genes that state the gene space in the walnut genome (Dvorak et al. 2008). The trimmed Illumina reads derived from each sample were assembled using Velvet v. 0.1.12, Oases v. 0.1.15 and CAP3 (Zerbino et al. 2009). The walnut contigs have also been annotated using Blast2GO software (Conesa and Gotz 2008; Gotz et al. 2008).

11.4.13.1 Functional Genomics: Transcriptomics

Several genomic techniques are employed to study transcriptome variation during development or as a response to biotic and abiotic stress.

cDNA-amplified fragment length polymorphism (AFLP) analysis by Bâaziz et al. (2012) was conducted on walnut leaves maintained under irradiance or in darkness to identify the early molecular events occurred during light-induced leaf hydraulic conductance (Kleaf). The results of this study showed that the most of transcript-derived fragments (TDFs) obtained via cDNA-AFLP correspond to genes whose protein products are involved in cellular regulation and global metabolism, respectively, 57.9 and 39.8% (Bâaziz et al. 2012). Availability of transcriptome information in walnut has increased the development of molecular markers like EST-SSRs that result from cDNA libraries. Available ESTs are used to both develop molecular markers and transcriptomics tools.

The first construction of walnut ESTs in *Juglans regia* from seed coat tissues by Muir et al. (2004) was submitted to the NCBI database (https://www.ncbi.nlm.nih.gov). In another study, functional genomic analysis to identify the genes involved in interactions between the walnut root and the nematode was done at UC Davis. In this project, at the first step, 13,559 expressed sequence tags (ESTs) were generated by sequencing cDNA libraries provided from *Pratylenchus vulnus* and infected and uninfected walnut (*J. hindsii* × *J. regia*) leaves and roots (Britton et al. 2007). In the next step, to identify and validate nematode and walnut genes associated with the infection analysis of gene expression between uninfected and infected plants. Walnut gene chips were used and confirmed by Taqman® real-time quantitative RT-PCR. Finally, functional analysis of nematode genes were done using RNA interference in vitro and *Medicago* root assays (Britton et al. 2009). Also in this project, 2733 *P. vulnus* genes and 8622 walnut genes were sequenced and displayed valuable information on the paths involved in the interaction between *P. vulnus* and a susceptible walnut rootstock. The result of gene expression indicated that a greater response was identified in the leaves than in the roots of plants inoculated with *P. vulnus*.

Zhang et al. (2010) obtained a total of 5025 walnut ESTs from the NCBI database and along with the SSR Hunter software, were used to analyze SSR motifs. Subsequently, a total of 123 primer pairs were designed from the non-redundant SSR-containing unigenes. The efficiency of candidate markers was examined by 7 DNA pools collected from different walnut accessions. Results revealed that 41 SSR primer sets with high polymorphic amplification products could be used for future genetic study in walnut (Zhang et al. 2010). In another study, 5213 EST sequences of walnut (*Juglans regia*) in NCBI were used for development of walnut EST-SSR markers, and 207 SSRs were obtained from the EST sequences (Feng et al. 2011)

A total of 7262 unigenes were obtained from 13,559 ESTs retrieved from the NCBI database and 309 EST-SSR primers were randomly designed. Finally, 13 highly polymorphic EST-SSRs were used for genetic analyses in *Juglans regia*, *J. nigra*, *Carya cathayensis*, *C. dabieshanensis* and an endangered species *Annamocarya sinensis* (Zhang et al. 2013). Also 40 polymorphic EST-SSR markers were developed by Zhao et al. (2015) in *J. regia*. Recently several transcriptome analysis have been performed in walnut which includes expression of the transcrip-

tion factor gene JrCBF involved in cold resistance mechanisms (Xu et al. 2014). In addition, expression of a large family of NBSLRR resistance genes in *J. regia* involved in plant-microbe interactions (Chakraborty et al. 2016) and transcriptome analysis of buds, leaves, female flowers and male flowers in *J. regia* were used to identify new EST-SSR markers (Dang et al. 2016). Also, Li et al. (2017) carried out comparative transcriptome analysis of genes involved in anthocyanin biosynthesis in leaf and peel color change in red and green *J. regia*.

11.4.13.2 Proteomics and Metabolomics

Transcriptomic information has facilitated the identification of candidate genes linked to agronomic traits of interest. However, the mechanisms of plant development stages and response to biotic and abiotic stress are complex due to the influence of multi-genes and post-transcriptional regulations. Therefore, functional genomics involving various proteomics and metabolomics approaches have been obligatory for understanding complex mechanisms. These approaches, integrated with genomics information, will accelerate identification of candidate genes and pathways involved in important agronomic traits that can be employed in plant genetic improvement programs.

Several proteomics and metabolomics studies have focused on nutritional properties and the beneficial effects of walnut consumption on health. Proteomics and metabolomics research carried out on *Juglans regia* has the following breeding purposes. One, proteomics studies such as protein markers development and assessment the genetic structure of Pakistan *J. regia* germplasm (Khan et al. 2010) and isolation of tyrosinase from *J. regia* leaves and identification as a PPO corresponding to the known *Jr*PPO1 sequence (Zekiri et al. 2014). And, two, metabolomics studies such as investigation of metabolite changes during kernel maturation using gas chromatography-mass spectrometry (GC-MS) (Rao et al. 2016), the study of biosynthesis of nonstructural polyphenols involved in pathogen resistance in *J. regia* (Colaric et al. 2005; Farooqui et al. 2015; Solar et al. 2006), identification of novel functions for the polyphenol oxidase enzyme in secondary metabolism and the regulation of walnut cell death by metabolite profiling (Araji et al. 2014; Escobar 2013).

Only a single PPO gene, *Jr*PPO1 has been identified in walnut by metabolite profiling. Metabolomics data integrated with information obtained from genome sequence, allow identification of second PPO gene, *Jr*PPO2 (Martínez-García et al. 2016). Also, PPO may be involved in pellicle color as an important commercial trait. Therefore, the results of this study are applicable to future walnut breeding.

11.4.14 Bioinformatics as a Tool for Walnut Research

Walnut databases that house genomics, transcriptomics, proteomics, metabolomics and phenomics resources provide an effective platform for walnut breeding programs. It is important for a breeder to understand how these data can be used for

genetic improvement of walnut. More recently, advances in NGS-Based genotyping and high-throughput phenotyping technology have revolutionized plant breeding, especially fruit tree genetic improvement, and turned it into an information-based science. All of this large-scale information requires appropriate analysis, storage and combination to enhance our understanding of genes underlying important traits to be utilized in further plant breeding program. Therefore, bioinformatic information and web sites have become an essential and integral part of plant genetic improvement programs (Mochida and Shinozaki 2010).

The walnut genome v1.0 sequencing projects was released by UC Davis on 2015 through NCBI (https://www.ncbi.nlm.nih.gov/) and (http://ucanr.edu/sites/wgig/) at Davis, California, providing access to walnut genomics and genetics information. Also, the Hardwood Genomics Project (https://www.hardwoodgenomics.org/organism/Juglans/regia) is a central repository of walnut genetics data.

A research report database for walnut (http://ucanr.edu/sites/cawalnut/) is under development by UC Davis to house and integrate growth and development, physiology, genomic, genetic, and breeding data for walnut management and genetic improvement. Initial transcriptome analyses in walnut focused on generating ESTs for identification of candidate genes involved in different stages of plant development and response to abiotic and biotic stresses. In 2004, the first walnut ESTs became available in the National Center for Biotechnology (NCBI) dbEST repository (https://www.ncbi.nlm.nih.gov), rising to over 21,000 sequenced ESTs by 2018. As of May 1, 2018, this search returned 1 genome match for the Persian walnut, 2 genome assembly information, 93 high-throughput DNA and RNA sequence read archive (SRA), 49,405 genome survey sequences (GSS), 173,178 DNA and RNA sequences (Nucleotide), 43,454 genes, 21,334 expressed sequence tag sequences (ESTs), 97 gene expression omnibus (GEO) datasets of expression and molecular abundance profiles on Persian walnut (https://www.ncbi.nlm.nih.gov/). Whole transcriptome shotgun sequencing (WTSS) or RNA-Seq is the latest powerful tool for transcriptome analysis. In walnut, RNA Seq technology is used to study walnut response to drought stress. Proteomics and metabolomics allow the parallel assessment of large-scale of proteins and metabolites in a biological sample. Many different methods are being taken to generate proteomics data; UniProt database (http://www.uniprot.org/) provides a comprehensive, high-quality and accessible resource of protein sequence and functional information. In total, 46,591 protein entries are available on the UniProt database for *Juglans* as follows: English walnut (45,744), *Juglans cathayensis* (103), *J. cinerea* (108), *J. mandshurica* (108) and *J. sigillata* (110). Currently (searched in May 2018), 45,764 protein entries are available for *J. regia* including; English walnut (45,744), *J. nigra* × *J. regia* (8) and *J. mandshurica* × *J. regia* (3) that all entries are in both Swiss-Prot and in the Translated European Molecular Biology Laboratory Nucleotide Sequence Database (TrEMBL). Since May 2018, 93,270 protein sequences from J. *regia* and 115 bioactivity screening studies of *J. mandshurica* and *J. regia* became available on the NCBI database (https://www.ncbi.nlm.nih.gov).

11.5 Genetic Engineering

Sometimes it is impossible to introduce specific traits into an existing cultivar by conventional methods, such as selection or hybridization. Consequently, an alternative method is direct gene transfer. Production of somatic embryos in walnuts was successfully reported in the 1980s (Tulecke and McGranahan 1985) and genetic engineering has been used successfully for walnut breeding. Walnut is one of the first woody plants to be transformed and to express foreign genes (Dandekar et al. 1988; McGranahan et al. 1988).

11.5.1 Tissue Culture

Traditionally, walnuts have been propagated by seeds and grafting onto seedling rootstock. In vitro propagation is important for the production of cultivars on their own roots, in vitro breeding (haploid induction etc.), disease-resistant rootstocks and development of transgenic walnuts (Dandekar et al. 2005; Sadat Hosseini Grouh et al. 2011; Vahdati et al. 2004). In addition, mature self-rooted clones in cv. Chandler exhibited more yield and superior vigor than grafted trees (Hasey et al. 2001).

The first reports of walnut micropropagation were published in the early 1980s (Chalupa 1981; Cossio and Minolta 1983; Driver and Kuniyuki 1984; Rodriguez 1982). Researchers have developed methods for rooting and acclimation of tissue-culture seedlings (Jay-Allemand et al. 1992; Navatel and Bourrain 2001; Ripetti et al. 1994; Vahdati et al. 2004). Driver and Kuniyuki (1984) developed DKW medium specifically for walnut micropropagation. The best explants for micropropagation of walnut are shoot buds, nodal segments and shoot tips (Gruselle et al. 1987; Rodriguez et al. 1993; Saadat and Hennerty 2002). A wide range of media (MS, DKW, WPM) and hormones (BAP, IBA, Kn, GA3, IAA) have been used for microshoot multiplication (Chalupa 1981; Revilla et al. 1989; Sommers et al. 1982). In the 1990–2000s, investigation of walnut micropropagation focused on rooting of microshoots (Jay-Allemand et al. 1992; Navatel and Bourrain 2001; Vahdati et al. 2004).

Rooting of commercial walnut cultivars, Chandler, Vina and Sunland, was accomplished using a two-phase procedure consisting of root induction (MS medium with 15 μM IBA in dark conditions) and root development (one-quarter of DKW medium and vermiculite (1:1.25, v/v). There was a positive relationship between vigor of cultivars and rooting ability, that is, the microshoots with expanded shoots root better. Rooting percentages were Chandler 55%, Vina 27% and Sunland 94%, and rooted plants were acclimatized successfully (Vahdati et al. 2004). This technique was extended to some commercial companies, (Fig. 11.7) but ex-vitro rooting methods are more commonly used commercially to reduce costs.

Fig. 11.7 In vitro rooting of Persian walnut cv. Chandler. (Photo by Kourosh Vahdati)

Acclimatization of micropropagated walnut is the most difficult phase because of susceptibility to abiotic stresses and rapid desiccation (Vahdati et al. 2004). Driver (1985, 1986) patented a method for the acclimatization of tissue-cultured cv. Paradox walnut propagules, which allows the direct field rooting and acclimatization of the propagules simultaneously (Driver 1985, 1986). He used polystyrene cups for acclimatization. The polystyrene cup(s) provide covering holding proper humidity (passive humidity) and adequate light. This system can be adapted to the greenhouse or directly in the field. It has shown value in acclimatizing small quantities rather than large, because of the logistics of handling larger quantities of cups. Clearly, walnuts require passive acclimatization and much in this area needs investigation.

Many recent studies have focused on acclimatization of micropropagated walnut (Fig. 11.8); Asayesh et al. (2017a, b) and Vahdati et al. (2017). Asayesh et al. 2017a compared the leaves of in vitro plants with those of greenhouse-grown plants. They reported that in-vitro plants had higher stomatal and epidermal densities and thinner leaves with larger stomata and pore area. Vahdati et al. (2017) also showed that increasing the CO_2 concentration of culture vessel headspace could be an efficient tool for improving acclimation of in vitro-grown cv. Chandler, because a higher CO_2 concentration resulted in a lower transpiration rate and a higher relative water content (RWC) during acclimatization. The results of this study and experiences at a commercial scale have consistently showed that using high-quality shoots with expanded leaves during the elongation stage will result in better rooting in walnut.

Fig. 11.8 Successful acclimatization of Persian walnut. (Photo by Kourosh Vahdati)

Fig. 11.9 Somatic embryogenesis in walnut on DKW medium under dark condition. (Source: Bahrami Sirmandi and Vahdati 2009)

11.5.2 Somatic Embryogenesis

Walnut somatic embryos are important for genetic improvement and mass clonal propagation (McGranahan et al. 1990; Vahdati et al. 2008), intergeneric hybridization (McGranahan et al. 1986) and generating triploid plants (Tulecke et al. 1988) (Fig. 11.9). The first somatic embryos of walnut were obtained from cotyledons and endosperm of *Juglans hindsii*, *J. regia*, and *Pterocarya* sp. (Cornu 1988, 1989; Cornu and Jay-Allemand 1989; Long et al. 1995; Tulecke and McGranahan 1985; Tulecke et al. 1988; Vahdati et al. 2006). Generally, somatic embryogenesis consists of four stages: induction, proliferation, maturation and germination (Hartmann

et al. 1997). For induction of somatic embryos, immature cotyledonary explants are harvested from developing nuts and cultured on conditioning medium for 2 to 4 weeks and then transferred to basal DKW medium (Polito et al. 1989; Tulecke and McGranahan 1985). For induction and maintenance, all cultures are maintained at room temperature in the dark. Germination efficiency of walnut somatic embryos is low (0–45%) (Deng and Cornu 1992; Lee et al. 1988; Vahdati et al. 2006). To solve this problem, studies have been conducted by various researchers. For example, gibberellic acid (GA_3), cold, and desiccation storage pretreatments and liquid germination medium were tested for promoting germination (Deng and Cornu 1992; Tang et al. 2001; Tulecke and McGranahan 1985). Vahdati et al. (2008) studied the effect of sucrose and abscisic acid (ABA) on maturation and germination of walnut somatic embryos; results showed that the best treatment was 2 mgl^{-1} ABA; sucrose had little influence on maturation of walnut somatic embryos.

11.5.3 Selection of Transformed Somatic Embryos

In most early *Juglans regia* transformation studies, β-glucuronidase (GUS) is used for marker selection. Escobar et al. (2000) used green fluorescent protein (GFP); while Zhang et al. (2015) employed a new red fluorescent protein from *Discosoma* sp. (DsRED), which was more stable and reliable. Liu et al. (2017) used this marker in confirming that genes were not translocated from rootstock to scion.

11.5.4 Rootstock Transformation

Tolerance to biotic and abiotic stresses is a primary goal in walnut rootstock improvement. Walnuts are quite susceptible to crown gall disease caused by *Agrobacterium tumefaciens*. Escobar et al. (2001) reported a strategy of gene silencing for production of plants resistant to crown gall disease. Their report was the first use of gene silencing for resistance to bacterial disease; Escobar et al. (2002) then used this method to produce gall-resistant walnut. In another study, in order to increase the rooting potential of Paradox hybrid (*J. hindsii* × *J. regia*), *rol*ABC genes (*rol*A + *rol*B + *rol*C), derived from the bacteria *A. rhizogenes,* were inserted to somatic embryos of walnut (Vahdati et al. 2002). Although the *rol*ABC genes induced a shorter internode length and a more fibrous root system, they did not increase rooting potential. Recently, Walawage et al. (2013) researched co-transformation using an RNAi inducing construct inserted into *A. rhizogenes* to silence Pv010 and a construct to silence the *iaaM* and *ipt* genes inserted into *A. tumefaciens.* The objective was to produce concurrent resistance to both the nematode *Pratylenchus vulnus* and crown gall in the same rootstock; combining the two bacterial strains at a 1:1 rather than a 1:3 ratio increased the cotransformation efficiency.

Flavonoid effects on the rooting ability of hybrid walnut were also investigated. Somatic embryos of hybrid walnut (*Juglans nigra* × *J. regia*) were transformed with an antisense construct including a 400 bp cDNA fragment of a walnut *chs* gene and CaMV-35S promoter. Resulting decreased flavonoid content in stems of antisense *chs* transformed lines was linked to improved adventitious rooting ability. Auxin content was determined during the latter phase of the in vitro propagation and no differences were identified between the control and antisense *chs* transformed lines. Transformed plantlets low in flavonoids were more sensitive to exogenous application of auxin (El Euch et al. 1998).

11.5.5 Scion Transformation

To produce plants resistant to the codling moth (*Cydia pomonella* L.), a major pest of walnut in California, Dandekar et al. (1994) inserted the *cry1A*(c) gene of *Bacillus thuringiensis* into walnut but, due to the low expression, this transformation was unsuccessful. In 1998, a synthetic *cry1A*(c) gene with altered codon bias corrected the problem. This resulted in levels of expression sufficient to obtain efficacious control of *C. pomonella* when fed on transformed embryos (Dandekar et al. 1998). Sheikh Beig Goharrizi et al. (2016) reported that transgenic walnut expressing the *fld* gene had increased tolerance to osmotic stress. A significant difference was observed between transgenic and non-transgenic somatic embryos exposed to 50 and 100 mM NaCl and 5 and 10% PEG (Fig. 11.10). Walnut transformation with a betaine aldehyde dehydrogenase (*badh*) gene is also under study Vahdati (2014).

Fig. 11.10 Wild (left) vs. transgenic (right) walnut, expressing the *fld* gene after 45 days on DKW medium containing 200 mM NaCl. (Source: Sheikh Beig Goharrizi et al. 2016)

11.5.6 Promoter Isolation

Studies of genetic engineering in walnut generally use 35S as a promoter to enhance expression of foreign genes in the gene cassette. Xu et al. (2018) tested a 1200 bp promoter fragment of vacuolar H^+-ATPase (V-ATPase). The G subunit of the *Juglans regia* (JrVHAG1) gene was identified from *J. regia* DNA and amplified by a PCR reaction. Cis-elements of this promoter were analyzed using the PLANTCARE database. Finally, to check the expression activity of the JrVHAG1 promoter, it was inserted into a pCAMBIA1301 vector to drive the expression of the GUS gene. Results showed that expression activity was enhanced significantly in *Arabidopsis* when subjected to the cadmium stress.

11.6 Mutation Breeding

Chemical and physical mutagenesis have successfully assisted in the development of improved and new cultivars in plant breeding programs (Parry et al. 2009). The number of physical and chemical mutagens used in mutation breeding is large and continues to increase (Mba 2013; Mba et al. 2010). The most powerful, effective, reliable and frequently used chemical is ethyl methane sulphonate (EMS). Gamma rays can successfully be used to develop new mutant varieties, especially in ornamental plants (Taheri et al. 2014).

11.6.1 Haploid Mutagenesis

Haploid refers to plants that contain a gametophytic chromosome number (n) in their somatic tissues; doubled haploid (DH) plants are generated by the spontaneous or induced doubling of their chromosome numbers. Szarejko (2012) found that haploid somatic embryos or callus can be induced in vitro from male or female gametes and regenerated into haploid plants with only one set of homologous chromosomes.

Haploid plants are normally sterile so their chromosomes are doubled to provide completely homozygous lines from heterozygous plant material. Therefore, *doubled haploidy* techniques are integrated into breeding programs of many horticultural crops, including major ornamental crops, vegetables, fruit crops and medicinal plants (Szarejko 2012). Researchers have tried to produce haploid plants in more than 250 plant species, but efficient and reproducible doubled haploidy production protocols are available for fewer than 30 of them (Maluszynski et al. 2003).

Szarejko (2012) described the development of three main methods of doubled haploid production: (1) androgenesis, (2) wide crossing followed by chromosome elimination and (3) gynogenesis.

One of the most important applications of radiation techniques in plant breeding has been irradiation to destroy pollen or egg cell nuclei. Parthenogenesis, the production of an embryo from an egg cell without the participation of the male gamete, is commonly used for haploid production in fruit crops, especially in those species in which in vitro pollen embryogenesis has not been applied successfully (Germanà 2012).

11.6.2 Mutant Selection

The ultimate goal of any mutation breeding program is selecting desired mutants. Characteristics of fruit crops, such as a long juvenile phase, a high degree of heterozygosis, and self-incompatibility, make it impossible to obtain homozygous lines by conventional inbreeding approaches. Therefore, techniques to produce doubled haploids are particularly useful for fruit trees breeding (Germanà 2012).

11.6.2.1 Ploidy Determination

In fruit crops, haploid, diploid, triploid or hexaploid regenerants are produced through parthenogenesis induced using irradiated pollen. Various methods, including chromosome counting, flow cytometry analysis, stomata size, number of chloroplast guard cells and nucleus size, have been used to determine ploidy level of mutants. Among these techniques, flow cytometry is gaining in importance because it allows rapid analysis of a large number of samples (Germanà 2012).

11.6.2.2 Molecular Techniques for Mutation Detection and Screening

Screening of novel induced mutations in plants has long been a major challenge. Mutant screening and confirmation (mutant validation) are the two major steps for identifying and selecting mutant plants with improved traits (Shu et al. 2012a; Wu et al. 2012a, b).

Since plant mutagenesis induced by chemical or physical mutagens is a random process, occurring at an extremely low frequency, previous studies have revealed that DNA markers, with the exception of functional markers, are not useful for screening or selecting induced mutants, (Wu et al. 2012a).

Techniques developed for detection of mutations in a gene of interest include: single-strand conformation polymorphism (SSCP), denaturing high-performance liquid chromatography (DHPLC), temperature/denaturing gradient gel electrophoresis (TGGE/DGGE), conformation-sensitive gel/capillary electrophoresis (CSGE/CSCE), mismatch cleavage and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) (Gady et al. 2009; Hestekin et al. 2006; McCallum et al. 2000; Shu et al. 2012a, b).

The advent of next-generation sequencing as an important tool for whole-genome sequencing and re-sequencing has revolutionized plant breeding. Recently, the efficiency of identifying DNA changes that generate a new trait has been increased tremendously by use of high-throughput mutation detection technologies (Quail et al. 2012) and reverse genetic techniques such as TILLING (targeting induced local lesions in genomes) (Taheri et al. 2017).

11.6.3 Practical Examples in Walnut

So far, few limited reports are available on walnut mutation breeding. Sadat Hosseini Grouh et al. (2011) reported the first successful production of haploid lines in Persian walnut through parthenogenesis, induced by gamma ray-irradiated pollen. In their study, female flowers of cvs. Hartley and Pedro and two native Iranian selections (Z63 and Z67) were pollinated with gamma ray-irradiated pollen from selections Z53 and Z30. The results revealed that using pollen irradiated at 300 and 600 Gy successfully generated haploid lines in Persian walnut. Simple sequence repeat (SSR) markers confirmed haploid plantlets. The techniques used in other fruit and nut breeding programs to induce haploids and other ploidy changes could be applied to Persian walnut to accelerate genome analysis (Sadat Hosseini Grouh et al. 2011). (Fig. 11.11).

Triploid walnut plants are produced by culturing endosperm from immature open-pollinated seed of *Juglans regia* cv. Manregian and inducing somatic embryos. The resulting plantlets were evaluated morphologically, and the roots were used for chromosome counting (Tulecke et al. 1988). When planted in the field, the resulting triploid trees produce abundant male flowers that mostly fail to produce pollen and rarely produce nuts. The few nuts observed have been very small and contained no kernel. These triploid plants represent novel germplasm that of possible use for breeding, following further ploidy manipulation.

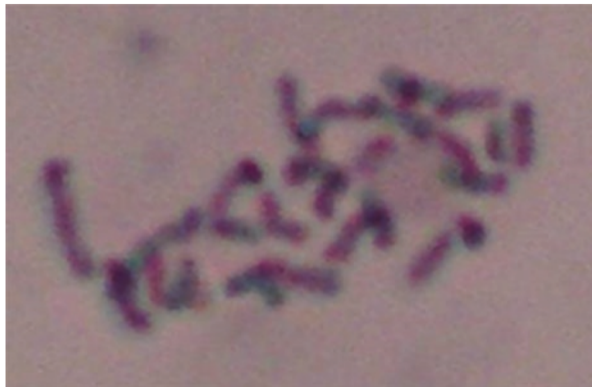

Fig. 11.11 Haploid set of chromosomes (n = x = 16) observed in a root apex of an embryo collected from Z63 genotype of Persian walnut

11.7 Hybridization

In natural populations, hybridization can be useful for the production of new lineages with adaptation to environmental changes, disease resistance and production of plants with uniform growth. Hybridization has always been an important walnut breeding strategy, leading to the release many varieties and rootstocks. A complete description of walnut breeding programs and varieties released based on hybridization is presented in Sect. 11.2.

11.8 Conclusions and Prospects

The high nutritional and economic value of walnut, along with significant improvements generated by walnut breeding and the release of high-quality and productive varieties, have led to its increased cultivation in recent years. High yield and quality have always been primary walnut breeding objectives. Lateral bearing, high nut weight and size, high kernel percentage, late leafing, early harvest date and light kernel color are the primary traits targeted for scion cultivars. Dwarfing, salt and drought tolerance and resistance to soil-borne pathogens such as *Agrobacterium*, *Phytophthora* nematodes and *Armillaria* are targeted traits for rootstocks. Breeders have used several strategies to achieve these goals, such as germplasm evaluation and selection, hybridization, genetic engineering, mutation breeding, genome sequencing, bioinformatics, marker-assisted selection, haploid and polyploid induction, proteomics and metabolomics. Current walnut breeding strategies focus on integrating molecular breeding (usage of genomic, transcriptomic, proteomic and metabolomics information) into traditional breeding programs. Climate change and global warming can severely impact global walnut production. Therefore, walnut breeding programs, especially in the leading and most active countries, are striving to generate cultivars quickly and efficiently that maintain and continually improve walnut production and quality. Accordingly, the topics listed below are priorities for future walnut breeding programs:

(a) Use of CRISPR-Cas9 systems for targeted-genome editing;
(b) Production of haploid, doubled haploid and wide-hybrid plants to enhance genome assembly quality;
(c) Combining of high-throughput phenotyping with GWAS and genomic selection to unlock genetic information coded in the walnut genome that controls complex traits;
(d) Simultaneous use of transcriptomics, metabolomics and proteomics for understanding drought and salinity tolerance mechanisms in walnut;
(e) Continued evaluation of genetic diversity to release and develop new cultivars and rootstocks;
(f) Release of new cultivars with low chilling requirement, early harvest and late-leafing date.

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Appendices

Appendix I: Some Important Research Institutes Relevant to Walnut

Country	Institution	Specialization and research activities	Contact information and website
Albania	Agricultural University of Tirana	Walnut breeding program based on selection	Prof. Endrit Kullaj ekullaj@ubt.edu.al http://ubt.edu.al/
Argentina	National Research Institute of Argentina (INTA-Catamarca)	Variety walnut breeding program based on hybridization and selection	Dr. Dante Carabajal Carabajal.dante@inta.gob.ar https://inta.gob.ar/
Belarus	Belarusian Research Institute for Fruit Growing	Walnut breeding program for early bearing, high yield, winter resistant, diseases tolerant	Dr. Vyacheslav A. Samus director@belsad.by http://www.belsad.by
Bulgaria	Fruit Growing Institute	Breeding and studies of walnut cultivars	Dr. Stefan Gandev sgandev@yahoo.com http://www.fruitgrowinginstitute.com/
China	Hebei Agricultural University (College of Life Science)	Walnut breeding based on classic and molecular breeding	Dr. Shugang Zhao zshug@126.com http://life-auh.com/Index.html
China	Mountainous Area Research Institute of Hebei	Walnut breeding based on selection and hybridization	Dr. Hongxia Wang whx@hebau.edu.cn http://shanyansuo.hebau.edu.cn/
China	Chinese Academy of Forestry (Research Institute of Forestry)	Walnut improvement program	Prof. Pei Dong peigu@caf.ac.cn http://www.caf.ac.cn
China	Pomology Research Institute, Shanxi	Walnut breeding program based on selection and hybridization	Prof. Jianbao Tian tianjb-001@163.com http://www.gfar.net/organizations/pomology-research-institute-shanxi-academy-agriculture-science
China	Liaoning Institute of Economic Forestry	Walnut breeding program for high yield, blight and coldness tolerant and rootstock selection	Prof. Baojun Zhao agroforestry@163.com liufeng0427@sina.cn http://www.lnly.gov.cn/lnly/kyyszw/sjjls/

Country	Institution	Specialization and research activities	Contact information and website
Chile	Instituto de Investigaciones Agropecuarias	Orchard management and plant material evaluation	Dr. Gamalier Lemus glemus@inia.cl http://www.inia.cl
France	French National Institute for Agricultural Research (INRA)	Walnut breeding program for late flowering, frost resistance, early bearing, high yield, blight tolerant, systematics and ecology of plant pathogenic bacteria	Dr. Sophie Cesbron sophie.cesbron@angers.inra.fr http://www.inra.fr
France	Centre Technique Interprofessionnel des Fruits et Legumes (CTIFL)	Walnut breeding program and study of the behavior of new INRA walnut varieties and rootstocks	Dr. Fabrice Lheureux lheureux@ctifl.fr http://www.ctifl.fr/
France	Station Expérimentale de la Noix de Creysse	Study on walnut orchard management and walnut quality, improving the efficiency of the walnut industry in the south west of France	Dr. Eloise Tranchand e.tranchand.creysse@orange.fr Dr. Fabrice Lheureux lheureux@ctifl.fr http://www.noixsudouest.fr
France	Station d'Expérimentation Nucicole Rhône-Alpes	Orchard management and plant material evaluation walnut industry efficiency in the south east of France	Dr. Agnès Verhaeghe Averhaeghe@senura.com http://senura.com/
Georgia	Georgian Research Institute of Horticulture, Viticulture and Oenology	Breeding new species and varieties of walnut through hybridization of different species and varieties	Dr. Zviad Bobokashvili bobokashvili@hotmail.com http://agruni.edu.ge
Georgia	Scientific-Research Center of Agriculture Georgia	Selecting and preservation of walnut varieties	Dr. Zviad Bobokashvili bobokashvili@hotmail.com Dr. Nugzar Shengelia shengelianugzar@gmail.com http://agruni.edu.ge
Germany	Hochschule Geisenhiem University	Breeding, selection and preservation of walnut varieties	Prof. Joachim Heller (Head) Joachim.Heller@hs-gm.de https://www.hs-geisenheim.de/en/research/departments/pomology/department-of-pomology/
Germany	State Education and Research Institute for Viticulture and Pomology Weinsberg	Breeding, selection and preservation of walnut varieties	obstbau@lvwo.bwl.de http://www.lvwo-bw.de/pb/,Lde/Startseite
Greece	Technological Educational Institute of Thessaly (TEI)	Walnut breeding program based on crossing of local genetical material with foreign cultivars	Prof. Alexandros Papachatzis papachad@teilar.gr http://www.teilar.gr

(continued)

Country	Institution	Specialization and research activities	Contact information and website
Greece	Technological Educational Institute of Peloponnese (TEI)	Walnut breeding program based on crossing of local genetical material with foreign cultivars	Prof. George Zakynthinos gzakyn@yahoo.gr http://www.teipel.gr
Greece	National Agricultural Research Foundation (NAGREF)	Walnut breeding program based on selection	Dr. Pavlina Drogoudi drogoudi@otenet.gr http://www.elgo.gr
Hungary	NARIC Fruitculture Research Institute	Walnut breeding program based on selection and hybridization	Dr. Geza Bujdosó resinfru@yahoo.com https://fruitresearch.naik.hu/en
Iran	University of Tehran, Aburaihan Campus, Center of Excellence in Walnut Improvement and Technology	Walnut breeding for late leafing, lateral bearing and high yield; rootstock breeding for drought tolerance and dwarfing using classic breeding and biotechnology; giving consultation for walnut commercial micropropagation labs and establishment of modern walnut orchards and nurseries	Prof. Kourosh Vahdati kvahdati@ut.ac.ir http://walnut.ut.ac.ir/ https://rtis2.ut.ac.ir/cv/kvahdati/?lang=en-gb
Iran	Horticultural Science Research Institute (HSRI)	Walnut breeding program for late leafing, lateral bearing, high yield.	Dr. Darab Hassani hassanida@gmail.com http://www.hsri.ir
Italy	University of Turin	Evaluation of the performance of Italian and foreign walnut cultivars and selections obtained by intraspecific hybridization	Prof. Roberto Botta roberto.botta@unito.it http://www.disafa.unito.it/do/home.pl
Italy	Istituto Sperimentale per la Frutticoltura	Evaluation of the performance of Italian and foreign walnut cultivars and selections obtained by intraspecific hybridization; improving rooting and disease resistance in walnut through tissue culture techniques	Dr. Pasquale Piccirillo pasquale.piccirillo@entecra.it http://sito.entecra.it/portale/index2.php
Kyrgyzstan	National Academy of Kyrgyzstan (Jalal-Abad Research Center)	Walnut breeding based on selection	Dr. D.K. Mamadjanov jangak@mail.ru http://www.nas.aknet.kg
Moldova	State Agrarian University of Moldova	Creation of the walnut assortment in the republic of Moldova	Dr. Valerian Balan v.balan@uasm.md http://www.uasm.md

Country	Institution	Specialization and research activities	Contact information and website
Moldova	Iargara State Forestry Service	Forestry management; walnut varieties improvement program	Ana Petrenco iargara@moldsilva.gov.md http://iargara.silvicultura.md/
Moldova	Institute for Horticulture and Food Technologies	Selection of walnut varieties within the existing local genetic resources	Constantin Dadu www.isphta.md
Morocco	Institut National de Recherches Agronomiques	Prospection in local walnut populations in the south of Morocco	Dr. Abdellah Kajji kajjiabdellah03@yahoo.fr https://www.inra.org.ma/
Pakistan	Arid Agriculture University Rawalpindi, Department of Horticulture	Walnut breeding based on selection within local genetic resource	Prof. Nadeem Akhtar Abbasi nadeemabbasi65@yahoo.com http://www.uaar.edu.pk/homeUaar.php
Pakistan	Ayub Agriculture Research Institute (AARI), Hill Fruit Research Station	Walnut breeding based on selection within local genetic resource	Dr. Muhammad Afzal mafzal834@gmail.com www.aari.punkab.gov.pk
Pakistan	University of Azad Jammu and Kashmir, Department of Botany	Walnut breeding based on selection within local genetic resource	Prof. Dr. Muhammad Qayyum Khan mqkhan2004@yahoo.com www.ajku.edu.pk
Pakistan	Agriculture Research Institute (North) Mangora	Walnut breeding based on selection within local genetic resource	Dr Khalil Ur Rehman khalilswat66@gmail.com http://agrires.kp.gov.pk
Pakistan	Hazara Agriculture Research Station	Walnut breeding based on selection within local genetic resource	Mr Akhtar Nawaz akhtarsaeed5650@gmail.com http://agrires.kp.gov.pk/page/hazaraagricultureresearchstation
Portugal	Direcção Regional de Agricultura da Beira Litoral	Behavior of some walnut-tree varieties in Região Agrária Da Beira Litoral	drapc@drapc.gov.pt http://www.drapc.min-agricultura.pt/drapc/contactos.htm
Romania	University of Craiova (Fruit Growing Research Station – SCDP Vâlcea)	Breeding new cultivars for high yield, intensive growing, nut quality, resistance to diseases, adapted to environmental conditions and rootstock selection; evaluation of the performance of Romanian and foreign walnut cultivars	Prof. Mihai Botu btmihai2@yahoo.com http://horticultura.ucv.ro/horticultura/en

(continued)

Country	Institution	Specialization and research activities	Contact information and website
Romania	Fruit Growing Research Station – SCDP Iaşi	Selection of walnut with high yield and fruit quality, resistance to late spring and winter frosts, resistance to diseases and pests, reduced vigor	Dr. Gelu Corneanu office@pomicolaiasi.ro www.pomicolaiasi.ro
Romania	Via Roots Srl	Study on walnut orchard management and walnut quality, study on lateral bearing in Romanian climate conditions	Iosif Kiss iosif.kiss@nucifere.com www.nucifere.com
Russia Federation	Nikita Botanical Gardens	Fundamental bases of management of selection process of creation of new plant genotypes with high economically valuable characteristics of productivity, resistance to biotic and abiotic stress	Dr. Sergei Khokhlov ocean-10@mail.ru http://www.nbgnscpro.com
Serbia	University of Priština, Faculty of Agriculture in Lešak	Biology of walnut flowering	Dr. Dragan Jankovic draganjankovickv@gmail.com Dr. Sladana Jankovic https://www.uni-pr.edu/
Slovenia	University of Ljubljana	Selection of walnut populations in Slovenia, quantitative analysis of genotypic diversity in tree architecture constitution, evaluation of the performance of foreign walnut cultivars	Dr. Anita Solar anita.solar@email.si https://www.uni-lj.si/academies_and_faculties/faculties/2013052914461802/
Spain	Institute of Agrifood Research and Technology (IRTA.)	Breeding and selection for walnut varieties and rootstocks, selection of basic materials for woodland.	Dr. Neus Aleta neus.Aleta@irta.es http://www.irta.cat
Spain	Bosques Naturales S. A.	Selection of genotypes for timber production, genotype × environment studies, genotyping by SSR markers	Dr. Ricardo Julian Licea-Moreno ricardolicea@bosquesnaturales.es https://bosquesnaturales.com
Switzerland	Nuss-Baumschule Gubler GmbH	Walnut breeding for high yield, lateral bearing, late leafing and disease resistance	Dr. Heini Gubler heini.gubler@skigubler.ch www.nussbaeume.ch
Tajikistan	Tajikistan Forestry Institute	Walnut breeding based on selection	Prof. Hafiz Muminjanov

Country	Institution	Specialization and research activities	Contact information and website
Turkey	Kahramanmaraş Sütçü imam University	Walnut cultivar and rootstock breeding based on classic (selection, hybridization) and molecular breeding program for late leafing, lateral bearing, high yield, nut quality	Dr. Mehmet Sütyemez sutyemez@ksu.edu.tr Dr. Akide Özcan akideozcan@ksu.edu.tr http://www.ksu.edu.tr
Turkey	University of Gaziosmanpasa	Walnut breeding for late leafing, lateral bearing, nut quality and blight resistance using intraspecific crosses, rootstock breeding for salt stress	Prof. Yasar Akca akcanut@gmail.com https://ziraat.gop.edu.tr http://www.ceviz.gen.tr/
Ukraine	Institute of Horticulture of the National Academy of Agrarian Sciences of Ukraine	Creation of the national genetic collection of Persian walnut of promising cultivars (breeding to combine precocity, high productivity, tolerance to diseases and high nut quality)	Dr. Igor V. Grynyk (Director) sad-institut@ukr.net http://sad-institut.com.ua/o_nas.html
United States	University of California, Davis, Department of Plant Science	Walnut cultivar and rootstock breeding based on classic and molecular breeding	Dr. Pat J. Brown pjbrown@ucdavis.edu https://pjblab.faculty.ucdavis.edu/ http://fruitsandnuts.ucdavis.edu/
Uzbekistan	Schroeder Uzbek Research Institute	Walnut breeding based on selection	Aziz Nurbekov a.nurbekov@cgiar.org
Uzbekistan	Uzbek Scientific Research Institute of Plant Industry (VIR)	Walnut breeding based on selection	Aziz Nurbekov a.nurbekov@cgiar.org

Appendix II: Some Walnut Genetic Resources

Cultivar	Important traits	Cultivation location
Gizavezhda	High yield, average nut, tender shell, very light kernel color, cold-hardy and resistant to pest and diseases	Albania
Leshnica	Medium yield, lateral bearing, large nut, light kernel color, aromatic	Albania
Smokthina	Medium yield, lateral bearing, average nut, light tasty kernel	Albania
Trompito	High yield, early leafing, medium kernel color	Argentina
Ivarto	Low yield, pollinizer for medium and late varieties	Argentina
Rote Donaunuss	Moderate yield, early to mid-early leafing, medium nut size	Austria
Weinberg 2	Early leafing, large nut, thin shell, light kernel color, good kernel quality	Austria
Axel	Medium yield, late leafing, large nut	Belgium/ Netherland
Dryanovski	Early flowering, medium nut size, light kernel color, high kernel quality	Bulgaria
Izvor 10	Moderate yield, medium nut size, thin shell, light kernel color	Bulgaria
Plovdivski	Large nut, high kernel percentage, high kernel quality, resistance to bacterial disease	Bulgaria
Proslavski	Large nut, good kernel quality, resistance to bacterial disease	Bulgaria
Silistrenski	Late flowering, medium nut size, frost resistant	Bulgaria
Broadview	Early leafing, precocious, homogamous, large nut, good kernel quality	Canada
Zha 343	High yield, lateral bearing, thin shell, light kernel color	China
Zanmei	High yield, lateral bearing, light kernel color	China
Jinlong 1	Moderate yield, terminal bearing, large nut, thin shell, light kernel color	China
Wen 185	High yield, lateral bearing, large nut, thin shell, light kernel color	China
Xiangling	High yield, lateral bearing, thin shell, light kernel color	China
Zhonglin 1	High yield, lateral bearing, thin shell, extra light kernel color	China
Liaoning 1	High yield, lateral bearing, thin shell, extra light kernel color	China
Jinboxiang1	High yield, lateral bearing, thin shell, light kernel color	China
Luguo 2	High yield, lateral bearing, thin shell, light kernel color	China
Luguo 7	High yield, thin shell, light kernel color, lateral bearing	China
Daixiang	High yield, thin shell, light kernel color, lateral bearing, dwarf	China

Cultivar	Important traits	Cultivation location
Xinfeng	High yield, thin shell, light kernel color, lateral bearing	China
Xinxin 2	High yield, thin shell, light kernel color, lateral bearing	China
Mars	High yield, late leafing, thin shell, good kernel quality	Czech Republic
Jupiter	High yield, large nut, thin shell, late leafing,	Czech Republic
Saturn	High yield, large nut, good kernel color	Czech Republic
Apollo	Good yield, terminal bearing, early flowering, large nut, good kernel color	Czech Republic
Sychrov	Medium nut size, red kernel color, thin shell, high kernel quality	Czech Republic
Franquette	Fair yield, terminal bearing, late leafing, good kernel quality, extra light kernel color, used as pollinizer for 'Chandler'	France
Lara	High yield, lateral bearing, medium leafing	France
Fernor	High yield, lateral bearing, late leafing, extra light kernel color	France
Fernette	Good yield, lateral bearing, extra light kernel color, used as pollinizer for 'Chandler'	France
Ferbel	High yield, lateral bearing, large nut, thin shell, good kernel quality	France
Ferouette	High yield, lateral bearing, extra light kernel color, large nut	France
Feradam	High yield, lateral bearing, extra light kernel color, medium leafing	France
Ferjean	High yield, lateral bearing, thin shell, extra light kernel color	France
Meylanaise	Moderate yield, late leafing, good kernel quality, Used as pollinizer	France
Ronde de Montignac	Late leafing, terminal bearing, high kernel quality, used as pollinizer,	France
Rubis	Early leafing, good kernel quality, red kernel color	France
Akura	Moderate yield, moderate lateral bearing, light kernel color	Georgia
Kaspura	High yield, moderate lateral bearing	Georgia
Avenisuri	Moderate yield, terminal bearing, large nut, thin shell	Georgia
Alazani	High yield, lateral bearing	Georgia
Aragvi	Moderate yield, terminal bearing, thin shell	Georgia
Atskuri	High yield, moderate lateral bearing, thin shell, frost resistance	Georgia
Drianovski	High yield, moderate lateral bearing, large nut	Georgia
Aufhausener Baden	Large nut, mid-early flowering, old favorite German cultivar	Germany

(continued)

Cultivar	Important traits	Cultivation location
Finkenwerder Deichnuss Royal	Large nut, precocious, thin shell, high kernel quality	Germany
Geisenheimer	Moderate yield, medium nut size, medium shell thickness	Germany
Kurmarker	Medium nut size, good kernel quality	Germany
Ledema	Protogynous, large and heavy nut,	Germany
Moselaner	Large nut, light kernel color, good kernel quality	Germany
Ockerwitzer Lange	High yield, large nut, frost resistant.	Germany
Seifersdorfer Runde	Good yield, early flowering, thin shell, light kernel color, frost resistant	Germany
Spreewalder	High yield, early leafing, precocious, light kernel color, good kernel quality	Germany
Weinheimer	Medium nut size, late leafing, high kernel quality, light kernel color	Germany
Weinsberg 1	Moderate to high yield, early flowering, large nut	Germany
Wunder von Monrepos	Medium nut size, late leafing, high kernel quality	Germany
ZP–1, 2, 3, 4, 5	‘Chandler’ hybrids, lateral bearing, blight resistant, moderate to late leafing, light kernel color.	Greece
Milotai Kései℗	Late leafing and flowering, lateral bearing, nut like Milotai 10, higher tolerance to blight compared to Milotai 10	Hungary
Alsószentiváni 117	Moderate yield, terminal bearing, light kernel color	Hungary
Alsószentiváni 118	High yield, mid-early flowering, large nut	Hungary
Milotai 10	High yield, moderate lateral bearing, light kernel color	Hungary
Tiszacsécsi 83	High yield, moderate lateral bearing	Hungary
Esterhazy II	Early leafing, medium to large nut size, light kernel color, good kernel quality	Hungary
Jamal	Moderate yield, terminal bearing, medium light kernel color	Iran
Damavand	Early leafing, used as pollinizer for Jamal	Iran
Sorrento	Moderate yield, terminal bearing	Italy
Malizia	High yield, moderate lateral bearing	Italy
Qingxiang	High yield, terminal bearing, light kernel color	Japan
Kyrgyzskya Bomba	Large nut, great kernel quality, light kernel color	Kyrgyzstan
Ak Terek	Medium nut, great kernel quality, light kernel color	Kyrgyzstan
Oshsky	Medium nut size, great kernel quality	Kyrgyzstan
Uygursky	Large nut, great kernel quality, light kernel color	Kyrgyzstan
Ostrovershinny	Large nut, great kernel quality	Kyrgyzstan
Immuniy	Medium nut, great kernel quality, light kernel color	Kyrgyzstan
Desertniy	Large nut, great kernel quality, light kernel color	Kyrgyzstan
Pescianski	High yield, partial lateral bearing, thin shell, frost resistant, extra light kernel	Moldova
Calarasi	High yield, terminal bearing, frost resistant, light kernel	Moldova

Cultivar	Important traits	Cultivation location
Ovata	Large nut, terminal bearing, very cold resistant	Moldova
Carpatica	Huge nut, terminal bearing	Moldova
Amphyon	High yield, high kernel quality, low susceptible to disease	Netherland
Dionym	High yield, high kernel quality, low susceptible to disease	Netherland
Big & Easy	Late flowering, thin shell, good kernel quality	Netherland
Blanco	Large nut, homogamous	Netherland
Coenen	Early flowering, large nut, thin shell	Netherland
Lange van Lod	Large nut, late leafing, high kernel quality, slight tolerance to late spring frost	Netherland
Rex	High yield, late leafing, light color	New Zealand
Shannon	High yield, light kernel color, blight resistant	New Zealand
Meyric	High yield, late leafing, thin shell, high kernel quality	New Zealand
Wilsons Wonder	Large nut, light kernel color	New Zealand
Valcor	High yield, terminal bearing, thin shell, light kernel color	Romania
Valmit (Verisval)	Thin shell, terminal bearing, light kernel color,	Romania
Valrex	High yield, terminal bearing, large nut, thin shell	Romania
Sibişel 44	Moderate yield, terminal bearing, large nut	Romania
Jupâneşti	Precocious, high yield, terminal bearing, thin shell	Romania
Velniţa	Precocious, high yield, terminal bearing	Romania
Valstar	Precocious, high yield, terminal bearing light kernel	Romania
Valcris	Precocious, high yield, terminal bearing light kernel	Romania
Timval	High yield, terminal bearing, large nut	Romania
Miroslava	Terminal bearing, large nut	Romania
Ovidiu	Terminal bearing, large nut	Romania
Anica	Terminal bearing, large nut	Romania
Sibişel 252	Precocious, high yield, terminal bearing	Romania
Ciprian	Precocious, high yield, terminal bearing, large nut	Romania
Claudia	Precocious, high yield, terminal bearing	Romania
Germisara	Moderate yield, terminal bearing, large nut	Romania
Şuşiţa	Precocious, high yield, terminal bearing, light kernel	Romania
Ronutex	High yield, terminal bearing, large nut	Romania
Belbeksky 70	Late leafing, light kernel color, moderate shell thickness	Russia Federation
Vynoslivy	High yield, light kernel color, resistance to low temperature	Russia Federation
Pervomaysky	Late leafing, thin shell, light kernel color	Russia Federation
Krymsky Urozhayany	High yield, thin shell, light kernel color	Russia Federation
Elit	Late leafing, precocious, light kernel color	Slovenia
Krka	Homogenous flowering, high yield, bright kernel	Slovenia

(continued)

Cultivar	Important traits	Cultivation location
Sava	Intermediate fruit-bearing, late leafing, moderate yield	Slovenia
Fischenthal	Medium nut size, thin shell, cluster bearing, homogamous	Switzerland
Giswill	Lateral bearing, interesting for wood production	Switzerland
Nyffenegger	Terminal bearing, thin shell, red kernel color	Switzerland
Rote Gubler	Medium nut size, light kernel color, forest resistant	Switzerland
Yalova 1	Fair yield, terminal bearing, large nut, thin shell	Turkey
Yalova 3	Fair yield, terminal bearing, thin shell, light kernel color	Turkey
Sebin	High yield, moderate lateral bearing, thin shell, light kernel color	Turkey
Bilecik	Moderate yield, terminal bearing	Turkey
Maras 18	Moderate yield, lateral bearing, light kernel color, high kernel percentage, very early harvest,	Turkey
Sütyemez 1	Moderate yield, lateral bearing, extra-large nut, light kernel color, very early harvest	Turkey
Kaman 1	High yield, lateral bearing, thin shell, light kernel color	Turkey
Maraş 12	High yield, terminal bearing, moderate nut, thin shell, light kernel color, extra high kernel percentage	Turkey
Diriliş	High yield, lateral bearing, late leafing, light kernel color, high kernel percentage, thin shell, early harvest	Turkey
15 Temmuz	High yield, lateral bearing, very late leafing, light kernel color, high kernel percentage thin shell, early harvest	Turkey
Bayrak	High yield, lateral bearing, thin shell, extra light kernel color, high kernel percentage	Turkey
Akça	High yield, lateral bearing, late leafing, good kernel quality, extra light kernel color, used as pollinizer for 'Chandler'	Turkey
Niksar 1	Moderate yield, lateral bearing, late leafing, used as pollinizer for cv. Chandler	Turkey
Eureka	Fair yield, terminal bearing, poor kernel color	USA
Scharsch Franquette	Late leafing, medium thin shell, light kernel color	USA
Hartley	Moderate yield, terminal bearing, light kernel color	USA
Payne	High yield, lateral bearing, precocious, early leafing, early harvest, light kernel color	USA
Vina	High yield, lateral bearing, poor color	USA
Pedro	High yield, lateral bearing	USA
Tehama	Moderate yield, lateral bearing, light kernel color, used as pollinizer for cv. Serr	USA
Serr	Moderate yield, moderate lateral bearing, light kernel color, thin shell, excellent kernel quality, susceptible to pistillate flower abscission (PFA)	USA
Chandler	High yield, lateral bearing, medium leafing, extra light kernel color, thin shell	USA
Howard	High yield, lateral bearing, medium leafing, large nut, thin shell, light kernel color	USA

Cultivar	Important traits	Cultivation location
Sunland	High yield but susceptible to nut drop, lateral bearing, large nut, thin shell, light kernel color	USA
Cisco	Moderate yield, terminal bearing, medium light kernel color, high susceptibility to blight, used as pollinizer for cv. Chandler	USA
Tulare	High yield, lateral bearing, light kernel color, susceptible to winter cold	USA
Robert Livermore	Moderate yield, lateral bearing, red kernel color	USA
Sexton	High yield, lateral bearing, very precocious, light kernel color	USA
Gillet	High yield, lateral bearing, large nut, light kernel color, low susceptibility to blight	USA
Forde	Good yield, lateral bearing, light kernel color, low susceptibility to blight	USA
Ivanhoe	High yield, lateral bearing, very precocious, very early harvest, thin shell, extra light kernel color	USA
Solano	High yield, lateral bearing, extra light kernel color	USA
Durham	Good yield, early harvest, lateral bearing, large nut, light kernel color	USA
Ideal	Small nut from secondary flower, high kernel quality, precocious, cluster bearing habit	Uzbekistan
Hybridiy	Medium size nut	Uzbekistan
Pioner	Large size nut	Uzbekistan
Kazahstansky	High kernel quality	Uzbekistan
Bostonliksky	Large nut	Uzbekistan
Rodina	Large nut, high kernel quality, high frost resistance, low susceptibility to anthracnose	Uzbekistan
Parkent	Large nut	Uzbekistan
Nani	Large nut	Uzbekistan
Gvardiesky	High kernel quality	Uzbekistan
Panfilovets	High kernel quality	Uzbekistan
Tonkoskorlupnii	High kernel quality	Uzbekistan
Ubilini	High yield, high kernel quality, resistance to spring frosts, precocious	Uzbekistan
Bostandik	Large nut, high kernel quality, resistance to spring frosts, precocious	Uzbekistan

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Part II
Beverage Crops

Chapter 12
Genetic Resources and Breeding of Coffee (*Coffea* spp.)

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Abstract Coffee is an important agricultural export commodity in many Asian, African and Latin American countries. It provides a livelihood for more than 125 million people worldwide. The genus *Coffea* comprises more than 125 species of which only 2 species *Coffea arabica* (arabica coffee) and *C. canephora* (robusta coffee) are commercially cultivated for beverage production. Climate change presents unprecedented challenges to sustainable coffee cultivation on a global scale. Besides, both arabica and robusta coffee are subjected to biotic and abiotic stress conditions that limit their production and productivity. Although conventional breeding approaches are followed to attenuate some of these problems, they were slow and time-consuming. Furthermore, arabica coffee has a narrow genetic base and needs to be addressed immediately by incorporating diverse germplasm with potential agronomic values, using focused breeding programs. In both arabica and robusta, the full potential of germplasm has not been exploited. Recent progress in the biotechnological field particularly on molecular markers and new generation sequencing platform hold great promise to discover new genes and accelerate coffee breeding programs. The progress achieved in coffee transgenic technology also has unparalleled opportunities to develop new cultivars with improved agronomic traits. Recent progress in gene editing techniques has a significant impact on the genetic improvement of coffee. This chapter provides current and innovative information about coffee's origin and distribution, genetic resource diversity and conventional breeding strategies and application. Current advances in the field of tissue culture, genetic transformation, gene editing and molecular breeding are also discussed.

Keywords Breeding strategies · Coffee · Genetic resources · Micropropagation · Molecular diversity · Transgenic technology

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12.1 Introduction

Coffee is one of the most popular beverages, consumed throughout the world. It is grown on 10.2 million ha of land spanning over 80 countries in tropical and subtropical regions, especially in Africa, Asia and Latin America (Mishra and Slater 2012). The economics of many coffee-growing countries depends substantially on the earnings from this crop. More than 125 million people worldwide derive their income directly or indirectly from the production of coffee. It is estimated that nearly 1.4 billion cups of coffee are drunk every day worldwide, making it the second most important commercial commodity in international trade, after crude oil.

The genus *Coffea* is in the family Rubiaceae, and consists of more than 125 species (Davis 2011; Davis et al. 2006; Razafinarivo et al. 2013). However, only 2 species *C. arabica* (arabica coffee) and *C. canephora* (robusta coffee) are commercially cultivated for beverage consumption and therefore have more economic significance. In 2017, global coffee production of coffee exceeded 159.66 million bags (60 kg capacity) comprising 97.43 million bags (61%) of arabica and 62.23 million bags (39%) of robusta coffee (ICO 2018). The total export value of coffee in 2017 was USD 32.7 billion in the international market. The top coffee producing countries and their export earnings in 2017 are given (Fig. 12.1, Table 12.1).

This chapter deals with origin and distribution, genetic resources and conservation strategies including cryopreservation techniques. Genetic improvement of coffee achieved during the last century using conventional breeding and improved cultivars cultivated worldwide is elucidated. Furthermore, advancements made in areas of coffee biotechnology especially micropropagation using somatic embryogenesis and transgenic technology, as well as new generation efforts carried out in genetic improvement of coffee are described.

12.2 Origin and Distribution

Among the coffee species discovered so far, *Coffea arabica* is the only self-fertile tetraploid ($2n = 4\times = 44$), whereas all other *Coffea* species are diploid ($2n = 2\times = 22$) and mostly self-sterile (Pearl et al. 2004) The center of origin and diversity of *C. arabica* is in the highlands of southwestern Ethiopia and the Boma Plateau of South Sudan, with wild populations also reported on Mount Marsalis in Kenya (Meyer 1965; Thomas 1942). Initial cultivation of *C. arabica* dates back 1500 years ago in southwestern Ethiopia from where it gradually spread to Yemen during the sixth century (Anthony et al. 2002). Two different genetic bases of Arabica, Typica and Bourbon, spread from Yemen and formed the base of present-day commercial arabica coffee cultivars grown worldwide.

In contrast to *Coffea arabica*, *C. canephora* has its origin in the tropical African rainforest, covering a large area from West Africa through Cameroon, the Central African Republic, mainly the Sudano-Guinean forest, Republic of Congo,

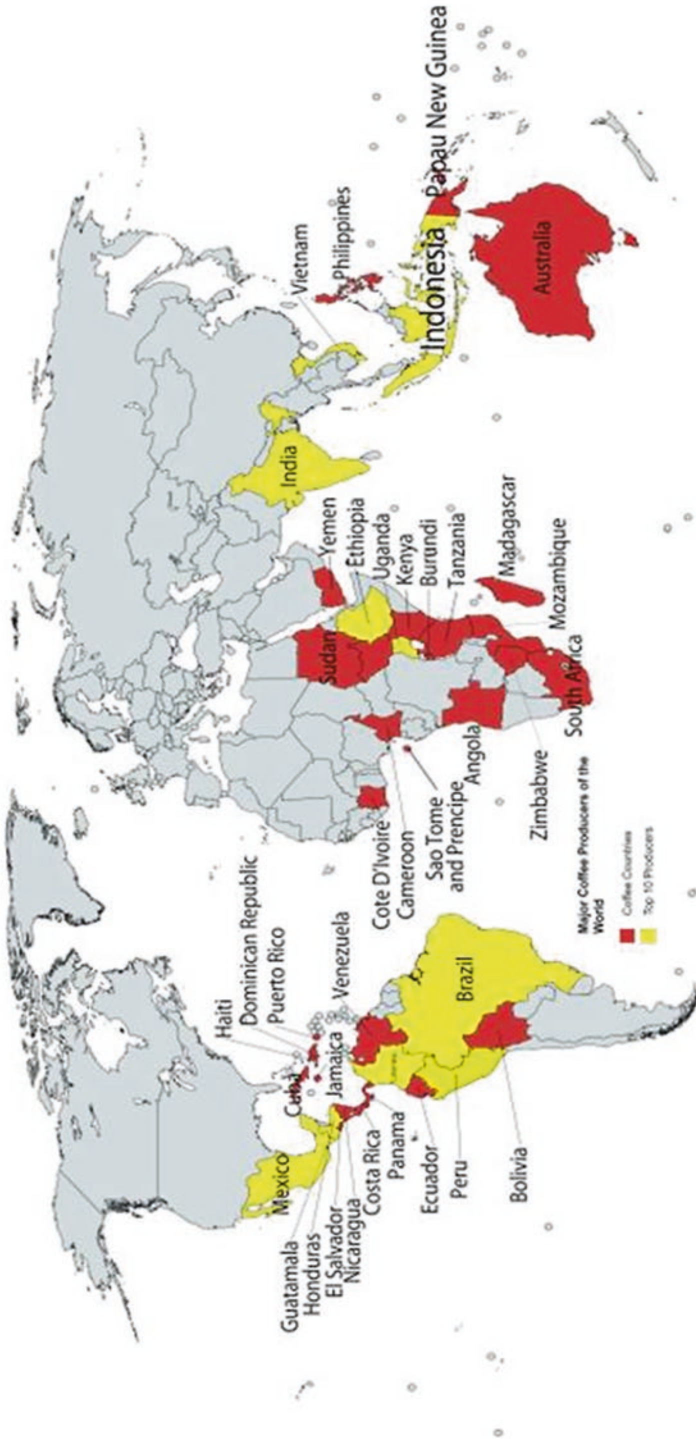

Fig. 12.1 Distribution coffee-producing countries in the world. Top 10 producers are marked yellow and others are marked red (Source: wired for coffee.com/where-is-coffee-grown-in-the-world/by Meredith as per 2016. Image is partially modified as per ICO Coffee Production Data, 2017)

Table 12.1 Top coffee-producing countries in 2017

Rank	Country	Cultivation area (in ha)	Production (in 1000 kg of 60 kg bags)	Export value (USD in millions)	% of world total
1	Brazil	2,339,630	51,000	4600	14.1
2	Vietnam	653,000	29,500	3500	10.7
3	Colombia	141,120	14,000	2580	7.9
4	Indonesia	1,230,495	12,000	1190	3.6
5	Honduras	308,000	8349	1160	3.6
6	Ethiopia	525,000	7650	938	2.9
7	India	423,270	5840	480.5	1.5
8	Uganda	352,000	5100	555.5	1.7
9	Peru	350,000	4300	707.2	2.2
10	Mexico	600,000	4000	433.6	1.3

Source: ICO (2018)

Democratic Republic of Congo, Uganda, and northern Tanzania up to northern Angola. Generally, wild populations of *C. canephora* exist in discontinuous patches with a small number of mother trees and offspring scattered over small areas (Musoli et al. 2009). Cultivation of robusta coffee started in Uganda and the eastern part of the Congo basin as early as the nineteenth century and during the early twentieth century; *C. canephora* was introduced to Java (Montagnon et al. 1998a, b, c). Subsequently, robusta coffee cultivation was spread to new regions of Asia, Africa and the Americas and gradually became established as the second largest cultivated coffee variety after arabica.

12.3 Genetic Resources Diversity and Conservation

12.3.1 Crop Genetic Resources Diversity

Crop genetic resources form the foundation of plant breeding programs and provide opportunities for breeders to develop improved cultivars with desirable agronomic traits. The evaluation and conservation of coffee genetic resources are considered to be of the urgent priority for sustainable coffee cultivation (Krishnan 2013). Commercial arabica cultivars currently grown worldwide are derived from two genetic bases known as Typica and Bourbon. Several researchers indicate that the Typica genetic base is derived from a single plant cultivated in Amsterdam in the early eighteenth century whereas the Bourbon genetic base consists of trees introduced to La Réunion (then Bourbon Island) from Mocha, Yemen in 1715 and 1718 (Krishnan 2013). The two subpopulations of wild coffee were originally introduced from Ethiopia to Yemen where it subsequently underwent successive reductions in genetic diversity. Introduction of coffee to Java, Amsterdam and La Réunion at the beginning of the eighteenth century led to further reductions in genetic diversity.

Fig. 12.2 Morphological diversity in plant habits among coffee species. (**a**) *Coffea canephora,* (**b**) *C. bengalensis,* (**c**). *C. canephora,* (**d**) *C. jenkinsii,* (**e**) *C. arnoldiana,* (**f**) *C. arabica,* (**g**) *C. stenophylla,* (**h**) *C. wightiana*, (**i**) *C. zanguebariae*

Due to the self-fertilization of *Coffea arabica*, extensive reduction in genetic diversity has taken place among commercial cultivars (Anthony et al. 2002). In contrast to *C. arabica*, huge genetic diversity exists among coffee species in terms of plant growth habits (Fig. 12.2) and fruit color (Fig. 12.3).

12.3.2 Genetic Resources Conservation

Conservation of coffee genetic resources is an established and recognized priority worldwide because of the high vulnerability of the genetic resources in the center of origin and diversity. In addition, the germplasm conserved in ex-situ gene banks also faces several challenges in the form of anthropogenic effects, climate change, lack of resources and injudicious planning, which can lead to the erosion of precious germplasm (Mishra 1998). The current system of conserving coffee germplasm is not sustainable, secure, cost-effective or rational and therefore should be considered as a global resource that will ensure the sustainability of coffee production for future generations (Krishnan 2018). At present two methods, in situ and ex situ, are used for germplasm conservation. However, recent advancements in

Fig. 12.3 Diversity in fruit color among coffee species. (**a**) *Coffea racemosa,* (**b**) *C. stenophylla,* (**c**) *C. canephora,* (**d**) *C. bengalensis,* (**e**) *C. arabica*, (**f**) *C. canephora,* (**g**) *C. wightiana,* (**h**) *C. arabica*, (**I**) *C. canephora*

cryopreservation techniques may also add it to the conservation strategies of this important crop.

12.3.2.1 In Situ and Ex Situ Conservation

Coffee genetic resources collection and conservation efforts were initiated during the 1950s, wherein germplasm exchanges between different counties took place. Subsequently, the Food and Agriculture Organization (FAO), various French organizations (ORSTOM, CIRAD) and IPGRI undertook extensive germplasm collecting missions in the 1960s, 1970s and 1980s in Ethiopia, Madagascar, Ivory Coast, Kenya, Congo and Yemen and collected several coffee species, and arabica and robusta genotypes (Charrier and Berthaud 1985) (Table 12.2). At present, field gene banks comprising *Coffea arabica* germplasm accessions are mainly available in Ethiopia, Kenya, Tanzania, India, Brazil, Columbia and Costa Rica and, similarly, robusta germplasm collections are held by Cameroon, Ivory Coast, Madagascar and India. Likewise, a large collection of diploid species is also available in the gene banks of Madagascar, Ivory Coast and India.

Table 12.2 Coffee genetic resources collecting missions

Year of collection	Participating organization	Countries explored	Type of germplasm collected	Countries possessing the germplasm
1964	FAO	Ethiopia	*Coffea arabica*	India, Brazil, Tanzania, Ethiopia
1966	ORSTOM	Ethiopia	*C. arabica*	Ethiopia, Cameroon, Ivory Coast, Madagascar
1960–1974	CIRAD, Museum of Natural History, France	Madagascar, Mauritius, Reunion Island, Comoro Islands	*Coffea* spp.	Madagascar
1975	ORSTOM	Central African Republic	*C. canephora*, *Coffea* spp.	Ivory Coast, the Central African Republic
1975, 1981	ORSTOM	Ivory Coast	*C. canephora*, *Coffea* spp.	Ivory Coast
1977	CIRAD, ORSTOM	Kenya	*C. arabica*, *Coffea* spp.	Kenya
1982	CIRAD, ORSTOM	Tanzania	*Coffea* spp.	Tanzania, Ivory Coast
1983, 1985, 1987	IPGRI, ORSTOM, CIRAD	Cameroon	*C. canephora*, *Coffea* spp.	Cameroon, Ivory Coast
1985, 1988	ORSTOM, IPGRI, CIRAD	Congo	*C. canephora*, *Coffea* spp.	Congo, Ivory Coast
1987	ORSTOM, CIRAD	Guinea	*C. canephora*	Guinea, Ivory Coast
1989	IPGRI, ORSTOM, CIRAD	Yemen	*C. arabica*	Brazil, Costa Rica

Source: Anthony et al. (2007) and Vega et al. (2008)

12.3.2.2 Cryopreservation

Coffee seeds are intermediate or recalcitrant in nature and therefore sensitive to both desiccation as well as storage. As a result of the difficulty to store coffee seed, even at low temperature, the main conservation strategy of coffee genetic diversity has been carried out ex situ by planting coffee trees directly in the field. However, this conservation strategy requires large areas, incurs high maintenance costs and is subject to many risks that can lead to the loss of genetic diversity (Valdes 2012). Cryopreservation is the storage of living biological material at ultralow temperatures. This technique is considered safe and economically viable for the long-term conservation of many plant species that are difficult to store using traditional methods. Cryopreservation has been investigated for coffee germplasm conservation by Dussert et al. (1997) and Santana-Buzzy et al. (2007).

In coffee, seeds, somatic and zygotic embryos are used in research for the development of cryopreservation techniques. Florin et al. (1995) evaluated three

preservation techniques for robusta somatic embryos and found that hydrated embryos can be preserved at 20 °C for up to 2 months. Similarly, partially-dehydrated embryos could be stored in liquid nitrogen for an indefinite period of time. These stored embryos have the same regeneration capacity as somatic embryos. Hatanaka et al. (1994) evaluated the survival rates of alginate-coated robusta somatic embryos, before and after freezing in liquid nitrogen, and found that critical dehydration was 13% and that below this level the embryos suffered desiccation injury. Somatic embryos which were cryopreserved for 8 months developed shoot roots directly within 50 days of incubating in the culture medium. Although, cryopreservation of somatic and zygotic embryos were successful, cryopreservation of coffee seeds appear to be practical for routine use in gene banks (Dussert and Engelmann 2006; Etienne et al. 2002).

In recent years, significant advances have been achieved in relation to use of the cryopreservation of coffee seeds (Dussert and Engelmann 2006; Dussert et al. 1997, 1998, 2000, 2003, 2012; Eira et al. 2005). However, the best cryopreservation results were obtained after drying seeds in saturated saline solutions. Recently, Coelho et al. (2017) suggested that rapid drying to 20% moisture content (dry basis), followed by direct immersion in liquid nitrogen and later reheating in a water bath for 2 minutes allowed effective cryopreservation of coffee seeds. Pinto et al. (2016) developed a protocol of cryopreserving coffee zygotic embryos using physical dehydration in silica gel, followed by osmotic rehydration after thawing. In another study, De Freitas et al. (2016) demonstrated the successful cryopreservation and germination of zygotic embryos of *Coffea arabica* by immersing the zygotic embryos in plant vitrification solution. These findings provide the technical advancement achieved in cryopreservation of coffee germplasm.

12.3.3 Genetic Resources Diversity Assessment

Assessment of genetic diversity is extremely important for effective conservation, as well as efficient utilization in breeding programs. In both *Coffea arabica* and *C. canephora*, germplasm diversity assessment was carried out using various techniques, such as (i) morphological, (ii) biochemical characterization/evaluation (allozyme and flavonoids), in the pre-genomic era, and (iii) DNA-based molecular marker analysis in the post-genomic era.

12.3.3.1 Morphological Markers

Morphological characters such as plant height, fruits and leaves color, leaf structure and venation pattern and seed size and quantitative agronomic parameters such as number of primary and secondary branches, fruits per cluster, productivity, disease and pest resistance and cup quality characteristics were studied in both arabica and robusta germplasm in India (Jamsheed Ahmad 1985; Mishra et al. 2011e;

Selvakumar and Sreenivasan 1986; Srinivasan 1969; Srinivasan and Vishveshwara 1981; Srinivasan et al. 1981; Thimma Reddy and Srinivasan 1979) and Brazil (Gaspari-Pezzopane et al. 2004; Medina-Filho et al. 2007; Silvarolla et al. 1999). However, morphological markers are inadequate to characterize all available genetic resources, either cultivated or wild, due to the intrinsic characteristics of coffee with a long juvenile period and low genetic diversity of commercial *Coffea arabica* plants (Lashermes et al. 1996; Maluf et al. 2005).

12.3.3.2 Biochemical Markers

Biochemical markers, particularly isozyme markers, which are co-dominant in nature with simple inheritance and detect diversity at functional gene level, are used in genetic diversity analysis of both *Coffea arabica* and *C. canephora* accessions. The analysis of six isozyme patterns in different *C. arabica* accessions revealed the absence of polymorphism, contrasting with the high level of morphological variation, and suggesting that isozymes are not appropriate for the study of genetic diversity and for *C. arabica* accession identification (Berthou and Trouslot 1977). In addition to isozymes, leaf flavonoid profiles and phenolic constituents of different arabica and robusta genotypes were studied by Mishra et al. (1993) and Saraswathi et al. (1991), which revealed that although biochemical markers can be helpful, they have limited discrimination abilities.

12.3.3.3 Molecular Markers

With the advent of DNA-based genetic markers, it became possible to identify large numbers of markers dispersed throughout the genetic material of any species of interest and use the markers for genotyping, varietal identification, germplasm diversity, construction of linkage maps, quantitative trait loci (QTL) identification and mapping in association with marker-assisted selection (MAS) breeding to develop genetically-improved crops with desirable traits. In coffee, the initial use of molecular marker analysis of germplasm was carried out using restriction fragment length polymorphism (RFLP) markers (Berthou et al. 1983; Lashermes et al. 1999, 2000). After the discovery of PCR technology, random amplified polymorphic DNA (RAPD) (Anthony et al. 2001; Lashermes et al. 1993, 1996; Mishra et al. 2011b, 2012; Orozco-Castillo et al. 1994, 1996) and amplified fragment length polymorphism (AFLP) markers (Anthony et al. 2002; Lashermes et al. 2000; Prakash et al. 2002, 2005; Steiger et al. 2002) are widely employed in coffee germplasm diversity analysis (Table 12.3). However, subsequently, the research focus shifted to more robust microsatellites or simple sequence repeat (SSR) markers and since then a large number of polymorphic SSR markers have been developed and utilized in germplasm assessment (Aggarwal et al. 2007; Baruah et al. 2003; Cubry et al. 2013; Moncada and McCouch 2004; Musoli et al. 2009; Poncet et al. 2004; Rovelli et al. 2000; Solorzanoet al. 2017; Sousa et al. 2017; Teressa et al. 2010;

Table 12.3 Molecular markers employed in genetic diversity analysis of coffee

Molecular markers employed	Genotypes/species analyzed	Inference	References
Chloroplast and mitochondrial DNA RFLP	*Coffea* spp.	RFLP analysis of chloroplast DNA could not distinguish between *Coffea arabica* and *C. eugenioides* as well as *C. canephora* and *C. congensis*	Berthou et al. (1983)
RAPD	Arabica accessions and *Coffea* spp.	Low genetic diversity among arabica and extensive variation among species	Lashermes et al. (1993)
RAPD	Robusta accessions, cultivated and wild arabica accessions and species	High genetic diversity obtained among the genotypes with species specific markers	Orozco-Castillo et al. (1994)
RAPD	*Coffea* spp.	Intra specific variation in arabica, robusta and high diversity among diploid species	Orozco-Castillo et al. (1996)
RAPD	Cultivated and wild arabica accessions	Low diversity obtained	Lashermes et al. (1996)
PCR-RFLP of Chloroplast DNA	*Coffea* spp.	Enzyme site polymorphism obtained	Orozco-Castillo et al. (1996)
Mitochondrial (ITS) PCR-RFLP	*Coffea* spp.	Intra species sequence variation in ITS region observed	Lashermes et al. (1997)
CP-DNA, ITS	*Coffea* spp.	Low sequence divergence among species	Cros et al. (1998)
RFLP	Arabica and robusta accessions along with 3*Coffea* spp.	Low genetic diversity in arabica was obtained	Lashermes et al. (1999)
AFLP	Cultivated and wild arabica as well as robusta accessions	High level of polymorphism in robusta compared to arabica was obtained	Lashermes et al. (2000)
SSR	Arabica cultivars	First report of microsatellites development in coffee	Rovelli et al. (2000)
SSR	Arabica and robusta accessions along with 15 *Coffea* spp.	Very low diversity in arabica and moderated diversity in robusta accessions	Combes et al. (2000)
RAPD	Wild and semi wild arabica cultivars	Low genetic diversity was obtained	Anthony et al. (2001)
AFLP	Arabica cultivars, robusta and diploid species	Very low genetic diversity among cultivated arabica genotypes	Steiger et al. (2002)
AFLP and SSR	Wild and commercial arabica cultivars and natural mutants	High genetic diversity among wild arabica compared to cultivated arabica genotypes was observed	Anthony et al. (2002)

(continued)

Table 12.3 (continued)

Molecular markers employed	Genotypes/species analyzed	Inference	References
AFLP	Introgressed hybrid arabica genotypes	Very low polymorphism in arabica with low alien genomic introgression	Prakash et al. (2002)
TBP	Arabica and robusta accessions	Tubulin-basedpolymorphism (TBP) showed no polymorphism in arabica and low polymorphism in robusta accessions	Bardini et al. (2004)
RAPD	Arabica cultivars	Low to moderated genetic diversity observed	Silveira et al. (2003)
RAPD	Forest arabica coffee from Ethiopia	Low to moderate genetic diversity was observed	Aga et al. (2003)
SSR	Arabica and robusta accessions along with diploid species	High genetic diversity in robusta and low diversity in arabica was observed	Baruah et al. (2003)
EST-SSR	Arabica and robusta accessions along with diploid species	Low genetic diversity in arabica and low to high diversity in robusta was observed	Bhat et al. (2005)
RAPD	Wild arabica accessions	High diversity in wild arabica was obtained	Chaparro et al. (2004)
SSR	Coffee species	High genetic diversity obtained	Poncet et al. (2004)
SSR	Wild and cultivated arabica genotypes along with robusta and diploid species	Diploid species and wild arabica had higher genetic diversity than cultivated arabica	Moncada and McCouch (2004)
RAPD, AFLP and SSR	Indian arabica and robusta cultivars	SSR displayed more polymorphism compared to AFLP and RAPD	Aggarwal et al. (2004)
AFLP	Robusta core collection from India	High level of genetic diversity was observed	Prakash et al. (2005)
ISSR	Ethiopian wild arabica	Moderate level of genetic diversity observed	Aga et al. (2005)
ISSR	Arabica accessions along with diploid species	Low genetic variability among arabica accessions	Masumbuko and Bryngelsson (2006)
SSR	Commercial arabica cultivars from the Americas, India and Africa	The genetic diversity within and between the Ethiopian populations is enormous	Tornincasa et al. (2006)
EST-SSR	Arabica and robusta accessions with diploid species	Low polymorphism obtained using EST-SSR	Aggarwal et al. (2007)

(continued)

Table 12.3 (continued)

Molecular markers employed	Genotypes/species analyzed	Inference	References
SSR	Arabica and robusta coffee with diploid species	Low to medium genetic diversity was observed	Hendre et al. (2008)
SSR	Wild and cultivated genotypes of *C. canephora* from Uganda	Higher genetic diversity was observed in cultivated genotypes and it was attributed to multiple origin	Musoli et al. (2009)
SSR	Arabica accessions from Ethiopia	High genetic variability was observed	Teressa et al. (2010)
ISSR and SRAP	*Coffea* spp.	Very high level of polymorphism was observed and specific markers were identified	Mishra et al. (2011a)
RAPD, ISSR and SRAP	Indigenous *Coffea* spp. from India	Species specific markers were obtained using all marker system. SRAP was found to be more suitable compared to RAPD and ISSR	Mishra et al. (2011b)
EST-PCR RFLP, SSR and SNP	*Coffea* spp.	Higher level of polymorphism was obtained in diploid species compared to arabica varieties	Mishra et al. (2011c)
RAPD, ISSR and SRAP	Indian coffee cultivars	SRAP marker system was more informative and amplified several cultivar-specific fragments	Mishra et al. (2012b)
SSR	*C. canephora* from the Guineo-Congolese region	High genetic diversity was observed	Cubry et al. (2013)
SSR	*Coffea* spp.	Genetic diversity in African and Indian Ocean *Coffea* is high and species of Madagascar region showed significant diversification	Razafinarivo et al. (2013)
SRAP, TRAP and SSR	Arabica genotypes from Yemen	moderate genetic diversity was obtained using combined marker analysis	Al-Murish et al. (2013)
SRAP	Ethiopian arabica germplasm	Substantial genetic diversity was obtained	Mishra et al. (2015)
SRAP	Dwarf arabica cultivars	SRAP markers amplified genotypic specific fragments	Hemavathi et al. (2015)
SNP	*C. canephora* genotypes from Vietnam and Mexico	Robusta genotypes from Vietnam and Mexico belongs to the Congo-Uganda group and closely related to each other	Garavito et al. (2016)
SSR	Arabica cultivars from Brazil	Markers were useful for DUS testing	Sousa et al. (2017)
SSR and AFLP	Arabica germplasm from Ethiopia	AFLP was more efficient than SSR in genetic diversity study of coffee	Desalegn (2017)

(continued)

Table 12.3 (continued)

Molecular markers employed	Genotypes/species analyzed	Inference	References
SSR	*C. canephora* germplasm from Ecuador	Wide genetic diversity was obtained	Solorzano et al. (2017)
SNP	*C. canephora* genotypes from Brazil	The markers were efficient in evaluating the genetic diversity and population structure of *C. canephora*	Alkimim et al. (2018)

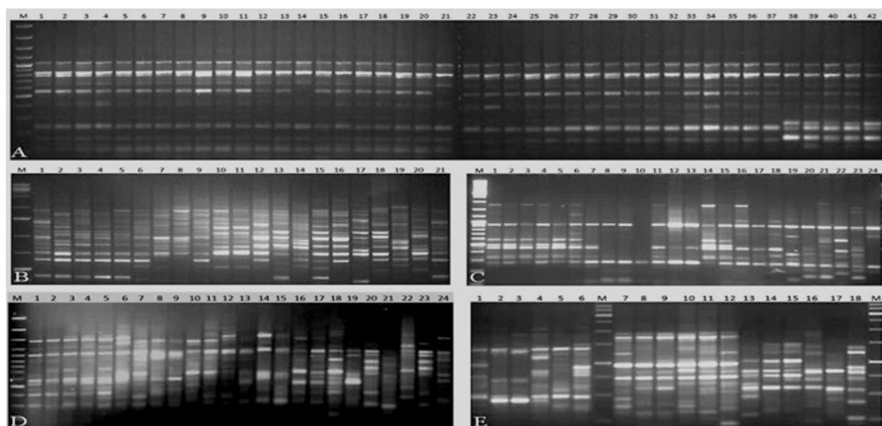

Fig. 12.4 Gel picture depicting genetic diversity among coffee germplasm and species using the various marker systems. (**a**) *Coffea arabica* germplasm diversity using SRAP markers, (**b**) Genetic diversity among *Coffea* spp. using SCoT markers, (**c**) Genetic diversity among *Coffea* spp. using SRAP markers (**d**) Genetic diversity among *Coffea* spp. using ISSR markers and (**e**) Genetic diversity among indigenous *Coffea* spp. using RAPD markers

Tornincasa et al. 2006). During the last few years, a new type of molecular marker known as a sequence-related amplified polymorphism (SRAP) marker was extensively used in genetic and diversity analysis of coffee (Fig.12.4) and was found to be extremely useful with better resolution compared to other molecular markers (Al-Murish et al. 2013; Hemavathi et al. 2015; Mishra et al. 2011a, b, 2012, 2015). More recently, a new generation of molecular marker known as single nucleotide polymorphism (SNP) is being used in coffee genome analysis (Alkimim et al. 2018; Garavito et al. 2016; Mishra et al. 2011c). More recently, another useful marker known as start codon targeted markers (SCoT) was used for molecular genetic analysis in coffee (Fig. 12.4). These markers provide valuable information regarding the association of genes with various agronomic or adaptive traits in plant species using strategies based on genetic mapping or association genetics studies and therefore likely to be the future marker of choice for genetic analysis of coffee.

12.3.3.4 Next Generation Sequencing

Next-generation sequencing (NGS) or second-generation sequencing technologies are revolutionizing the study of variation among individuals in a population (Govindaraj et al. 2015) and therefore have tremendous scope in genomics-assisted breeding in coffee. Most NGS technologies reduce the time as well as cost required for sequencing, compared to the Sanger method of sequencing (first-generation sequencing). Currently, there were several NGS technologies available such as the Roche/454 FLX, the Illumina/Solexa Genome Analyzer, the Applied Biosystems SOLiD System, the Helicos single-molecule sequencing and Pacific Biosciences SMRT instruments. These techniques can be effectively used to unravel the genetic diversity among closely-related germplasm and can also be used in important trait dissection studies. The combination of genomics-assisted breeding along with conventional breeding will greatly enhance the scope of genetic improvement of coffee.

12.4 Breeding Objectives and Limitations

The genetic improvement of coffee is largely restricted to the two economically-important species, *Coffea arabica* and *C. canephora*, which dominate world coffee production. The major objectives of arabica breeding, during the initial years, included increasing productivity and resistance to leaf rust and later on, during the 1990s, other objectives like improving quality, resistance to pests and drought were pursued. Unlike *C. arabica*, genetic improvement of *C. canephora* was inconspicuous until the 1950s, but gained momentum thereafter and in recent years much emphases has been given to robusta improvement.

For the last 80–100 years, genetic improvement of coffee was primarily effectuated using conventional breeding in many coffee-growing countries around the world. However, there are several bottlenecks that limit the use of conventional breeding strategies. First of all, the long process of traditional breeding involving several different techniques, such as parental selection, hybridization and progeny evaluation require at least 30 years to develop a new cultivar. Furthermore, the long generation time of the coffee tree, the high cost of field trials, the lack of accuracy of the breeding process, the differences in ploidy level between *Coffea arabica* and other diploid species and their incompatibility, are major limitations associated with conventional breeding (Mishra and Slater 2012). In addition to these, genetic resistance to important pest and disease such as coffee white stem borer (*Xylotrechus quadripes*), a devastating pest on *C. arabica,* as well as coffee berry disease (CBD) caused by the fungal plant pathogen *Colletotrichum kahawae* are not available in the *C. arabica* gene pool and therefore difficult to be incorporated into a breeding program. Similarly, the source of resistance to coffee berry borer caused by *Hypothenamus hampei*, drought and cold tolerance and herbicide resistance are not readily available in the coffee gene pool.

Another factor that usually impedes arabica coffee genetic improvement programs using traditional breeding techniques is the difficulty in selecting parental lines for hybridization, as well as confirmation of hybrid genotype at the early stages of plant growth based on morphological traits (Mishra et al. 2011d). This is because most of arabica genotypes are morphologically indistinguishable from each other. Uniformity of morphological traits in *Coffea arabica* could be attributed to the origin of the species, its narrow genetic base and its self-fertile nature. In view of the above, it is indispensable to develop the biotechnological tools to complement conventional breeding efforts in coffee. The last few years have seen a technical upsurge in the coffee biotechnological field especially in micropropagation, genetic transformation and functional genomics fronts.

12.5 Biotechnological Approaches

12.5.1 In Vitro Plant Regeneration

Establishment of an efficient regeneration system is important not only for more rapid multiplication of superior genotypes but also indispensable for developing genetic transformation protocols for coffee. Various in vitro multiplication methods such as somatic embryogenesis, meristem and axillary bud culture, and induction of adventitious buds have been reported using different types of explants in various coffee species (Carneiro 1999; Kumar et al. 2006).

12.5.1.1 Somatic Embryogenesis

The initiation and development of embryos from somatic tissues without the involvement of sexual fusion is known as somatic embryogenesis. In coffee, induction of somatic embryogenesis and plant regeneration was first reported in *Coffea canephora* using intermodal explants (Staritsky 1970). Sharp et al. (1973) successfully induced callus from seeds, leaves and anthers of two different cultivars, i.e. Mundo Novo and Bourbon amarelo of *C. arabica*. In the past 40 years, a number of improved protocols for somatic embryogenesis have been developed for various coffee genotypes (Santana-Buzzy et al. 2007). Somatic embryogenesis in coffee can be achieved using a single-step protocol (Yasuda et al. 1985) or by culturing in different media in a sequential manner (Dublin 1984; Neuenschwander and Baumann 1992; Sondahl and Sharp 1977; Zamarripa et al. 1991). However, in all cases, the availability of auxins is critical for the induction of embryogenic calli (Shondhal and Sharp 1977). In coffee, both high-frequency somatic embryogenesis (HFSE) and low-frequency somatic embryogenesis (LFSE) have been achieved. Using 2, 4-D in combination with other auxins/cytokinins strongly influences HFSE in primary cultures, whereas IBA and NAA combined with K increases LFSE. In coffee,

somatic embryogenesis follows two distinct developmental patterns: (1) direct somatic embryogenesis, where embryos originate directly from the explants and (2) indirect somatic embryogenesis, where embryos are derived from embryogenic dedifferentiated tissue (callus). However, both direct and indirect somatic embryos of coffee formed from leaf segments and callus, respectively, have a unicellular origin (Quiroz-Figueroa et al. 2002). In India, high frequency somatic embryogenesis and plant regeneration was achieved in various cultivars belonging to *C. arabica* and *C. canephora* using leaf explants (Fig. 12.5).

Somatic embryo induction and plantlet regeneration in coffee is time-consuming and often takes more than a year. Various attempts have been made to reduce the time needed for embryogenesis and increase the embryogenesis frequency in coffee. However, this situation has not yet been resolved completely. Triacontanol, silver nitrate ($AgNO_3$), salicylic acid, thidiazuron and 6-(3-methyl-3-butenylamino) purine (2ip) are the widely used growth regulators in coffee embryogenesis. Interestingly, picomolar concentrations of salicylates are reported to induce cellular growth and enhance somatic embryogenesis in *Coffea arabica* tissue culture (Quiroz-Figueroa et al. 2001). Similarly, triacontanol, as well as silver nitrate, at a low concentration in combination with indole-3-acetic acid (IAA) and benzyladenine (BA) induced direct somatic embryogenesis in *C. arabica* and *C. canephora* (Giridhar et al. 2004a, b). Additionally, thidiazuron (TDZ) also induced direct somatic embryos from the cultured leaf explants of *C. canephora* cv. CxR (Giridhar et al. 2004c).

The production of somatic embryos on an industrial scale was achieved by incubating somatic embryos of *Coffea arabica* in liquid medium using bioreactors

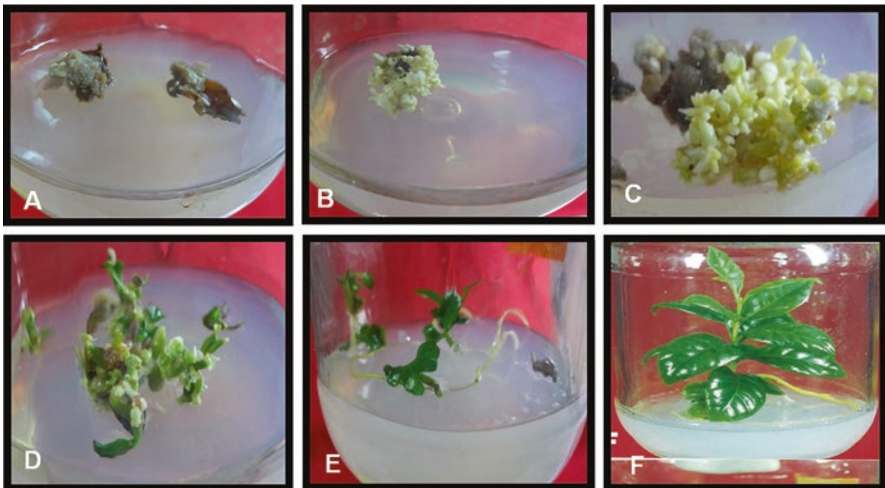

Fig. 12.5 In vitro regeneration of *Coffea arabica through* somatic embryogenesis. (**a**) Callusing leaf explants, (**b**) Initiation of somatic embryogenesis, **c** Somatic embryo maturation, (**d**) Germination of somatic embryos, (**e**) Developing plantlets, (**f**) In vitro plantlet ready for hardening

(Zamarripa et al. 1991). Subsequently, various other researchers also reported the production of somatic embryos for industrial use (Ducos et al. 1999; Noriega and Sondahl 1993); efficiency of conventional and temporary immersion system for coffee somatic embryo production (Albarran et al. 2005; Etienne and Berthouly 2002). Somatic embryogenesis has been effectively optimized commercially in *C. arabica* (Bobadilla Landey et al. 2013). The CIRAD-ECOM group consortium has been producing 1–2 million intraspecific F_1 hybrid plants per annum in Nicaragua and Mexico since 2007 (Georget et al. 2010). However, one of the major obstacles associated with the commercial production of somatic embryos is the poor conversion of somatic embryos to plantlets and very low percent of survival of plantlets in the nursery during acclimatization. Recently Georget et al. (2017) developed an efficient propagation protocol of somatic embryo-derived plantlets using horticultural rooted mini-cuttings.

12.5.1.2 Micropropagation

The coffee plant has a single apical meristem with each leaf axil producing 4–5 orthotropic buds and two plagiotropic buds. The plagiotropic buds only start development from the 10 to 11th node. For apical meristem culture and the culture of dormant buds, both orthotropic and plagiotropic buds are cultured to obtain plantlets. Microcuttings or nodal culture comprise a tissue culture approach which involves culturing nodal stem segments carrying dormant auxiliary buds and stimulating them in vitro to develop plantlets. Each single segment can provide 7–9 microcuttings every 80 days. Most of these studies carried out in the 1980s are reported by Carneiro (1999).

Several research studies have been carried out with a view to micropropagating superior coffee genotypes using apical or axillary meristem culture and nodal culture (Carneiro and Ribeiro 1989; Ribeiro and Carneiro 1989; Zok and Dublin 1991). A maximum of nine shoots was obtained per explant (Carneiro and Ribeiro 1989). However, in a temporary immersion system, culture of microcuttings resulted in a six-fold increase in the multiplication rate, in comparison with the solid medium (Berthouly et al. 1995; Teisson et al. 1995).

12.5.1.3 Genetic Fidelity and Field Performance of Tissue Culture Plants

Micropropagation through somatic embryogenesis involving a prolonged callus phase is considered unreliable due to genomic changes of in vitro raised plantlets (Jain 2001; Rani et al. 2000). Thus, the genetic fidelity of in-vitro derived plantlets should be tested as early as possible, especially in a woody perennial like coffee which has a long life cycle. Several strategies have been employed to assess the genetic fidelity of regenerated plants, of which DNA-based molecular markers have gained paramount importance in recent years. Previous studies have demonstrated culture-induced variation and regeneration of somaclonal variants in coffee obtained

through direct and indirect somatic embryogenesis (Loyola-Vargas et al. 1999; Sanchez Teyer et al. 2003; Sondahl and Lauritis 1992). Landey et al. (2013) analyzed the genetic fidelity of in vitro-propagated plants of *Coffea arabica* using AFLP and SSAP markers and confirmed that genetic variation observed in micropropagated plants are caused by abnormal chromosome numbers. However, they could not detect genetic variability among micropropagated plants using AFLP markers. Muniswamy et al. (2017) analyzed the genetic fidelity of micropropagated plants of *C. canephora* using SRAP markers and obtained 95% genetic similarity between somatic embryo derived plants and the mother plants indicating a high degree of genetic fidelity. We routinely evaluate genetic fidelity of the regenerated plants of *C. arabica* and *C. canephora* cultivars by using SRAP and SCoT markers and obtained more than 95% genetic similarities between the somatic embryogenesis derived plants with the mother plant. This clearly demonstrates the importance of culture conditions employed during somatic embryogenesis.

Reported field performance of somatic embryo derived plants showed a normal response in terms of physiology and yield. The genetic fidelity of micropropagated plants of *Coffea canephora* obtained through somatic embryogenesis was assessed in a large-scale field trial (Ducos and Pétiard 2003). A total number of 5067 trees regenerated from 5- to 7-month-old embryogenic cell suspension cultures were planted in the Philippines and in Thailand for comparison with control plants derived from auxiliary budding in vitro. No significant differences in yield and morphological features were observed between the somatic seedlings and microcutting-derived trees (Ducos and Pétiard 2003). Muniswamy et al. (2015) compared the performance of in vitro-derived plants and the seedlings obtained of a F_1 hybrid of *C. arabica* and observed that the growth of TC plants was found to be almost on a par with the seedlings of the respective mother plant without any segregation of traits. Furthermore, a significant increase in yield was observed in TC plants at one location. However, at the other locations, the mean fruit yield in TC plants was slightly higher compared to seedling progenies, although statistically there was no significant variation. In another study, Muniswamy et al. (2017) compared the vegetative vigor and yield of tissue cultured derived plants along with seedlings and concluded that the average yield obtained between tissue culture plants and seedlings they were statistically nonsignificant.

12.5.2 Transgenic Technology

Genetic transformation technology has potential applications in coffee agriculture by incorporating important agronomic traits such as disease and insect resistance, drought and frost tolerance and herbicide resistance. Transgenic technology can also be used to increase nutritional value and improve cup quality, produce varieties with caffeine-free beans and to produce hybrid crops for molecular farming. However, identification of target-specific genes is a prerequisite for developing transgenic crops. The availability of a large number of expressed sequence tags

(EST) sequences and the recent coffee genome sequencing data, and use of the next generation sequencing (NGS) platform may speed up gene discovery and accelerate transgenic research efforts in coffee.

12.5.2.1 Candidate Genes

In coffee, many candidate genes have been identified and some cloned and are currently being characterized. These include a caffeine biosynthesis gene (Ogita et al. 2004; Satyanarayana et al. 2005), a sucrose synthase gene (Leroy et al. 2005), osmotic stress response genes (Hinniger et al. 2006) genes for seed oil content (Simkin et al. 2006), isoflavone reductase-like protein in *Coffea arabica* (Brandalise et al. 2009), *C. arabica* BURP domain-containing gene for abiotic stress tolerance (Dinh and Kang, 2017), *CaSMP*, a seed maturation protein gene from *C. arabica* (Quintero et al. 2018) and several pathogen resistance genes such as *Mex-1* gene (Noir et al. 2003) and the *Ck-1* gene to CBD (Gichuru et al. 2006). However, an efficient genetic transformation protocol is required to validate the structural and functional aspects of these intrinsic genes. Furthermore, genes isolated from heterologous sources as well as from coffee were used in coffee transformation experiments. Some of the genes isolated from coffee used in transformation experiments include a theobromine synthase gene (*CaMXMTI*) for suppressing caffeine biosynthesis (Ogita et al. 2004) and an *ACC oxidase* gene involved in ethylene biosynthesis (Ribas et al. 2005). Genes introduced to coffee from heterologous sources include a *cry1Ac* gene from *Bacillus thuringiensis* targeted against leaf miner (Leroy et al. 2000) and the *α-AII* gene from common bean for imparting resistance to coffee berry borer (Cruz et al. 2004).

12.5.2.2 Transformation Systems

Direct and indirect DNA delivery systems have been used to transform coffee by various techniques which are described below.

12.5.2.3 Electroporation

Electroporation is a technique in which an electrical field is applied to cells in order to increase the permeability of the cell membrane, allowing chemicals or DNA to be introduced into the cell. Barton et al. (1991) used this technique to deliver a transgene into protoplast of *Coffea arabica*, but the regenerated plant failed to survive due to a weak root system. In another experiment, Da Silva and Yuffa (2003) delivered the *uidA* gene through electroporation using torpedo stage somatic embryos. Kumar et al. (2006) studied the expression of *uidA* gene driven by the N-methyltransferase (NMT) promoter in coffee endosperm by electroporation.

12.5.2.4 Microprojectile Bombardment

The first report of successful regeneration of transgenic coffee using particle bombardment of embryogenic calli was demonstrated by Cunha et al. (2004) in *Coffea arabica*, using kanamycin selection. Ribas et al. (2005) demonstrated the effectiveness of the *bar* gene as a selectable marker in microprojectile-mediated gene delivery. Gatica-Arias et al. (2008) bombarded the secondary somatic embryogenic suspension culture of *C. arabica* with a high regenerative capacity but could not regenerate the transgenic plants due to damage to the bombarded tissue. The successful regeneration of transgenic *C. arabica* was reported using bombardment of embryogenic calli followed by kanamycin selection (Albuquerque et al. 2009). The authors reported normal growth of the transgenic plants and obtained T1 progeny presenting 3:1 segregation of the *uidA* transgene. Transgenic *C. arabica* plants carrying α-amylase inhibitor-1 gene (α-*AI1*) from the common bean, *Phaseolus vulgaris*, under control of the seed-specific phytohemagglutinin promoter (PHA-L) were regenerated using microprojectile bombardment of embryogenic calli (Barbosa et al. 2010). The presence of the α-*AI1* gene in six regenerated transgenic T1 coffee plants were identified by PCR and Southern Blotting.

12.5.2.5 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens-mediated transformation technique has been widely used for genetic transformation of coffee. The first successful *A. tumefaciens* transformation and regeneration of transgenic plant was achieved in *Coffea canephora* (Hatanaka et al. 1999) and subsequently in *C. arabica* (Leroy et al. 2000). Mishra et al. (2002) reported that in *C. canephora,* pre culture of explants prior to *Agrobacterium* cocultivation, the addition of acetosyringone to co-cultivation medium and the duration of the cocultivation period significantly influenced the T-DNA delivery to coffee tissue. In a separate study, Mishra et al. (2008) compared the efficiency of four different *Agrobacterium* strains (*LBA 4404*, *EHA101*, *EHA105*, *AGL1*) in coffee transformation using *pBECKS2000* vector constructs carrying *uidA* and *gfp* reporter genes and concluded that *EHA 105* and *EHA101Agrobacterium* strains were more efficient compared to LBA4404 in T-DNA delivery and transgenic plant regeneration. Based on this improved protocol, mass production of transgenic coffee plants of both *C. arabica* and *C. canephora* was achieved (Fig. 12.6). Several workers have incorporated sonication and vacuum infiltration methods during *A. Tumefaciens* co-cultivation (Canche-Moo et al. 2006; Ribas et al. 2005). In most *A. tumefaciens* mediated transformation experiments, embryogenic tissues and/or somatic embryos were used as the target material for cocultivation. In *C. canephora,* a highly efficient *A. tumefaciens* transformation and regeneration protocol was established using hypocotyl explants as the target material (Mishra and Sreenath 2004). In *C. canephora*, the collar region of the hypocotyls was found to be most suitable for *A. tumefaciens* transformation (Sridevi et al. 2010). However, in *C. arabica,* embryogenic calli were found to be more suitable for *A. tumefaciens*

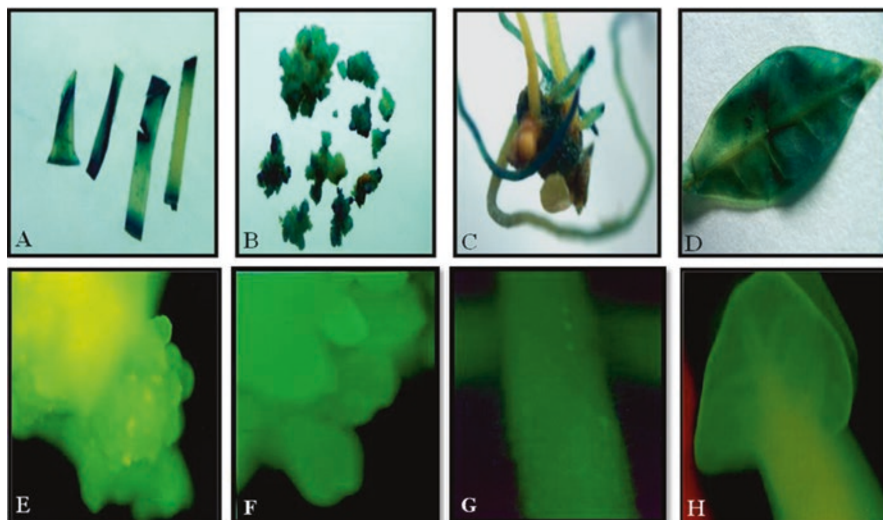

Fig. 12.6 Expression of *gus A* (**a, b, c, d**) and *sgfp* gene (**e, f, g, h**) in different tissue of coffee following *Agrobacterium tumefaciens*-mediated transformation

transformation (Ribas et al. 2011). The methodology for genetic transformation of *Coffea* using *Agrobacterium* was described in detail by Leroy and Dufour (2004) and Mishra and Slater (2012).

Successful recovery of stable transformants of coffee depends upon the appropriate selective agent, its optimal concentration, timing and frequency of selection. For regenerating transgenic coffee, various selectable marker genes (*hpt* hygromycin-R, *nptII* kanamycin-R, *csr1-I* chlorosulfuron-R, *ppt* phosphinothricine-R) were used and their efficacy evaluated by Van Boxtel et al. (1995) and Mishra et al. (2002). There are several reports of successful selection of transformed coffee plants using kanamycin as the sole selective agent (Albuquerque et al. 2009; Cunha et al. 2004). Besides kanamycin, hygromycin at concentrations of 20–100 mg/L was also used successfully for selection of transformants (Hatanaka et al. 1999; Mishra et al. 2002; Ogita et al. 2004). In addition to antibiotics, several other types of selection markers such as herbicide selection and positive selection have also been used in coffee transformation. The reliability of chlorosulphuran (*csr1-I*), phosphinothricin (*ppt*) and ammonium glufosinate (*bar*) as selection markers to regenerate transformed tissue are confirmed in both *Coffea arabica* and *C. canephora* using various transformation methods (Cruz et al. 2004; Leroy et al. 2000; Ribas et al. 2005). Furthermore, positive selection marker genes such as phosphomannose isomerase (PMI) and xylose isomerase (*xylA*) have also been used for coffee transformation, producing transformants able to grow in the presence of mannose and xylose, respectively, without an additional carbohydrate source. The study confirmed that compared to mannose, xylose is an effective selective agent for coffee transformation (Samson et al. 2004).

Regenerated transgenic crops carrying antibiotic and herbicide resistance genes have generated public concern about food safety and environmental impact. This has stimulated research into utilizing visual selection markers instead of antibiotic and/or herbicide selection markers. In coffee, green fluorescent protein, (*gfp*) and red fluorescent protein (*DsRFP*) were used for visual selection of transformed tissue following *Agrobacterium tumefaciens* mediated transformation (Canche-Moo et al. 2006; Mishra et al. 2008; Ogita et al. 2004). Recently, Mishra et al. (2010) regenerated transgenic plants of *Coffea canephora* and *C. arabica* by employing green fluorescent protein as the sole visual selection marker following *A. tumefaciens* mediated transformation (Fig. 12.6).

12.5.2.6 Gene Editing

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas9) is a powerful genome editing tool that has been widely adopted in model organisms including plants. In a recent study, Breitler et al. (2018), developed a genomic web tool and identified all gRNA target sequences in the *Coffea canephora* draft genome sequences. In all, 8,145,748 CRISPR guides were identified corresponding to 5,338,568 different sequences and, of these, 4,655,458 were single and 514,591 were covering exons. The proof of concept of CRISPR-associated (Cas9) gene editing was demonstrated by targeting the phytoene desaturase gene (*CcPDS*) utilizing the *Agrobacterium tumefaciens* transformation technique and the somatic embryogenesis plant regeneration protocol. The analysis of the RNA-guided genome-editing events revealed that 22.8% of the regenerated plants were heterozygous mutants and 7.6% were homozygous mutants. Mutation efficiency at the target site was estimated to be 30.4%. The study demonstrated that genome editing by the CRISPR/Cas9 method is an efficient and reliable way of knocking out genes of agronomic interest in the coffee.

12.6 Conventional Breeding

Coffee genetic improvement using conventional breeding techniques is mainly restricted to *Coffea arabica* and *C. canephora* because of their economic significance. However, several other species such as *C. liberica*, *C. excelsa*, *C. racemosa* and *C. zanguebarie* have contributed to the gene-pool enrichment either artificially or spontaneously through interspecific hybridization. In *C. arabica*, systematic breeding initiatives began as early as 1920 in different countries. Until today, most traditional arabica cultivars selected during the period 1940–1970 are still commercially cultivated and much of the world’s coffee is derived from these cultivars. These cultivars including Kents, S.288, S.795 from India, Mundo Novo, Caturra and Catuai from Brazil and Blue Mountain from Jamaica, are still under commercial cultivation.

Unlike *Coffea arabica*, genetic improvement of *C. canephora* was inconspicuous until the 1950s, but gained momentum thereafter and in recent year's considerable emphases has been given to robusta improvement.

12.6.1 Conventional Breeding Strategies

The strategies applied in conventional breeding depend primarily on the mating system of arabica (self-fertilized) and robusta (outbreeding) coffee. The breeding methods adapted to coffee have been extensively reviewed by many researchers (Carvallho 1985; Charrier and Berthaud 1985; Eskes and Leroy 2004; Van der Vossen 2001). A schematic representation of the breeding process followed in arabica and robusta coffee is given in Figs. 12.7 and 12.8, respectively. Coffee cultivars released for commercial cultivation using these techniques are shown in Table 12.4.

Historically, arabica coffee breeding was directed towards achieving tolerance to coffee leaf rust, higher productivity and improved quality. Subsequently, breeding objectives were expanded to include regional/country specific breeding goals, like insect pest resistance, CBD and nematode resistance and hybrid vigor. In robusta, breeding objectives are primarily focused on high yield. Recently, the development of drought tolerance, improved cup quality and hybrid vigor has been added.

12.6.2 Production and Productivity

The importance of hybrid vigor (heterosis) has long been realized to improve the production and productivity of arabica coffee. Generally, F_1 crosses involving distantly related genotypes of *Coffea arabica* produced a significant increase in yields of coffee in many countries such as India (Sreenivasan and Santa Ram 1993; Srinivasan and Vishveshwara 1978), Central America (Bertrand et al. 1997), Ethiopia (Ameha 1990) and Kenya (Gichimu 2012). Substantial transgressive yield increase (20–40%) in the hybrids was projected to be a probable utility model for enhancing coffee production. Furthermore, it was also observed that arabica coffee hybrids have higher yield stability and lower genotype x environment interaction.

In robusta coffee, experimental evidence for significant heterosis for yield was documented in the progenies involving intra/inter varietal crosses in several countries such as India (Dharmaraj and Sreenivasan 1992), Ivory Coast (Leroy et al. 1994, 1997; Montagnon et al. 1998a, b). Progenies obtained from the F_1 hybrid involving intergroup (Congolese x Guinean) crosses recorded 40% increase in yield compared to the parental genotypes. In India, best-combining clones of robusta were identified for establishing the biclonal/polyclonal garden for maximizing yield. In India, dwarf mutants of robusta with short internodes and an interspecific hybrid of *Coffea congensis* and *C. canephora* (CxR) opens up new avenues of high density planting and a way forward for robusta coffee development.

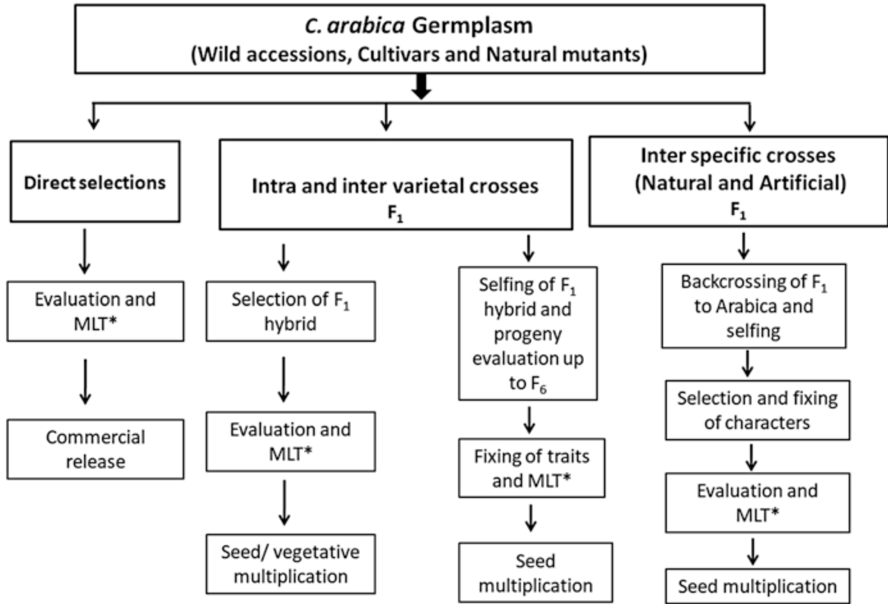

Fig. 12.7 Schematic representation of breeding strategies for genetic improvement of *C. arabica* (∗ multi location trials)

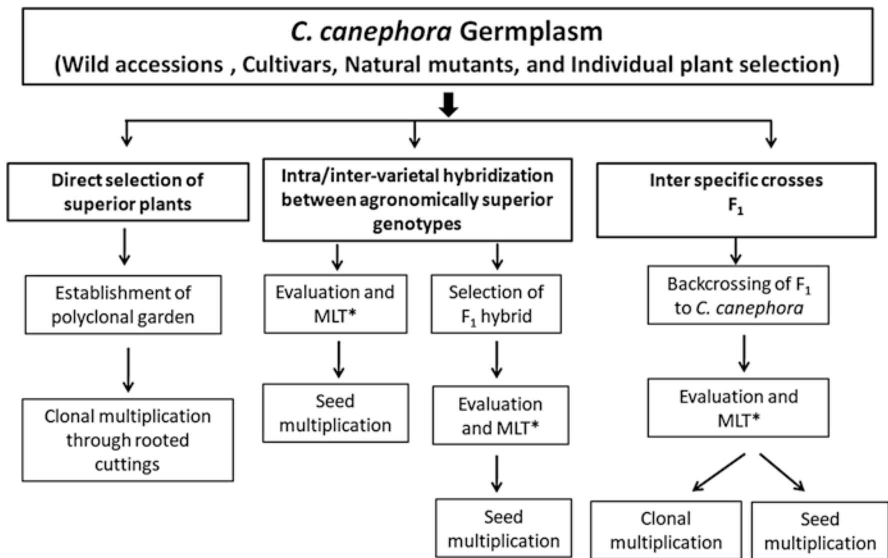

Fig. 12.8 Schematic representation of breeding strategies for genetic improvement of *C. canephora* (∗ multi location trials)

Table 12.4 List of breeding strategies followed for coffee improvement and cultivars released

Breeding Strategies	Techniques involved	Commercialization strategy	Material released
Arabica			
Pure line selection	Selection and selfing	Seed multiplication	Kents (India), SL.28 (Kenya), Java (Cameroon), Blue Mountain (Jamaica), Tekisic (El Salvador), Caturra (Brazil), Venecia (Costa Rica)
Hybridization followed by pedigree selection	Crossing, selfing and selection	Seed/clonal multiplication	Catuai, Tupi (Brazil) Catimor, Sarchimor (Costa Rica), S.795, Chandragiri, Sln.5A, Sln5B, Sln.9 (India), Catisic, Cuscatleco (El Salvador), Limani (Puerto Rico), Marsellesa (Nicaragua)
	Multiparental crosses		Sln.7.3, Sln.10 (India), Ruiru 11, Batian (Kenya), Ababuna (Ethiopia), Centroamericano (Costa Rica), Anacafe (Guatemala), Evaluna (Central America), Nayarita, Starmaya (Nicaragua)
Inter specific hybridization	Hybridization followed by backcrossing and pedigree selection	Seed	Sln.6, Sln.11 (India), Icatu (Brazil)
Robusta			
Mass selection	Selection and multiplication	Seed	S.274 (India), Nemaya (Central America), Apoata (Brazil)
Clonal selection	Selection of multiple clones	Seed	BR Series (India), SA and BP selection, BP39, BP42 (Indonesia), IF 126, 202,461 clones (Ivory Coast)
Inter specific hybridization	Hybridization, open pollination	Seed/clonal	Arbusta (Ivory Coast)
	Hybridization and backcrossing		CxR (India)

12.6.3 Resistance to Coffee Leaf Rust

Coffee leaf rust (CLR), popularly known as orange rust, is caused by the biotrophic fungus *Hemileia vastatrix* Berk. & Br. It is one of the major diseases in coffee species reported in almost all coffee-growing countries of the world. It has been estimated that at a severe incidence level, the yield is reduced 30–80% in the case of arabica coffee which accounts for a more than USD 2 billion losses per year. Resistance to CLR is conditioned primarily by a number of major genes (SH) and coffee genotypes are classified in resistance groups according to their interaction with the virulent genes (V) associated with different races of rust pathogen. The

Rust Research Centre (CIFC), Oeiras, Portugal, has identified more than 47 *H. vastatrix* races across the coffee-growing countries of the world of which 39 races (26 designated + 13 new races) were isolated from India (Bhat et al. 2013). India has been the forerunner in breeding coffee varieties resistance to leaf rust. The Indian cv. Kent, which contains the dominant S_H2 resistance gene, was selected by a farmer. Similarly, S_H3 resistance gene was also obtained from S.795 a popular Indian arabica cultivar derived from *Coffea liberica* introgressed lines. S_H3 gene is not present in any commercial cultivar grown all over the world. Similarly, Catimor having "A" type of resistance (resistance to all races) fell susceptible in India within 10 years of its cultivation. Inoculation tests carried out at CIFC Portugal confirmed that Catimor lines with A type of resistance clearly indicates the unidentified S_H genes and the presence of the new type of virulence genes in unidentified rust isolates. In India, the current breeding focus is primarily based on pyramiding the rust resistance genes, especially the introgression of S_H3 gene in the different genetic background in semi-dwarf arabica cultivars using marker-assisted selection (MAS) approach (Prakash et al. 2011). Several new breeding lines introgressed with S_H3 gene are currently under field trials in India. In addition to this, several Indian arabica cultivars derived from Devamachy (an indigenous spontaneous robusta x arabica tetraploid hybrid similar to Hibrido de Timor) crosses have shown high level of field tolerance to coffee leaf rust for nearly 50 years now. Another arabica selection (Sln.6) which is developed in India by inter specific hybridization of robusta x arabica and backcrossed to arabica followed by pedigree selection has shown a high tolerance to CLR with good yield and satisfactory quality (Srinivasan et al. 1999).

12.6.4 Resistance to Coffee Berry Disease

Coffee berry disease (CBD) is caused by the fungus *Colletotrichum kahawae* on developing berries of arabica coffee in Africa. The disease has direct impact on yield and quality of the coffee as it affects the harvestable crop. Host resistance to CBD is mainly conditioned by three major genes (dominant R, codominant T and recessive k genes) in addition to several recessive genes which provide additive effects (Bellachev 1997; Walyaro 1997). Resistance to CBD has been observed in indigenous species and transferred to cultivated varieties by hybridization. Java, which is an Ethiopian landrace, shows considerable tolerance to CBD and is cultivated in Cameroon. In Kenya, a new composite variety known as Batian has been developed recently which shows tolerant to CBD. Another cultivar K7 which is cultivated in Kenya and Tanzania shows variable tolerance to CBD.

12.6.5 Resistance to Nematodes

Arabica cultivars are susceptible to both root-knot (*Meloidogyne* spp.) and root-lesion (*Pratylenchus* spp.) nematodes. Variable levels of resistance have been observed in diploid species such as *Coffea canephora, C. liberica* and *C. excelsa*. Therefore diploid species are used as rootstocks for arabica cultivars in nematode-infested areas. In India, topee stage grafting with robusta rootstocks and arabica scions is being practiced on a large scale to evade nematode problems. Similarly, the cultivars Nemaya in Central America and Apoata in Brazil are being cultivated because of good yield potential and good cup quality.

12.6.6 Resistance to Insect Pests

Although several insect pests are known to attack coffee, only two have considerable economic significance: coffee berry borer caused by *Hypothenemous hampei* and coffee white stem borer caused by *Xylotrechus quadripes*. It has been observed that a resistance source to coffee berry borer is not available in either primary or secondary gene pools of coffee and the best control strategy of this insect pest is achieved by IPM methods.

White stem borer is the major pest on arabica coffee in India. The annual loss incurred due to WSB infestation is estimated atUSD17.5–26 million (Venkatesha 2010). Identification and synthesis of a male sex pheromone of the white stem borer has helped in designing the pheromone traps which are used to reduce WSB incidence (Vinod Kumar PK et al. 2000). Recently breeding efforts have been initiated in India to develop WSB-tolerant arabica cultivars by introgressing genes from diploid sources (Prakash et al. 2016). A spontaneous amphidiploid of *Coffea liberica* x *C. eugenioides*, which was released as cv. Sln.11,showed a high level of field tolerance to WSB and therefore taken further for breeding refinement by crossing it with HDT. The population S.4595 derived from this cross manifests a high level of tolerance to WSB under field conditions and therefore has the potential for commercial cultivation.

12.6.7 Drought Tolerance

Arabica coffees are generally more tolerant to moisture stress as compared to robusta coffees because of a more extensive and deeper root system. However, genotypic differences in drought tolerance were observed between different arabica cultivars. An empirical study on stomatal features and leaf venation pattern of several Indian cultivars have shown that Sln.5, Sln.7.3, Sln.9 and Sln.11 cvs. are more tolerant to drought compared to others (Mishra et al. 2011e). Field performance

data also indicated the adaptation of Sln.5, Sln.9 and Sln.11 in drought-prone areas (Sreenivasan 1985). In India, several robusta accessions were collected from drought-prone areas and are being evaluated for drought tolerance. Among the Indian robusta cultivars, CxR appears to have better drought tolerance as compared to S.274 and BR series cultivars and therefore is cultivated in wider geographic zones.

12.7 Conclusion and Prospects

Coffee is the most important agricultural commodity and popular beverage consumed throughout the world. Therefore, sustainable cultivation of coffee has practical significance both for the producers and consumers. The conventional breeding procedure of coffee is a lengthy process often stretching 20–30 years for evaluating a variety suitable for cultivation. In recent years, germplasm of a number of crop plants species was analyzed using new generation sequencing (NGS) platforms. NGS methods also provide a powerful basis for rapid mapping and identification of genes underlying quantitative traits (e.g. Abe et al. 2012; Gao et al. 2012; Schneeberger et al. 2009). Furthermore, future studies on coffee will shed light on the association between genotypic and phenotypic variability with the variability of SNP frequency which will give very precise insights into genetic diversity at the sub genomic level. Taking the above into consideration, it is all-important to analyze the coffee germplasm using modern genotyping tools. Trancriptomic analysis is likely to facilitate and speed up the conventional breeding process by enhancing the availability of potential genes from different cultivars and ecotypes of crops. This development provides a greater venue for faster integration of improved crops for commercial exploitation (Mittler and Shulaev 2013). Therefore, studies on functional genomics of coffee hold great significance in understanding the various mechanisms associated with different physiological processes, biotic and abiotic stress tolerance, improved productivity and quality which have potential implications for the genetic improvement of coffee (Barreto et al. 2012; Lima et al. 2002). Furthermore, a repository of robusta genotypes in the form of clones encompassing improved traits will greatly facilitate the robusta improvement programs.

Appendices

Appendix I: Research Institutes Relevant to Coffee

Institution	Specialization and research activities	Contact information and website
Central Coffee Research Institute, Coffee Board, India	Agronomy, soil sciences, plant protection, plant breeding and genetics, Biotechnology and tissue culture, crop physiology, postharvest technology and coffee quality analysis	Central Coffee Research Institute, Balehonnur, Dist Chikamagalur-577,117 Karnataka, India Phone: 08265-243029 Fax 08265-243143 https://www.indiacoffee.org/
CeniCaffe Colombia	Agronomy, soil sciences, plant protection, biotechnology	National Center for Coffee Research – Cenicafé Planalto Head quarters,via Chinchiná-Manizales. Manizales (Caldas) – Colombia Tel .: PBX +57 (6) 8506550 Fax +57 (6) 8504723 AA 2427 Manizales cenicafe@cafedecolombia.com
Kenya Agricultural & Livestock Research Organization	Agronomy, plant protection, plant breeding and coffee quality analysis	Kenya Agricultural and Livestock Research Organization, Kattegat Rd, Loresho Nairobi Kenya PO Box 57811, City Square, NAIROBI, 00200, Kenyahttp://www.kalro.org/crops_contacts
Coffee Institute of Costa Rica (Icafe)	Sustainable coffee production	ICAFE 400 meters north of the San Pedro de Barva Catholic Church, Heredia. 280-3011, Barva, Heredia, Costa Rica Tel: +506 2243-7800; Fax: +506 2243-7554 http://www.icafe.cr/contactenos/
Indonesian Coffee and Cocoa Research Institute	Agronomy, soil sciences, plant protection, tissue culture, machinery, processing, and quality control	Gebang, Nogosari, Rambipuji, Jember Regency, East Java 68175, Indonesia http://iccri.net/
Jimma Agricultural Research Center, Ethiopia	Plant protection, soil fertility, crop production and integrated watershed management	http://www.eiar.gov.et/jarc/ Jimma Agricultural Research Center Tel: (+251) 471-128020; Fax: (+251) 471-111999 PO Box 192, Jimma Zone,Oromia Region
National Coffee Research Institute (NaCORI), Uganda	Plant breeding and evaluation, micropropagation and germplasm conservation	National Coffee Research Institute Katosi Road PO Box 185, Mukono, Uganda 256-414-697659 www.nacori.go.ug/

(continued)

Institution	Specialization and research activities	Contact information and website
Embrapa Café Brazil	Agronomy, plant protection, breeding and genetics , biotechnology and tissue culture, crop physiology, postharvest and quality analysis	Park Biological Station, PqEB, Brasília, DF 70770-901, Brazil Tel: +55 61 3448-4433; Fax: +55 61 3448-4890 https://www.embrapa.br/en/cafe
Centre de coopération internationale en recherche agronomique pour le (CIRAD) , France	Sustainable development of tropical and Mediterranean regions	Avenue Agropolis, 34398, Montpellier Cedex 5, France Tel: +33 4 67 61 58 00 https://www.cirad.fr/en
IRD, France	Coffee biotechnology	Florence Morineau IRD 911 avenue Agropolis, BP 64501 34394 Montpellier cedex 5 Tel : +33 (0)4 67 41 61 00 Fax : +33 (0)4 67 41 63 30 Courriel : delegation.occitanie@ird.fr http://www.ird.fr/
University of Trieste	Molecular biology, functional genomics and physiology	Department of Life Science, University of Trieste,Piazzale Europa, 1 – 34127 – Trieste, Italia https://www.units.it

Appendix II: Genetic Resources of Coffee

Cultivar	Important traits	Cultivation location
S.795	Tall arabica variety with very high yield potential. Susceptible to leaf rust but produces excellent cup quality	India
Sln.5A	Tall arabica hybrid involving Ethiopian/Sudanese variety with good yield potential. Produces more B grade beans of high cup quality. Shows field tolerance to leaf rust	India
Sln.5B	Tall arabica variety with very good yield potential. Exhibit tolerance to leaf rust with good cup quality	India
Sln.6	A robusta arabica hybrid with vigorous growth and good yield potential. Show tolerance to leaf rust and CBD. Produces good cup quality	India
Sln.9	Tall arabica hybrid involving HDT and Tafarikela with early ripening and good yield potential. Produces very good cup quality	India

(continued)

Cultivar	Important traits	Cultivation location
Chandragiri	Hybrid between Villasarchi and HDT this dwarf arabica hybrid with good yield potential and high tolerance to leaf rust. Produces big size beans with good cup quality	India
Kents	A natural mutant of Typica discovered in India. High productivity but susceptible to leaf rust. Very good cup quality	India, Kenya
SL28	Tall, drought tolerant and very good cup quality potential, but susceptible to major diseases	Kenya, Malawi, Uganda, Zimbabwe
SL34	Tall arabica cultivar with high yield potential and exceptionally good cup quality but susceptible to major diseases	Kenya
Ruiru11	High yielding, dwarf hybrid tolerant to coffee leaf rust and resistant to CBD. Produces good cup quality	Kenya
Batian	High yields, tolerance to coffee leaf rust, resistance to CBD, bold bean with good cup quality	Kenya
Caturra	Natural bourbon mutant, dwarf variety with good yield potential. Susceptible to leaf rust and CBD but with good cup quality	Brazil, Central America
Catuai	Dwarf compact arabica variety with good yield potential. Highly susceptible to leaf rust and CBD. Produces good cup quality	Brazil, Costa Rica. Guatemala, Honduras
Mundonovo	A natural hybrid between Bourbon and Typica the variety produces vigorous and productive plant with good quality cup but susceptible to major diseases	Brazil
Obata	Hybrid between HDT and Villasarchi this is known as Sarchimor elsewhere. Moderate to high yield with tolerance to leaf rust	Brazil, Costa Rica
IAPAR 59	Dwarf arabica hybrid with good yield potential. Show tolerance to leaf rust and nematodes but susceptible to CBD. Cup quality is low	Brazil
Catimor	A dwarf hybrid between HDT and Caturra developed in Portugal in 1959 and cultivated in many countries with very high yield potential. Show variable resistance to leaf rust in different countries. Good cup quality	India, Brazil, Colombia, Costa Rica
Tekisic	A variety selected from bourbon known for excellent cup quality in the highest altitudes	El Salvador, Guatemala
Catisic	High High yields, tolerance to coffee leaf rust, adaptable to warm and acidic soil, susceptible to CBD and poor cup quality	El Salvador
Cuscatleco	Dwarf compact arabica variety with high yield potential. Show resistance to leaf rust and nematode. Produces good cup quality	El Salvador
Centroamericano	Dwarf F_1 arabica hybrid with very high yield potential Exhibit resistance to leaf rust and tolerance to CBD. Produces good cup quality	Costa Rica, El Salvador, Guatemala and Honduras

(continued)

Cultivar	Important traits	Cultivation location
RAB C15	High yielding tall variety selected from Indian cultivar Sln.6. Show resistant to rust and coffee berry disease	Rwanda
Castillo	High yielding coffee variety resistant to coffee leaf rust	Colombia
Anacafe	Dwarf high yielding hybrid between Catimor and Pacamara with good cup quality. Plants show resistance to leaf rust but susceptible to CBD	Guatemala
Evaluna	Dwarf introgressed F_1 arabica hybrid with very good yield potential. Susceptible to leaf rust but tolerant to CBD. Produces very good cup quality	Central America
Nayarita	Dwarf F_1 hybrid involving Ethiopian arabica with high yield potential. Show resistance to CBD but susceptible to coffee leaf rust. Very good cup quality	Nicaragua
Starmaya	Dwarf arabica hybrid involving Marsellesa and Ethiopian arabica with very high yield potential with resistance to leaf rust. Very good cup quality	Nicaragua
Marsellesa	Introgressed dwarf Sarchimor variety with very good yield potential. Resistant to leaf rust and tolerant to CBD. Produces good cup quality with high acidity	Nicaragua
Limani	Introgressed dwarf Sarchimor variety with good yield potential. Tolerant to leaf rust and produces good cup quality	Puerto Rico
Bourbon	Genetically important arabica variety, with medium yield potential, susceptible to both leaf rust and CBD. Produces excellent cup quality	El Salvador, Guatemala, Honduras and Peru
Panamian Geisha	Tall arabica landrace with medium yield potential. Tolerant to leaf rust but susceptible to CBD. Produces exceptionally good cup quality	Panama
K7	Tall arabica variety with good yield potential. Show tolerance to leaf rust and CBD with good cup quality	Kenya, Tanzania
KP 423	Selected from Kent, this tall arabica variety has high yield potential. Show tolerance to drought and leaf rust but susceptible to CBD. The cup quality is poor	Uganda

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Chapter 13
Advances in Tea [*Camellia sinensis* (L.) O. Kuntze] Breeding

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Abstract Tea is the most popular beverage, prepared with leaves of *Camellia sinensis* (L.) O. Kuntze, consumed by almost all communities and has economic, medicinal and cultural importance. The tea plant originated in southwestern China around 5000 years ago and is now grown in over 52 countries. Tea-breeding activities first started in ancient China and the selection of promising plants from natural variability of heterogeneous populations was practiced in the early years. This chapter highlights limitations in the traditional crop improvement programs and the necessity for adopting novel biotechnological tools to overcome them. The role of tea genetic resources in crop improvement, an overview of tea genetic resources in the world and conservation attempts made to date are discussed. Molecular markers have been used to improve the crop in tea-growing countries. The progress and significant achievements of marker-assisted selection for important traits are highlighted. A comprehensive account is given of genetic linkage maps constructed. The recent developments in functional genomic research and the progress of the *omics* approaches in tea are discussed. Several attempts have been made to identify functional genes associated with important traits. The progress of transgenic research in tea during the last two decades is summarized. The first genome sequence of tea was reported in 2017, which is a milestone in genome research. Mutation breeding has the potential to be used as an alternative tool to develop new cultivars in tea. The progress on controlled hybridization between selected parents, the most preferred strategy for tea crop improvement to date, is discussed. The chapter provides an overview of the current status and future prospective on the application of new technologies to combat global climate change.

Keywords Controlled hybridization · Genomics · Marker-assisted selection · Tea genetic resources · Tea breeding.

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13.1 Introduction

Apart from water, tea is the most popular beverage consumed today and has a rich cultural history dating back 5000 years. Also, tea plays an important role in agricultural economies of many tea-growing countries, making significant contributions to their gross domestic product. The tea industry provides a vital source of employment along the value chain impacting millions of livelihoods worldwide.

The focus of the chapter is on how current cultivation practices of tea are challenged and possible improvement strategies through crop improvement. Conventional tea-breeding strategies and their limitations are discussed and the role of biotechnological tools in resolving them are highlighted. The chapter provides an overview of tea genetic resources in the world and conservation attempts made to date. Molecular markers have been widely used in tea-crop improvements in all countries and the chapter discusses application of marker-assisted selection for important traits. Also, a comprehensive account of genetic linkage map construction is given. Further, the chapter reviews recent developments in functional genomic research and the progress of *omics* approaches in tea. The chapter describes the progress of transgenic research in tea over the last two decades. Mutation breeding has the potential to be used as an alternative tool to develop new cultivars in tea and the chapter explains methodologies and success stories. Conventional hybridization is the main method of tea cultivar development and the progress of activities are discussed in detail. The chapter provides an overview of the current status and discusses research initiatives taken for combating global climate change. Finally, some recommendations for future research are given.

13.1.1 Tea

Tea, *Camellia sinensis* (L.) O. Kuntze, is the source of an aromatic beverage, consumed by almost all communities. The tender shoots of the tea plant, comprising two or three of the topmost, immature leaves and bud, referred to as the *flush*, are harvested or plucked for processing into *made tea* (Modder and Amarakoon 2002). Tea is the second most consumed beverage in the world – water being first – well ahead of coffee, beer, wine and carbonated soft drinks (Costa et al. 2002). Tea is also consumed as a therapeutic drink for many illnesses. It is estimated that over one-half a billion cups of tea are consumed daily worldwide and a per capita mean consumption of 120 ml/day (Mc Kay and Blumberg 2002).

Today, tea has received considerable attention due to the health benefits of the compounds it contains. Over 700 chemical constituents including flavonoids, amino acids, vitamins, alkaloids and polysaccharides (Thomas et al. 2006) and over 500 flavor compounds have been identified in tea leaves (Rawat and Gulati 2008). Most of them are purported to be antidiabetic, anti-cancer, anti-obesity, anti-ageing and antihistamin (He et al. 2006; Sharangi 2009).

Approximately 76–78% of the tea consumed is black, 20–22% is green and less than 2% is oolong tea (Mc Kay and Blumberg 2002). Depending on the manufacturing process, teas are classified into three major types:

(a) Nonfermented green tea, produced by drying or steaming the fresh leaves to inactivate the polyphenol oxidase and thus, arrest oxidation;
(b) Semifermented oolong tea (produced when the fresh leaves are subjected to partial fermentation before drying);
(c) Fermented black and red (*pu-erh*) teas which undergo postharvest fermentation before drying and steaming, respectively. Although the fermentation of black tea is due to an oxidation catalyzed by polyphenol oxidase, fermentation of *pu-erh* tea is attained by using microorganisms (Cabrera et al. 2006).

13.1.2 Botany

Tea is a woody perennial shrub or small tree that grows well in tropical climates. The leaves are glossy green with serrated edges. When allowed to flower the plant produces small white flowers with bright yellow stamens. The fruit that develops has a hard green shell and a single, round, brown seed. The seeds can be used to make tea oil.

The tea plant, *Camellia sinensis,* originated in southwestern China around 5000 years ago (Chen and Chen 2012). Sealy (1958) described 82 species of *Camellia* while Mondal in 2002 recognized more than 325 species within the genus. The botanical classification of tea has been approached differently by various authors. Linnaeus originally called tea *Thea sinensis* and later included two more species: *T. bohas* and *T. viridis.* After much debate, tea was listed as *Camellia sinensis* in Index Kewensis (Mandal et al. 2011) and since then the name has remained unchanged. The botanical classification of *C. sinensis* (L.) O. Kuntze is given below (Chase and Reveal 2009):

Kingdom: Plantae
Sub kingdom: Tracheobionta
Super division: Spermatophyta
Division: Magnoliophyta
Class: Equisetopsida
Sub class: Magnoliidae
Order: Ericales
Family: Theaceae
Genus/Species: *Camellia sinensis* (L.) O. Kuntze

Tea is generally a diploid (2n = 30; basic chromosome number, × = 15) and occasional polyploidy has been reported (Bezbaruah 1971). Tea plants show a high degree of self-sterility and the mechanism of self-incompatibility remains a challenge to plant improvement.

There are three varieties within the species of *Camellia sinensis*. They are *C. sinensis* var. *assamica* (Assam plant) and *C. sinensis* var. *sinensis* (China type) (Sealy 1958). Third variety referred as Cambod or Southern form as a subspecies of *C. assamica* was recognized by Wight in 1962. Recently, Das and Ghosh (2016) proposed that the name for the Cambod variety of tea should be corrected as *C. sinensis* var. *lasiocalyx*.

Characteristics of the style can be used to differentiate between varieties (Wight 1962). According to Wight's classification, China types are having geniculate styles (free for greater part of their length) and Cambod types are ascending type in nature (free for about half their length). Wight (1962) further stated that styles of Assam are characteristic with united style (united for greater part of their length).

13.1.3 Origin and Domestication

The history of the discovery of the tea plant and the drinking of tea as a medicinal beverage in China dates back to 2737 BC (Yamanishi 1995), during the Shen-Nong era (Chen and Chen 2012). It is believed that tea originated in China, but the exact area of origin is not clear (Meegahakumbura et al. 2016). Also, there are no records of wild populations of tea and the identity of the exact species first used first domestication (Kingdon-Ward 1950). A recent study by Meegahakumbura et al. (2016) proposed southern China (e.g. Fujian, Jiangxi, Hunan, Guizhou) as the most likely area of origin of China type tea. The domesticated China type tea has been dispersed and planted in other provinces along the Yangtze River region. The findings of Meegahakumbura et al. (2016) revealed that Assam tea found in India is distantly related to Assam tea in China, suggesting that Indian Assam tea originated and was domesticated independently from China Assam tea. This differs from the earlier assumption of the introduction of Assam tea to India from Yunnan Province, China through Myanmar (Das et al. 2012b). It is believed that the Cambod type tea originated through hybridization between China and Assam types and shows mixed genetic composition. Meegahakumbura et al. (2016) also reported that the Cambod type tea did not form a distant grouping pattern in their study.

Tea cultivation until the mid-nineteenth century was exclusive to China (Raina et al. 2012). Today, tea is grown in over 52 countries in Asia, Africa, South America, and limited areas in North America, Australia and Europe (Chen and Chen 2012) within the latitudinal range of 45° N–34° S (Mondal 2014).

13.1.4 Selection and Early Improvement

Tea breeding activities began in ancient China thousands of years ago (Chen and Chen 2012). Successful development of vegetative propagation in the 1780s in China (Yao and Chen 2012) accelerated selection and breeding of tea.

Selection of promising plants from the natural variability of heterogeneous wild populations (open-pollinated progenies) was practiced in the early years and is still considered an effective tea-breeding strategy. Genetic material selected for high yield, high quality, and pest and disease resistance, later became cultivars using vegetative propagation. The value of selection from the wild should not be underestimated, as many popular tea cultivars in China, India, Sri Lanka, Japan and Kenya were based on such selections. In China, 76% of the released cultivars, including Longjing 43, Anhui 1 and Yunkang, were bred by individual selection (Yao and Chen 2012). Out of the 64 tea cultivars recommended in Sri Lanka, 14 were selected from old seedling tea fields by mass selection and are still very popular in tea plantations (Gunasekare 2012). Among them, high-quality cultivars DT 1, PK 2, N2 and H 1/58 are popular to date. Tea breeding in Japan began in the nineteenth century by selecting elite cultivars from original Japanese tea plants; major tea cultivars in Japan, Yabukita, Asatsuyu, Asahi and Samidori, are selections made from original Japanese teas (Tanaka 2012).

Later, controlled hybridization between selected parents became the most preferred strategy for tea crop improvement. Also, non-conventional approaches such as polyploid breeding and mutation breeding have been used in tea crop improvement.

13.2 Cultivation and Traditional Breeding

13.2.1 Current Cultivation Practices

Tea can be grown under a wide array of soil and environmental conditions; the optimum temperature range is 18–25 °C and well-distributed annual rainfall of 2500 mm is required. Tea plants prefer acidic (pH 4.5–5.5) deep, permeable soils (Wijeratne 2018).

Seed and vegetatively-propagated plants are the two planting methods available for tea cultivation. Seeds were the only planting material initially, but the area planted with clonal tea has gradually increased, as shown in Table 13.1.

Table 13.1 The ratio of clonal tea plantations in major tea-growing countries

Country	Clonal tea percentage (year)
China	46 (2010)
Japan	92.1 (2004)
North India	60 (2011)
Sri Lanka	55 (2004)
Kenya	60 (2011)

Source: Chen and Chen (2012)

Plant density and spacing of bearing tea plants vary from country to country, depending on slope, type of harvesting, etc. (Wijeratne 2018). Tea is a shade-loving plant and the canopy should be light saturated at around 60–70% of full sunlight. Harvesting or *plucking* can commence 18–24 months after planting. Although, manual harvesting is preferred, the plucking operation has been mechanized in many countries due to labor cost and scarcity (Wijeratne 2018). Tea shoots can be harvested year around in tropical conditions, maintaining 5–7 days intervals manually; the frequency may extend to 2–3 weeks under machine plucking. In temperate climate conditions, machines are mostly used and harvesting is done only 3–4 times during the growing season, at a frequency interval of about 45 days (Wijeratne 2018).

The practice of removing branches and training the tea bush to maintain the canopy is termed *pruning* in tea cultivation. The height and the frequency of pruning depend on the cultivar, soil conditions and climatic factors (Wijeratne 2018). The economic life span of a tea bush is 50–75 years, depending on the cultivar and environmental factors.

13.2.2 Current Agricultural Problems and Challenges

Today the impact of global climate change in tea-growing regions is considered one of the major challenges faced by tea growers. A reduction of tea yield has been projected due to rising temperatures and drier weather conditions. Increasing temperatures and the occurrences of extreme weather events (prolonged droughts, sudden downpours) can cause severe soil degradation and reduce soil fertility (Wijeratne 2018). Also, it is predicted that the impact of global climate change may increase the incidence of pests, diseases and weeds in these areas (Lal 2005). Moreover, in warmer tea-growing regions, insufficient exposure to low temperatures during winter months may disrupt the synchronization of budding at the first flush (Tanaka 2012). Synchronization of budding is important in maintaining the quality of first flush leaves. Hence, it is necessary to develop new tea cultivars suited to varying ecological conditions and new cropping patterns (Wijeratne 2018).

Tea cultivation practices are labor intensive and the availability of a skilled workforce is declining rapidly in tea-growing regions (Wijeratne 2018). Tea cultivation practices such as land preparation, harvesting and pruning, are now being mechanized in most tea-growing countries. Hence, tea cultivars amenable to mechanization are in high demand and crop improvement programs need to be attuned to this.

The demand for a pure beverage among tea consumers has been growing and continuous; the application of agrochemicals is discouraged all over the world. Tea breeders are entrusted with the challenge of developing new tea cultivars with multiple resistances to pest and diseases without compromising quality. Continuous and excessive application of inorganic fertilizer affects soil health over time (Wijeratne 2018) and leads to ground water pollution (Tanaka 2012). Hence, development of new tea cultivars with higher natural fertilizer use efficiency needs to be researched.

Furthermore, the trend toward organic tea farming has increased substantially over the last few years due to environmental protection and health concerns (Hajra 2018). Therefore, to reap the benefits of organic agriculture, there is the need to develop suitable tea cultivars for organic farming.

13.2.3 Traditional Breeding Methodologies and Limitations

Traditional tea breeding and selection programs are multistage and comprised of the generation of variation, individual selection of promising genotypes, evaluation and screening for yield, quality, biotic and abiotic stresses, local adaptability testing and cultivar releasing, all of which can take 20–25 years (Gunasekare 2012; Yao and Chen 2012). According to Mondal (2014), the major limitations in a traditional tea-breeding program are as follows:

(a) Woody perennial nature
(b) Long gestation period
(c) High inbreeding depression
(d) Self-incompatibility
(e) Unavailability of distinct mutant of different biotic and abiotic stress
(f) Lack of early selection criteria for traits
(g) Low success rate of hand pollination
(h) Seasonal short-flowering time
(i) Long duration for seed maturation
(j) Nonoverlapping of flowering times of different cultivars
(k) Slower rates of propagation by cuttings
(l) Poor survival rates of cuttings/poor rooting of cultivars
(m) Unavailability of planting materials due to climatic factors, i.e. winter, drought
(n) Season dependent rooting of different tea cultivars
(o) Unavailability of wild and non-related germplasm for crop improvement (in some countries)
(p) Pre- and post-zygotic fertilization barriers for wide hybridization

13.2.4 Role of Plant Tissue Culture

Tea researchers have been working on adopting advances in biotechnology to overcome some of the limitations mentioned above. The progress made on other biotechnological applications in tea, i.e. molecular markers, recombinant DNA technology and functional genomics, are discussed in subsequent sections. Here, the focus is on overcoming limitations in vegetative propagation of tea through the development of protocols for *in vitro* multiplication or micropropagation. Initial attempts at developing protocols began in the early 1980s and continues to date.

Mondal et al. (2004) and Mondal (2014) revived the efforts made to develop different protocols for in vitro shoot multiplication, rooting, somatic embryogenesis, organogenesis, etc. Concurrently, research has been geared more towards protoplast culture (Bagratishvili et al. 1979), cryopreservation (Chaudhury et al. 1990) and anther culture (Katsuo 1969).

13.2.4.1 Micropropagation

Micropropagation of tea is useful in the rapid multiplication of elite tea cultivars and to produce quality planting materials to cater to the high demand in tea plantations. Initial attempts were focused on establishing aseptic in vitro cultures, optimization of explants type, nutrient composition of culture media and further multiplication (Mukhopadhyay et al. 2015). Different explant types were used for micropropagation such as nodal segments (Agarwal et al. 1992), shoot tips (Banerjee and Agarwal 1990), zygotic embryos (Iddagoda et al. 1988; Ranaweera et al. 2013; Seran et al. 2006), embryogenic axes (Seran et al. 2006), epidermal layers of stem segments (Kato 1985), axillary buds (Nakamura 1990) and leaves (Sarwar 1985). Different basal media formulations such as Murashige and Skoog (1962), White's medium (White 1963), Woody Plant Medium (WPM) (Lloyd and McCown 1980) and Schenk and Hildebrandt (1972), supplemented with various concentrations of growth regulators like BAP, Kinetin, NAA, GA3, IBA, Zeatin and TDZ (Das et al. 2012b) for multiple shoot proliferation. However, no single universal medium and hormone combination was found for micropropagation, and results varied widely with the tea cultivar used. Das et al. (2012b) reported that the shoot proliferation rate of Assam type cultivars is significantly higher than in the China and Cambod types.

The success of tea micropropagation relies on an efficient rooting and acclimatization program. Initial attempts were mainly concentrated on in vitro rooting and later ex vitro rooting was the focus due to the low cost of production and high success rate. As summarized by Mondal (2014), in vitro rooting of tea microshoots can be done by either long-time exposure of low auxin concentration or being subjected to an *auxin-shock* treatment via initial culturing in a high auxin medium, followed by transfer into an auxin free medium. Ex vitro rooting of tea microshoots is influenced by the pH of the hardening media and relative humidity of the hardening chamber (Mondal 2014).

Ranaweera et al. (2013) optimized an *ex vitro* rooting protocol for tea microshoots with simultaneous acclimatization that reduced the cost of production of micropropagated plants by 71%, compared to that via in vitro rooting. Microshoots were subjected to a *pulse treatment* of 50 mg L^{-1} IBA for 3 h and transferred to a medium containing top soil: sand: coir dust (1:1:1) for rooting. Figure 13.1 shows rooted tea microshoots after 2 months, which are ready for transplanting into normal nursery mixtures.

Fig. 13.1 (**a**) *Ex vitro* rooted tea microshoots, (**b**) Tea plant ready for transplanting into normal nursery medium

13.2.4.2 Somatic Embryogenesis

Somatic embryogenesis in tea is an alternative mass-propagation method for rapid production of healthy plantlets within a short time (Fig.13.2). The advantages of somatic embryogenesis in tea are maintaining genetic fidelity of plants (Bano et al. 1991), production of synthetic (artificial) seeds (Mondal et al. 2000) and genetic transformation (Mondal et al. 1999). Various factors affect success of somatic embryogenesis in tea i.e. explant type, physiological stage of the explant, cultivar and culture media composition (Mondal 2014).

Different explant types have been used for induction of embryogenesis in tea i.e. pollen (Chen and Liao 1983), immature cotyledon (Abraham and Raman 1986; Bano et al. 1991; Nakamura 1988), decotyledonated embryos (Mondal et al. 2000; Nakamura 1985; Paratasilpin 1990), de-embryonated cotyledons (Mondal et al. 2001c; Rajkumar and Ayyappan 1992), nodal cuttings (Akula and Akula 1999; Akula and Dodd 1998), leaves (Seran et al. 2007) and leaf stalks (Hua et al. 1999).

Mondal et al. (1999) and Nakamura (1988) confirmed that somatic embryogenesis of tea can be affected by the maturity stage of the cotyledons used. Paratasilpin (1990) reported higher somatic embryogenesis (28%) in China type tea cultivars than in Assam type (2%). Also, the influence of genetic variation on somatic embryogenesis was reported by Kato (1996), confirming the highest frequency of embryogenesis in cultivar Yabukita.

MS medium has been widely used for induction of somatic embryos in tea and a few reports are available for other media i.e. WPM (Tahardi et al. 2003) and Nitsch and Nitsch (Nakamura 1985). The influence of plant growth regulators has been extensively studied and generally a high cytokinin-to-low auxin ratio or a singular cytokinin at low concentration has resulted in a higher frequency of somatic embryogenesis (Mukhopadhyay et al. 2014).

Secondary embryogenesis via repetitive (recurrent) embryogenesis has been commonly reported in tea and is useful in transgenic plant production (Mondal 2014; Mondal et al. 2001b). Kato (1986), Jha and Sen (1992), Balasubramanian

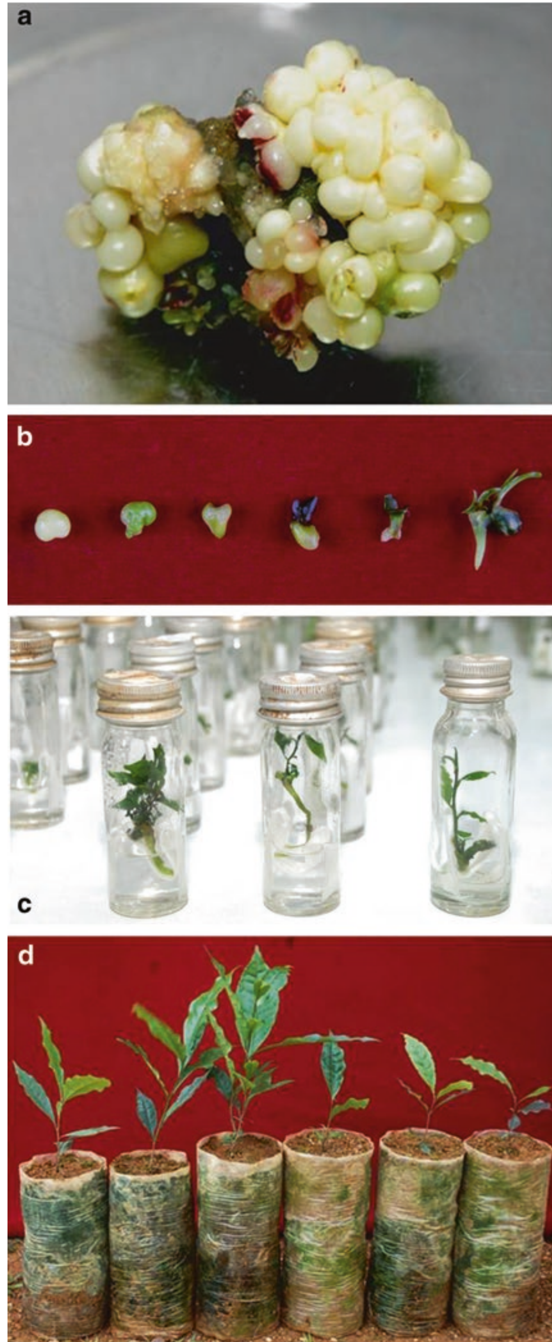

Fig. 13.2 Development of tea plants through direct somatic embryogenesis. (**a**) Somatic embryos produced from embryogenic axis, (**b**) Somatic embryos development stages (globular, heart, torpedo, cotyledonary from left to right) and germination, (**c**) In vitro plantlet development, (**d**) Ex vitro rooting and hardening of tea plants

et al. (2000b) and Mondal et al. (2001a) demonstrated successful attempts of secondary somatic embryogenesis in tea. Recurrent embryogenesis in tea was successfully utilized for bioreactor technology by Akula et al. (2000). Globular embryos induced from nodal explants of cultivar TRI 2025 were used and a 24-fold multiplication for secondary embryos reported using a modified temporary immersion system.

13.2.4.3 Anther Culture

Production of homozygous lines of diploid plants in tea is difficult due to its highly heterozygous and heterogeneous nature (Mondal 2014). Hence, production of haploid plants using the anther-culture technique is immensely useful in tea crop improvement (Seran 2007). Haploid plant production depends on a number of factors which influence androgenesis viz. donor plant, pretreatment of buds, microspore stage and culture medium (Seran 2007). Initial attempts at anther culture of tea were made by Katsuo (1969) and by Okano and Fuchinone (1970); success was limited to production of roots from anther-derived callus. The first tea plant produced through anther culture was accomplished by Chen and Liao (1982, 1983) using cultivar Fuyun-7. Later, Raina and Iyer (1992) and Shimokado et al. (1986) also reported haploid plant production from true pollen-derived embryos. Seran et al. (1999, 2007) reported production of embryogenic calli from leaf explants of tea and observed a high frequency of haploid cells (68%). However, plantlets could not be regenerated from these microcalli.

13.2.4.4 Embryo Culture and Embryo Rescue

Embryo rescue is important in tea-crop improvement for developing new hybrids using interspecific hybridization among different species of *Camellia*. Natural hybridization among these species is not common. However, no successful embryo rescue technique for tea is available to date. Hence, development of a successful protocol for embryo rescue will be immensely useful.

The Tea Research Institute of Sri Lanka (TRISL) recently integrated an in vitro protocol using embryo culture and micropropagation into their conventional breeding program to reduce by 5–6 years the evaluation cycle (Ranaweera et al. 2013). This is the first record of integrating an in vitro protocol to supplement a conventional breeding program of tea. Currently, TRISL practices this protocol (Fig. 13.3) which saves time, money and resources required for cultivar development.

In China, a combined approach of marker-assisted selection and micropropagation technique has been adopted to develop efficient tea breeding program (Chen et al. 2007).

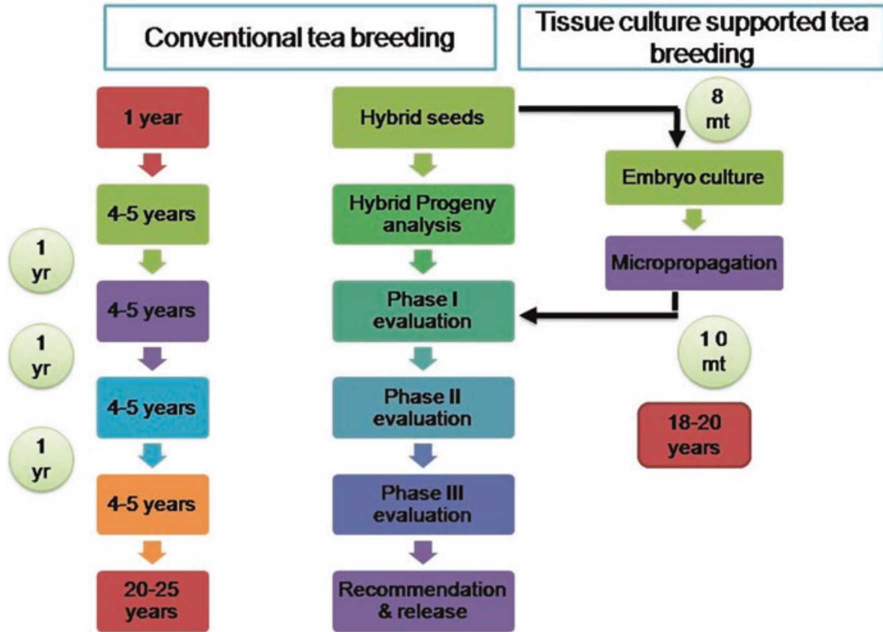

Fig. 13.3 Tissue culture supported conventional tea breeding program of Sri Lanka

13.3 Germplasm Biodiversity and Conservation

13.3.1 Global Tea Germplasm Diversity

As the origin center of tea (*Camellia sinensis*), China has the world's richest germplasm collections and total tea genetic resources preserved in national and provincial tea germplasm repositories, containing more than 10,000 accessions (Chen et al. 2012). The permanent ex situ conservation facility, the China National Germplasm Tea Repository (CNGTR), including the Hangzhou Tea Repository (CNGHTR) in the Tea Research Institute, Chinese Academy of Agricultural Sciences (TRICAAS) and the Menghai Tea Repository Branch (CNGMTRB) in the Tea Research Institute, Yunnan Academy of Agricultural Sciences (TRIYAAS), were established in 1990. By the end of 2010, around 3000 accessions including wild tea plants, landraces, improved cultivars, introduced cultivars and related species had been registered in the CNGTR (Yao and Chen 2012).

In India, two germplasm repositories are maintained and the largest collection of about 2532 accessions is maintained at the Tocklai Experimental Station of the Tea Research Association, Jorhat, Assam (Sharma 2011). The collection includes primitive seed sources, improved cultivars, polyploids, natural variants, breeding stocks and wild and related *Camellia* spp. The second germplasm repository is the United

Planters Association of Southern India, Tea Research Institute (UPASI-TRI), Valpari, Coimbatore District, Tamil Nadu has about 440 accessions including selections from old seedlings and cultivars (Sharma 2011).

The Institute of Vegetable and Tea Science (NIVTS) of the National Agriculture Research Organization (NARO), Japan holds a tea collection within the National Institute of Agrobiological Sciences (NIAS) Genebank; the collection was started 100 years ago (Taniguchi 2014). This is one of the largest tea germplasm collections and began with the objective of introducing tea seeds from China and India for crop improvement programs.

Collection of tea genetic resources in Vietnam was initiated by a French scholar named Baux (1885); subsequently collected accessions were maintained in the Phu Ho station (La 1998). Currently 180 accessions are maintained in the Phu Ho and Ha Giang stations, which include both seedlings and cultivars (Ngoc 2012).

In Sri Lanka, conservation of tea germplasm, as an ex situ gene bank living collection, was initiated in 1986. Currently, about 600 accessions are being maintained at the TRISL, Talawakelle and some accessions are maintained in duplicate at Ratnapura and Passara regional stations (Gunasekare et al. 2012). Sri Lankan tea germplasm can be broadly categorized into two groups, beverage type and nonbeverage type. Beverage type comprises all tea accessions including introductions, estate selections and improved cultivars, based on available pedigree data and breeding history (Ranatunga and Gunasekare 2008). The nonbeverage types are ornamentals, including *Camellia rosaeflora*, *Pyrenaria barringtoniaefolia*, *Gordonia zeylanica* cv. Elliptica, and various *C. japonica* varieties. A few examples of the unique germplasm available in different countries are given in Fig.13.4 and Table 13.2.

13.3.2 Cultivar Descriptors

A *descriptor* is defined as an attribute, characteristic or measurable trait that is observed in an accession of a gene bank that is used to facilitate data classification, storage, retrieval, exchange and use (Bioversity International 2007). Descriptors are important in the characterization of crop germplasm. Tea descriptors were prepared in 1997 by studying over 250 accessions comprising all three varieties (*sinensis*, *assamica* and *cambodiensis*). The descriptors are organized into four categories: passport, management, environment and site, and characterization. A descriptor list provides an international format and thereby provides a standardized language for tea genetic resources data (IPGRI 1997).

Chen et al. (2005) proposed 111 descriptors for tea germplasm characterization including 26 passport data, 45 morphological traits and biological characteristics, 29 quality attributes, 8 biotic and abiotic resistance traits, and 3 other chromosomes, ploidy, DNA fingerprinting and remarks, respectively.

Fig. 13.4 Unique tea germplasm accessions. (**a**) TRI 2043, (**b**) Zijuan, (**c**) Anji-Baicha

Table 13.2 Unique germplasm accessions released as cultivars

Cultivar	Special character(s)	Originator	References
TRI 2043	High anthocyanin pigmentation and pubescence density, exclusively for silver tips	TRISL, Sri Lanka	Gunasekare (2012)
TRI 9	Nonfermenting	TRISL, Sri Lanka	Ramaswamy (1960)
Zijuan	High anthocyanin content (0.5–1%), suitable for purple tea	TRIYASS, China	Jiang et al. (2013)
Sunrouge	High anthocyanin content, suitable for purple tea	NARO, Japan	Saito et al. (2011)
TRFK 306	High anthocyanin content, suitable for purple tea	Tea research foundation, Kenya	Kamunya et al. (2012)
Anji-Baicha	Albino plant	China	Shin et al. (2018)
Zhonghuang 2	Light sensitive, suitable for yellow tea	China	Wang et al. (2014)

13.3.3 Genetic Resource Conservation Approaches

13.3.3.1 Core Tea Collections

The core collection approach developed by Frankel (1984) can play an effective role in providing access to a wide range of tea genetic resources, bringing their desirable gene(s) into cultivated species to meet future challenges. This strategy was introduced with the intention of minimizing the cost of genetic conservation, while ensuring representation of maximum genetic variation; moreover, it allows rapid evaluation of germplasm and better access to the base collection.

Li and Jiang (2004) established a preliminary core collection of 615 tea accessions based on 10 traits, including place of origin, tree form and variety type data. Recently, another core collection for Chinese tea germplasm was established with 532 accessions selected to capture diversity (Wang et al. 2011). Around 2665 tea accessions conserved at the CNGTR, China were characterized using 33 passport data and phenotypic traits, including agronomical, cup tea quality and main chemical components, to establish the core collection. First, accessions were clustered using Ward's method and when selecting entries for core collection, approximately 20% of the accessions were randomly selected from each cluster; some germplasm with special traits, such as white young shoot and zigzag growth, were directly selected into the core collection. The core collection was validated comparing statistics of the 9 quantitative traits used.

Raina et al. (2012) developed a core collection of 105 accessions, 52 of which were selected by using AFLP genotype data and 53 by phenotypic data, using accessions and clones of Indian hybrid tea. A total of 1587 tea accessions (single plant selection from the wild of elite-looking single plants) and the 57 commercial clones (widely cultivated in tea plantations) conserved at the Tocklai Experimental Station

and UPASI Tea Research Institute, were used in the study. Two data sets were used to establish the core collection; AFLP marker data of 1644 accessions and phenotypic details for 26 traits (18 categorically and 7 quantitatively scored) for 698 accessions. The core collection was validated with generated AFLP data and the core collection captured 98% of the diversity.

Taniguchi (2014) evaluated a total of 788 accessions from 14 origins to reveal the genetic diversity of tea genetic resources and develop a worldwide core collection of tea. Out of the 7800 accessions at NIVTS, 788 were selected for SSR-marker genotyping, based on available passport data (including country of origin and collection site), the origin (wild, landrace, breeder's line), and previous knowledge about genetic diversity (Matsumoto et al. 2002; Takeda 2002), to capture as much variation as possible. Four core subsets of 192, 96, 48, and 24 accessions were selected. Core collections were validated using floral morphology and the chemical composition of first-flush young shoots.

Ranatunga (2016) developed the first core collection of tea germplasm in Sri Lanka with 64 accessions representing the diversity of the germplasm. Out of the 600 accessions conserved at TRISL, Sri Lanka, 93 were selected as the preliminary core collection based on breeding history and pedigree data (Ranatunga and Gunasekare 2008). The preliminary core germplasm was analyzed using 13 floral traits, 13 biochemical parameters and 6 SSR markers. The core collection developed using biochemical parameters and the preferred sampling method was found to be the best method for selecting entries for the final core collection. The final core collection consists of 24 estate selections, 18 introductions, 17 improved cultivars, 1 unknown accession and 4 nonbeverage type accessions.

13.3.3.2 Cryopreservation

Cryopreservation is a long-term preservation method for germplasm stock; several attempts have been made to establish protocols for this type of storage of tea tissue i.e. seeds, shoot tips, stems, roots, in vitro plants etc. Kuranki and Sakai (1995) standardized two cryopreservation techniques, namely vitrification and alginate-encapsulation dehydration, for tea shoot tips. Kuranuki (2006) confirmed that the vitrification method is suitable for preserving terminal buds and shoot tips which can be preserved using the alginate-encapsulation dehydration technique. Kim et al. (2002) demonstrated the possibility of using cotyledonary embryonic axes of tea seed for cryopreservation.

13.3.4 Cytogenetics

13.3.4.1 Karyotype

Cultivated tea plants are mostly diploids with the chromosome number $2n = 2 \times = 30$. As revealed by Kondo (1975), relatively little intraspecific karyotypic variations were observed for the cultivated *Camellia* species. The karyotype of most species were found to have M (metacentric) and SM (submetacentric) chromosomes. Only a few species were recorded with ST (subtelocentric) chromosomes and generally the number order was M>SM>ST (Mondal 2014).

13.3.4.2 Polyploids

Polyploids are generally found in cultivated *Camellia sinensis* and *C. assamica* and naturally-occurring polyploids have been reported (Bezbaruah 1971; Janaki Ammal 1952). Some natural triploids are used as popular cultivars in India (Sundaram, UPASI 3, UPASI 20, TV 29), Sri Lanka (HS 10A, GF 5/01) and Kenya (382/1) (Gunasekare and Ranatunga 2003).

13.4 Molecular Breeding

Conventional tea-breeding programs are complex due to self-incompatibility, and the woody and perennial nature of the crop. Hence, several attempts have been made to accommodate recent developments of molecular biology in tea crop improvement programs.

13.4.1 Genetic Linkage Maps

Establishment of a high-density genetic linkage is a prerequisite in accelerating marker-assisted selection (MAS) in tea plants (Wang et al. 2018c). Genetic linkage maps are essential for the mapping of genes and quantitative trait loci (QTLs), developing MAS methods, map-based cloning and crucial tools for the assembly of the genome sequence and comparative genomic analyses (Ma et al. 2015). Several genetic maps of the tea plant have been generated with various types of molecular markers during the past two decades. The early maps were constructed using randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR) and amplified fragment length polymorphisms (AFLP) markers. Later sequence-tagged site (STS), cleaved amplified polymorphism sequences (CAPS) and simple sequence repeats (SSR) were used. Recently, next-generation sequencing (NGS)

technologies have accelerated the process of large-scale single nucleotide polymorphism (SNP) discovery and SNPs are being used for high-density genetic map construction in the tea plant (Ma et al. 2015) due to its abundance, uniform genome distribution and cost-effectiveness (Ganal et al. 2009). A summary of genetic linkage maps constructed for tea plant is given in Table 13.3.

13.4.2 Marker-Assisted Selection (MAS)

13.4.2.1 MAS for Development of Biotic and Abiotic Resistant Cultivars

White peach scale, *Pseudaulacapsis pentagona* (Tarigoni) is a serious pest of tea in Japan (Tanaka 2012) and in 2012 a resistant cultivar Nanmei, which is the world's first tea cultivar developed by MAS, was released (Taniguchi et al. 2018). This tea cultivar was selected using the peach-scale resistant QTL *MSR-1,* a gene that confers resistant to the insect. The resistant locus was identified using the linkage map of the resistant cultivar Sayamakori, constructed using RAPD and SSR marker systems (Tanaka 2012). As illustrated in Fig. 13.5, *MSR-1* marker band can be observed in Sayamakori resistant parent to white peach scale (P1) and resistant cultivar Nanmei in lane 12.

Suganthi et al. (2014), identified three RAPD markers (OPW-03725, OPT-01625, OPG-11750) linked to tea-mosquito resistance. Tea mosquito (*Helopeltis theivora*) is a serious pest in tea causing crop loss. These markers were converted into codominant sequence-characterized amplified region (SCAR) markers (Rht1, Rht2, Rht3) and are now available for MAS for tea-mosquito resistance cultivars.

Work is continuing to identify markers for blister blight disease in Sri Lanka (Mewan et al. 2009) and *Anthracnose* in Japan (Tanaka 2012).

Koech et al. (2018) reported identification of 3 QTLs for percent of relative water content influencing drought stress traits of the tea plant using a consensus genetic map constructed using the DArTseq platform. Kamunya et al. (2010) identified 13 QTLs for drought tolerance using a map constructed with 250 RAPD primers, 96 AFLP primer combinations and 15 SSR primers pairs. These validated molecular markers will contribute greatly to adoption of MAS for drought tolerance in future tea-crop improvement.

13.4.2.2 MAS for Quality Improvement and Agronomic Traits

Jin et al. (2016) conducted a study to investigate the genetic relationship between the tea caffeine synthase 1 (*TCS1*) gene and the caffeine content of the tea plant and its related species, using the association mapping approach. They identified CAPS markers developed from sequence variations SNP4318 associated with caffeine content of tea plants and the markers can potentially be applied for future MAS to improve tea quality. Also, Koech et al. (2018) reported identification of 6 caffeine

Table 13.3 Genetic linkage maps constructed for tea

Marker type/s	Mapping population	Map details	References
RAPD + AFLP	90 F1 individuals	Total markers 126	Hackett et al. (2002)
	SFS150 and TN14/3	Total map length 1349.7 cM	
		Average inter-locus distance 11.7 cM	
RAPD + AFLP + SSR	42 F1 clonal progeny	Total markers 100	Kamunya et al. (2010)
	TRFCA SFS150 and AHP S15/10	Total map length 1411.5 cM	
		Average interlocus distance 14.7 cM	
RAPD + AFLP + SSR	79 F1 individuals	Total markers 678	Chang et al. (2017)
	Fushun and *Kemsull*	Total map length 1441.6 cM	
		Average interlocus distance 4.7 cM	
SSR + RAPD + CAPS	54 F1 clones	Total markers 441 SSRs, 7 CAPS, 2 STS and 674 RAPDs	Taniguchi et al. (2012)
	Sayamakaori and *Kana-Ck17*	Total map length 1218 cM	
		Average interlocus distance 4.35 cM	
SSR	183 F1 individuals	Total markers 406	Ma et al. (2014a)
	Yingshuang and *Beiyue Danzhu*	Total map length 1143.5 cM	
		Average interlocus distance 2.9 cM	
SSR	170 F1 individuals	Total markers 483	Tan et al. (2016)
	Longjing 43 and *Baihaozao*	Total map length 1226.2 cM	
		Average interlocus distance 2.5 cM	
SSR	148 F1 individuals	Total markers 190	Mewan et al. (2009)
	TRI 2043 and TRI 2023	Total map length 2543.3 cM	
		Average interlocus distance 12.8 cM	
		Total markers 146	
		Total map length 1018.1 cM	
		Average interlocus distance 7 cM	
SNP	148 F1 individuals	Total markers 6448	Ma et al. 2015
	Yingshuang and *Beiyue Danzhu*	Total map length 3965 cM	
		Average inter-locus distance 1.0 cM	
Diversity Arrays Technology (DArTseq)	261 F1 clonal progeny	Total markers 1421	Koech et al. (2018)
	TRFK St 504 and TRFK St 524	Total map length 1260.1 cM	
		Average interlocus distance 1.1 cM	

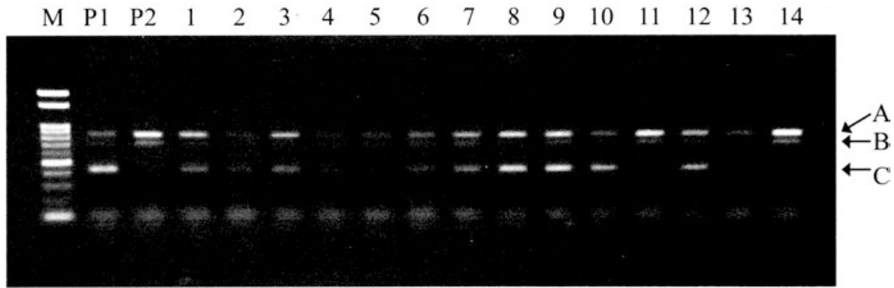

Fig. 13.5 DNA marker-assisted selection for the MSR 1 gene. **M** size standard, **P1** Sayamakaori, **P2** Makurazaki 13, (Lanes 1–14, F1 plants Nanmei in lane 12), **A** Positive, control band, **B** Nonspecific band, **C** MSR 1 marker band. (Source: Taniguchi et al. 2018)

QTLs, 25 catechins QTLs, 3 theaflavins QTLs and 9 QTLs for tea-taster scoring using the DArTseq platform.

The timing of spring bud flush (TBF) in tea is an important agronomic trait for tea grown in temperate regions and Tan et al. (2018) validated two QTLs which could be used in MAS for TBF.

13.4.3 Tea Genome

Sequencing of the tea genome uncovers molecular mechanisms underlying important desirable traits, i.e. quality, biotic and abiotic stress tolerance etc., thus contributing to developing better tea cultivars in the future. A high-quality genome assembly further facilitates genomic, transcriptomic and metabolomic analyses of these traits. Sequencing of the tea genome is a critical step toward enabling the use of modern molecular breeding methods in cultivar development, such as marker-assisted selection and genomic selection that would speed up tea breeding.

Xia et al. (2017) sequenced the first tea tree genome using the cultivar Yunkang 10 (*Camellia sinensis* var. *assamica*) collected from Menghai County, Yunnan Province, China. A whole-genome shotgun sequencing analysis with the Illumina next-generation sequencing platform (HiSeq 2000 sequencing system) was performed and the genome size estimated as 3.02-Gb. Xia et al. (2017) report identification of 24 characteristic and important metabolite-related genes encoding enzymes involved in the biosynthesis of catechins, theanine and caffeine in cultivated tea. These novel findings are important in further metabolomic and functional genomic refinement of characteristic biosynthesis pathways which may useful in developing diverse tea flavors with enhanced quality.

A high-quality draft genome assembly of cultivar Shuchazao (*Camellia sinensis* var. *sinensis*) was reported by Wei et al. (2018) using both Illumina and PacBio

sequencing technologies. The genome size is 3.1 Gb and 64% consists of repetitive sequences and rest yields 33,932 high-confidence predictions of encoded proteins. The genome sequence can serve as a vital resource for studying the genetic bases of major plant metabolic pathways and for germplasm utilization to breed improved tea cultivars.

Therefore, tea can be categorized as a large-genome species and diploid genome consisting of 15 chromosome pairs (2n = 30) (Wang et al. 2018c). This information could be immensely useful in future tea improvement activities.

13.4.4 Functional Genomics Studies

Functional genomics facilitates identification of gene functions and the interactions between genes in regulatory networks related to desirable traits (Akpinar et al. 2013) and they can be exploited in crop improvement programs. Initial attempts on identification of functional genes of tea plant were based on gene cloning and the homologous cloning approach (Wang et al. 2018c). Takeuchi et al. (1994) reported the first isolation of three full length cDNA encoding chalcone synthase (CHS, EC 2.3.1.74) followed by b-primerverosidase gene (Mizutani et al. 2002), tubulin encoding gene (*Tua1*) (Fang et al. 2006), w-3 fatty acid desaturase genes (Ma et al. 2014b) and anthocyanadin reductase gene (*CsANR*) (Thirugnanasambantham et al. 2014), using the same technique.

Later, sequencing technology medium and high throughput technologies were used in identification and cloning of functional genes. Thereafter, many genes related to the beverage quality, yield and stress have been cloned and information summarized by Mukhopadhyay et al. (2015). The tea leaf contains a large number of biochemicals and catechins; theanine and caffeine are important for beverage quality. Genes encoding key enzymes of the catechin biosynthetic pathway have been cloned. Information on phenylalanine ammonia lyase (PAL) (Singh et al. 2009), chalcone synthase, flavanone 3-hydroxylase, anthocyanidin synthase (Rani et al. 2012) and flavonol synthase (SuXia et al. 2009) genes are available to utilize in tea crop improvements. Caffeine is the most abundant alkaloid responsible for briskness of tea; caffeine biosynthetic pathways are controlled by S-adenosylmethionine (SAM), synthase, caffeine synthase and 7-N-methyltransferase genes. These genes have been isolated and characterized by Kato et al. (2000).

Also, functional genomics studies are useful in understanding molecular mechanisms of desirable traits of the tea plant such as dormancy of apical buds (Krishnaraj et al. 2011), response to low temperature (Wang et al. 2009), response to drought (Das et al. 2012a) and cold acclimatization (Ban et al. 2017; Yue et al. 2015).

13.4.4.1 Transcriptomics

The advent of high-throughput sequencing technologies facilitates understanding of complex traits and gene discovery in plant species, especially when full genome information is unavailable. This is a robust approach to understanding global transcript changes under a biotic or abiotic stress, cell type, tissue or developmental stage of a plant. Expressed sequence tags (ESTs) from protein-coding mRNA sequences are generated for gene discovery. The use of next-generation sequencing (NGS) technologies and gene discovery RNA sequencing (RNA-seq) have become a rapid and cost-effective approach, and provide very high throughput by generating millions of reads in a single sequencing run (George et al. 2012).

Several attempts have been made to illustrate complex biological traits in tea using the omics approach and useful results have been generated as discussed below (Table 13.4). These studies focused on identification and characterization of genes and differentially-expressed genes (DEGs) related to growth and development, biotic and abiotic stress factors and major biosynthetic pathways of tea plant.

13.5 Genetic Engineering

Genetic engineering could be an effective biotechnological tool to overcome limitations in conventional tea breeding. Transgenic technologies offer several advantages for perennial crops like tea. Development of an effective transformation system is crucial in any transgenic work.

Two different transformation methods were attempted for genetic transformation of tea; (a) using explants in cocultivation with *Agrobacterium* Ti/Ri plasmid-based binary/co-integrate vectors and (b) particle gun bombardment (biolistics) (Mukhopadhyay et al. 2015). Several attempts were made using strains of *A. tumefaciens* by Matsumoto and Fukui (1998) and Mondal et al. (1999, 2001b). Zehara et al. (1996), Konwar et al. (1998) and John et al. (2009) attempted induction of hairy roots in tea using *A. rhizogenes*. Attempts were made to standardize the biolistics mediated transformation protocol for tea by Akula and Akula (1999) and Wu et al. (2003).

Mondal et al. (2001b) produced the first transgenic tea plants from somatic embryos transformed by disarmed *A. tumefaciens* strains, EHA105 and LBA4404, both carrying a binary plasmid construct p35SGUSINT with *nptII* gene and gus-intron. They confirmed the stable integration of transgene by the Southern hybridization technique and reported 80–90% survival of transgenic plants in the greenhouse.

Sandal et al. (2007) reported production of transgenic tea plants from in vitro leaf explants. Tender leaves were bombarded with plasmid DNA containing *gus* and *nptll* genes. However, they reported that the survival rate of the transgenic seedlings was poor.

Table 13.4 Progress of transcriptomics research in tea

Trait/function	Major findings and related genes	References
Dormancy of bud	30 genes involved in gibberellin (GA) and abscisic acid (ABA) metabolism and signaling play crucial roles in bud activity-dormancy transition	Yue et al. (2017)
Dormancy of bud	16,125 DEGs identified	Hao et al. (2017)
Plant development and responses to stresses	8 genes encoding germins and germin-like proteins (GLPs) – *CsGLP1–8*	Fua et al. (2018)
	CsGLP2 increased at bud sprouting stage-potential marker for bud germination	
	CsGLP1 and *CsGLP2* induced by geometrid feeding, indicating may be involved in insect resistance	
Thermotolerance	Calcium-dependent protein kinases (CDPKs) regulate plant growth, development, and stress response.	Wang et al. (2018a)
	CsCDPK20 and *CsCDPK26* may act as positive regulator in response to heat stress	
Metal stresses	PP2AA3 and 18S rRNA genes stably express for heavy metal exposure	Wang et al. (2017)
Water-deficit stress	Heat shock proteins (HSP70) and superoxide dismutase (SOD) – highly expressed in drought tolerant cultivars	Tony et al. (2016)
L-Theanine	*CsTS2*, *CsGS1* and *CsGDH2* genes – positive correlation with the theanine contents	Liu et al. (2017c)
Catechin synthesis and accumulation	Chalcone synthase 1(CHS1) – important in catechin (C) biosynthesis than CHS2 and CHS3	Zhang et al. (2016)
	Anthocyanidin reductase (ANR) – regulate the conversion of C	
	ANRs and leucoanthocyanidin reductase –biosynthesis of EGCG and ECG	
Catechin metabolic pathways	*CsCHIc*, *CsF3'H* and *CsANRb* expression levels are associated with the concentration of soluble proanthocyanidin (PA)	Wang et al. (2018b)
	Expression levels of CsPALc and *CsPALf* with the concentration of insoluble PA	
Anthocyanin and proanthocyanidin biosynthesis	*CsMYB5a* reduce the accumulation of anthocyanin and promote the accumulation of PAs	Jiang et al. (2018)
	Genes related to PA and anthocyanin biosynthesis pathways markedly up regulated	
	3 UGTs and 4 GSTs involved in anthocyanin glycosylation and transportation	
Flavonoid accumulation	A *R2R3-MYB* transcription factor (*CsMYB6A*) and a novel UGT gene (CsUGT72AM1) –highly expressed in purple tea leaves	He et al. (2018)
	CsMYB6A activate the expression of flavonoid-related structural genes, especially CHS and 3GT	
	CsUGT72AM1 has catalytic activity as a flavonol 3-O-glucosyltransferase, and displayed broad substrate specificity	

(continued)

Table 13.4 (continued)

Trait/function	Major findings and related genes	References
Catechin production	1196 (DEGs) affect *chloroplast organization* and *response to high light*	Liu et al. (2017b)
	Flavonol synthase and flavanone/flavonol hydroxylases – positively correlate with quercetin accumulation	
	Leucoanthocyanidin reductase, anthocyanidin reductase and synthase – negatively correlate leading to catechin biosynthesis	
Flavonoid and carotenoid	207 DEGs – 110 up-regulated and 97 downregulated	Song et al. (2017)
	DEGs – involve in protein and ion binding and oxidoreductase activity	
	Shading inhibited the expression of flavonoid biosynthesis-associated genes and induced carotenoid biosynthesis-related genes	
Anthocyanin biosynthesis	Activation of the R2R3- MYB transcription factor (TF) anthocyanin1 (*CsAN1*) specifically upregulated the *bHLH TF CsGL3* and anthocyanin late biosynthetic genes (LBGs) to confer ectopic accumulation of pigment in purple tea	Sun et al. (2016)
	CsAN1 interacts with bHLH TFs (*CsGL3* and *CsEGL3*) and recruits a WD-repeat protein CsTTG1 to form the MYB-bHLH-WDR (MBW) complex that regulates anthocyanin accumulation.	
	The hypomethylation of a CpG island in the CsAN1 promoter is associated with the purple phenotype.	
Temporal aroma accumulation	*CsOCS2* – a key gene involved in terpenoid biosynthesis	Xu et al. (2018)
Anthracnose-resistant	Genes encoding a MADSbox transcription factor, NBS-LRR disease-resistance protein, and phenylpropanoid metabolism pathway components (CAD, CCR, POD, beta-glucosidase, ALDH and PAL) were among those differentially expressed in ZC108	Wang et al. (2016)
Blister blight (BB)	Differentially expressed, 149 defense related transcripts/ genes, defense related enzymes, resistance genes, multidrug resistant transporters, transcription factors, retrotransposons, metacaspases and chaperons were observed in RG, suggesting their role in defending against BB	Jayaswall et al. (2016)
	RPM1, RPS2 and RPP13 in quantitative RT PCR indicate salicylic acid and jasmonic acid – induce synthesis of antimicrobial compounds, required to overcome the virulence of *Exobasidium vexans*	
Flower development	207 flowering-associated unigenes identified	Liu et al. (2017a)
	Transcription factors, such as WRKY, ERF, bHLH, MYB and MADS-box showing upregulated in floral transition, which might play the role of progression of flowering	

Although transgenic research in tea began in the mid 1990s, slow progress has been observed due to (a) difficult transgenic protocol, (b) low competence for transformation and regeneration in tea and (c) lack of funding for transgenic research at the public tea research institutes. Despite these constrains, the commercialization of transgenic tea is hindered by poor public acceptance to genetic modification technology among consuming countries. The primary focus of resistance to GM foods and crops among the general public is on human and environmental safety, labeling and consumer choice, intellectual property rights, ethics, food security and poverty reduction (Bawa and Anilakumar 2013). Tea is being promoted as a natural beverage and currently increasing demand can be seen for GMO-free tea in many countries. Hence, tea breeders are facing enormous challenges with any move forward with the transgenic technology. To date, no transgenic tea plants have been developed for commercial cultivation (Mukhopadhya and Mondal 2018).

13.6 Mutation Breeding

13.6.1 Mutation Induction

Induced mutations are useful in improving crops with economically-important traits such as high yield, and biotic and abiotic stress resistance. In tea, mutation induction work began in India (1967–1968) at the Tocklai Experimental Station (Mondal 2014). Tea seeds and cuttings were treated with different concentrations of X-ray and gamma radiation but little success was achieved. Later, irradiation techniques for induction of desirable mutations using γ-rays were standardized (Ann Sci Rpt 1968–1970, 1974–1975, 1979–1980). It has been reported that the maximum dosage of γ-rays of 2 Kr resulted in a higher survival rate of stem cuttings of tea and mutation induction. Studies also showed significant differential response of tea cultivars to the dose of γ-radiation. Tocklai cultivars TV1 and TV2 reported tolerance for higher dosages beyond 2 Kr; the lowest response was observed in TV 23 cultivar (Das et al. 2012b).

In China, a new national tea variety Zhongha 108 was bred by the Tea Research Institute of Hunan Province, using Co60 γ-rays as a radiation source (Yang et al. 2003). This variety has selected from irradiated cuttings of Longjing 43 and characteristics of early sprouting, high cup quality, and high resistant to pest and diseases (Yao and Chen 2012). Another national variety Wannong 111 and two provincial varieties Fufeng and Dongtingchun were also released in China in 2002, 1997 and 2010, respectively. All were bred by irradiating with Co60 γ-rays as the radiation source (Yao and Chen 2012).

Preliminary work on mutation breeding using ionizing radiation in Sri Lanka was conducted and the LD50 value has been determined for nodal cuttings of tea cultivar TRI 2025 (Sarathchandra and Pieris 2001).

Reports on mutation induction using chemicals are limited; some preliminary attempts have been made in India (Das et al. 2012b) and China (Yao and Chen 2012). Colchicine and ethyl methylsulfonate could be used as chemical mutagens; however, no success has been reported (Yao and Chen 2012).

13.6.2 Albino Tea Plants

Albino tea plants are mutants that produce white young leaves owing to the lack of chlorophyll under certain environmental conditions (Du et al. 2006). According to Wang et al. (2015), two types of albino tea plants are available: temperature sensitive and light-sensitive. In China, mostly temperature regulated albino tea cultivars are grown and they produce white shoots due to the reduction of chlorophyll biosynthesis during early spring, when air temperatures are below 20–22 °C (Du et al. 2006). However, when the air temperature exceeds 22 °C during the summer and autumn, chlorophyll biosynthesis resumes and the leaves turn green. As explained by Du et al. (2006) light-sensitive albino tea plants produce white shoots during the summer and autumn under intensive sun light. Some popular temperature-regulated cultivars in China are Xiaoxueya and Baiye-1 (former White leaf No. 1) and light-sensitive albino tea cultivars Yujinxiang, Huang-2, Huang-8 and Huang-13 (Shin et al. 2018). Tea cultivar Huangjinya is an albino field clone released in 2008 in Zhejiang Province (Yao and Chen 2012) and Anji Baihca is the most famous and widely grown albino tea cultivar in China (Wei et al. 2012).

Albino tea cultivars are characterized by high amino acid content that leads to the umami (savory) taste and fragrant aroma of green tea (Li et al. 2016). Albino tea germplasm is unique due to their special flavor and white leaf color. Tea produced from albino shoots have a characteristic brisk taste and fragment aroma which fetches higher market prices than green teas.

13.7 Hybridization

13.7.1 Conventional Hybridization

Tea plants show a high degree of self-sterility and the mechanism of self-incompatibility remains a challenge for crop improvement. Two selected parents are crossed and the F1 progeny will be screened and evaluated for development of new tea cultivars in subsequent stages. However, controlled hybridization is the predominant method of crop improvement in all tea-growing countries. In China, 22.2% of registered cultivars in 2002 were bred using controlled hybridization (Chen et al. 2007). Out of the 64 tea cultivars recommended in Sri Lanka, 24 (37.5%) were developed from controlled hybridization (Gunasekare 2012). In

Japan, out of the 10 major varieties, 3 were bred using controlled hybridization. Also, 52% of the registered tea cultivars in Japan were developed through hybridization (Tanaka 2012). In Vietnam, new promising tea cultivars LDP1, LDP2, PH8 and PH9 were developed by controlled hybridization (Ngoc 2012). The Bangladesh Tea Research Institute has released 17 cultivars and 6 of them were developed by controlled hybridization (Khan 2012). Sriyadi et al. (2012) reported that all 11 GMB (Gambung) series cultivars released in Indonesia were generated from controlled hybridization of first-generation cultivars.

The progress of conventional hybridization of tea cultivars is hindered by low success rate, seasonal flowering and short availability (2–3 months), longer seed maturation time (8–12 months) and variability of flowering time (Mondal 2014).

13.7.2 Somatic Cell Hybridization

To overcome problems of conventional hybridization and facilitate distant hybridization between different *Camellia* spp., cell culture techniques have tremendous potential. Protoplast culture and somatic cell hybridization (or cybridization) techniques are useful in the improvement of tea. Somatic cell hybrids are formed through fusion of different somatic cells of the same or different species. In tea, somatic hybridization can be used to incorporate agronomically-important biotic and abiotic stress tolerance traits of wild relatives into cultivars (Mondal 2014).

Several attempts have been made to develop necessary protocols for somatic hybridization *via* protoplast fusion. Bagratishvili et al. (1979) established cell suspension culture protocols and Nakamura (1983) developed protocols for isolation of protoplasts from tea leaves and flower petals. Later Kuboi et al. (1991) obtained 87% *via*ble protoplast from young leaves of the Yabukita tea cultivar. Gunasekare and Evans (1998) optimized a protocol for obtaining adequate yield of *via*ble protoplasts from leaf mesophyll cells. Balasubramanian et al. (2000a) evaluated different explants and reported that *in vitro* leaves are the best explants for a higher yield of viable protoplasts. However, it is difficult to regenerate plants from protoplast (Balasubramanian et al. 2000a). Hence, it is necessary to remove bottlenecks in protoplast isolation and regeneration for producing somatic hybrids.

13.8 Conclusions and Prospects

13.8.1 An Overview of Current Status

Conventional tea-breeding programs continue in all tea-growing countries focusing on further improvements of breeding and selection activities. Few successful attempts have been made to overcome limitations in the conventional programs,

accommodating novel biotechnological tools, induced mutations and polyploidy breeding for cultivar development during recent decades.

Considerable progress has been made on tea germplasm conservation and characterization over past decades that increase their utilization in crop improvement programs. Attempts have been made to accommodate novel techniques to resolve taxonomic confusion in the genus. Also, new information has been generated on origin and domestication events of the tea plant which is useful in effective conservation efforts.

Significant advances in molecular-marker development and utilization in tea have been reported. Several genetic linkage maps have been constructed and are successful in identification of a few closely-linked markers for MAS in tea breeding and selection. The progress of functional genomics and omics research in tea has been very limited during the last two decades. The first whole genome sequence of the tea plant (cultivar Yunkang 10) was reported and the information on genome assembly and gene annotation is available. The genome provides the foundation for revealing the genetic basis of important traits and accelerates future functional genomics research in tea.

The protocols for genetic transformation in tea have been standardized; however, not much progress can be observed in transgenic research in tea compared to other crops due to difficult protocols and limited funding availability. Hence, it is necessary to focus on this area in the future.

13.8.2 Current Research Initiatives to Combat Global Climate Change

As tea is grown under a rainfed monocultural cropping system, its ecosystem is vulnerable to global climate change. Rising temperatures and elevated CO_2 levels, extreme weather events i.e. drought, heavy precipitation and frost may adversely affect the growth and development and quality of tea. Hence, it is necessary to focus on developing management strategies to combat climate change and to promote sustainable development in the tea industry.

Thus, breeders have been entrusted with developing new resistant tea cultivars with high tolerance to heat, drought and cold stress. It is anticipated that, occurrences of pest and diseases may increase with the climate change and cultivars with multiple resistance will be preferred. Also, in response to elevated CO_2 levels, development of cultivars with high net photosynthesis rate and high nutrient use efficiency would be useful in the future. Tea researchers in temperate regions have been developing new cultivars with early sprouting ability which may be preferred.

Being a monoculture, the agrobiodiversity within the tea ecosystem is narrow. Hence, it is necessary to create more diverse tea ecosystems with shade trees and integrated farming approaches which could be useful in maximizing adaptation to climate change. Furthermore, in many countries, excessive cultivation of few

selected vegetatively-propagated cultivars further reduces the agrobiodiversity in the ecosystem. Hence, introduction of diverse tea cultivars and improved tea seedlings with high vigor is immensely desirable.

13.8.3 Recommendations of Future Research and Utilization

There are number of tea germplasm collections available in different countries; however, there has not yet been an effort to assess the security of conservation as well as promote exchanges of germplasm. Thus, it is necessary to develop a global strategy for the long-term conservation of tea genetic resources to allow researchers and users to identify key vulnerabilities, future opportunities, and a framework for action to ensure the secure conservation and use. Development of public databases and data-sharing systems within the tea research community would facilitate exploitation of the full potential of global genetic resources in developing elite tea cultivars to cater to multistakeholder needs. Furthermore, it is important to develop formal mechanisms of germplasm exchange among researchers to utilize genetic resources to develop new tea cultivars to face emerging challenges due to the global climate change.

The first genome sequence of tea in 2017 represents a milestone in genome research in tea. With the fast development of sequencing technologies (i.e. third generation sequencing) this sequence could be further upgraded to a high quality reference sequence. However, to accelerate tea plant genome research, it is necessary to link tea genomic research centers worldwide and for them to work together as was done in the human genome project. Also, it is important to maintain a tea genome data repository with easy access.

Many genetic linkage maps have been established; however, utilization of them in MAS is limited due to the unavailability of tightly-linked markers. Hence, development of saturated maps would be useful in the future. Also, it is worth exploring possibilities for alternative approaches like association mapping. The tea industry is yet to benefit from the developments of transgenic technologies. Thus, intensive research with adequate funding in this line of research is recommended.

Appendices

Appendix I: Research Institutes Relevant to Tea

Institution	Specialization and research activities	Contact information and website
The Tea Research Institute of the Chinese Academy of Agricultural Science (TRICASS)	Basic science, technological innovation, market analysis	9 South Meiling Road, Hangzhou 310008, China
		Tel: +86-571-86650444; Fax:+86-571-86650056
		Website: www.tricaas.com
The Tocklai Experimental Station (TES) of the Tea Research Association (TRA)	All technological aspects of from tea cultivation to processing especially for North Indian tea plantations	Tea Research Association, P.O. Jorhat-785008, Assam
		Tel:+91-0376-2360973/974; Fax: +91-0376-2360474
		Website: www.tocklai.org
The United Planters' Association of Southern India (UPASI)	All technological aspects of from tea cultivation to processing especially for South Indian tea plantations	The United Planters' Association of Southern India
		Glenview, Coonoor – 643 101, Nilgiris, Tamilnadu, India
		Tel: (0423) 2230270 or 2232030
		Website:www.upasi.org
The Institute of Himalayan Bioresource Technology – Council of Scientific and Industrial Research (CSIR-IHBT)	Molecular biology, biotechnology, genomic research	Post Box No. 6 Palampur (H.P.) 176061, India
		Tel: +91-1894-230411; Fax: +91-1894-230433
		Website: www.ihbt.res.in
The Institute of Fruit Tree and Tea Science, NARO, Japan	All technological aspects of from tea cultivation to processing	NIFTS Tsukuba Headquarter, 2-1 Fujimoto, Tsukuba, Ibaraki 305-8605, Japan
		Tel: +81-29-838-6416; Fax: +81-29-838-6437
		Website: www.naro.affrc.go.jp
The Tea Research Institute of Sri Lanka	All technological aspects of from tea cultivation to processing	Tea Research Institute of Sri Lanka, Talawakelle 22100, Sri Lanka
		Tel: +94522258201; Fax: +94522258229
		Website: www.tri.lk

(continued)

Institution	Specialization and research activities	Contact information and website
The Tea Research Institute (former Tea Research Foundation of Kenya)	All technological aspects of from tea cultivation to processing	PO Box 820, Kericho 20200, Kenya
		Tel: +254-52-20598, 20599, +254-722-209915
		Website:www.kalro.org/tea
Tea Research Institute of the Yunnan Agricultural Sciences	All technological aspects of from tea cultivation to processing	Yenhai County, Xishuangbanna, Yunnan Province, China
		Tel: 0691 5170139
		http://www.yntri.com.cn
The Centre for Tea Science and Development of the Northern Mountainous Agriculture and Forestry Research Institute	All technological aspects of from tea cultivation to processing	Phu Ho – Phu Tho town – Phu Tho province – Vietnam
		Tel: +84 210 3865 073; Fax: +84 210 3865 073
		Website: www.nomafsi.com.vn
Mokpo Experiment Station, National Institute of Crop Science, Rural Development Administration	All technological aspects of from tea cultivation to processing	181, Hyeoksin-ro, Iseo-myeon, Wanju-Gun, Jeollabuk-do 55365, South Korea
		Tel: +82-63-238-5000; Fax: +82-63-238-5191
		Website: www.nics.go.kr/english/
Bangladesh Tea Research Institute	All technological aspects of from tea cultivation to processing	Bangladesh Tea Research Institute, Srimangal-3210, Moulvibazar
		Tel: +880862671225; Fax: +880862671930
		Web: www.btri.gov.bd
Indonesia Research Institute for Tea and Cinchona	All technological aspects of from tea cultivation to processing	PO Box 1013, Bandung, Gambung 40010, Jawa Barat
		Tel: +6222 5928185, 5928782; Fax:+62 22 5928186
		Website: www.bdg.centrin.net.id/gambung
Cocoa Research Institute of Nigeria	All technological aspects of from tea cultivation to processing	Ijebu Ode Road, Idi-Ayunre, Oluyole, Oyo, Nigeria
		Tel: +2348078467448
		Website: www.crin-ng.org

(continued)

Appendix II: Genetic Resources of Tea Sorted by Cultivation Location and Cultivar Name

Cultivation location	Cultivar	Important traits
Bangladesh	BT 18	Black tea, above average quality, drought tolerant
	BT 1	Black tea, above average quality
	BT 10	Black tea, average quality
	BT 11	Black tea, above average quality, drought tolerant
	BT 12	Black tea, above average quality, high drought tolerant
	BT 13	Black tea, above average quality, drought tolerant
	BT 14	Black tea, above average quality, high drought tolerant
	BT 15	Black tea, excellent quality, drought tolerant
	BT 16	Black tea, average quality
	BT 17	Black tea, above average quality, drought tolerant
	BT 2	Black tea, above average quality, flavor
	BT 3	Black tea, above average quality
	BT 4	Black tea, excellent quality, high drought tolerant
	BT 5	Black tea, above average quality
	BT 6	Black tea, above average quality, high drought tolerant
	BT 7	Black tea, above average quality, high drought tolerant
	BT 8	Black tea, above average quality, high drought tolerant
	BT 9	Black tea, above average quality
China (Anhui)	Anhui 1	Green tea
	Anhui 3	Green tea
	Anhui 7	Green tea
	Fuzao 2	Green tea
	Huangshang Zhong	Landrace
	Qimenzhong	Landrace
	Shifocui	Field clone
	Shuchazao	Field clone
	Wancha 91	Field clone
	Wannong 111	Mutated
	Wannong 95	Green & black tea
	Yangshuling 783	Field clone
China (Chongqing)	Nanjiang 1	Green & black tea
	Nanjiang 2	Green & black tea
	Shuyong 1	Green & black tea
	Shuyong 2	Green tea
	Shuyong 3	Black tea
	Shuyong 307	Black tea
	Shuyong 401	Black tea

(continued)

Cultivation location	Cultivar	Important traits
	Shuyong 703	Black tea
	Shuyong 808	Black tea
	Shuyong 906	Black tea
	Yinghong 1	Black tea
	Zaobaijian 5	Black tea
China (Fujian)	Baxiancha	Field clone
	Benshan	Oolong tea
	Chunlan	Field clone
	Dangui	Field clone
	Daye Wulong	Green tea
	Fu'an Dabaicha	White tea
	Fuding Dabaicha	Green tea, high quality, high aroma
	Fuding Dahaocha	Brown blight resistant
	Fujian Shuixian	Oolong tea
	Fuyun 10	Green tea
	Fuyun 6	Green & black tea
	Fuyun 7	Green tea
	Huangdan	Oolong tea
	Huangmeigui	Oolong tea
	Huangqi	Selected from open pollinated progeny
	Jinmudan	Selected from controlled pollinated progeny
	Maoxie	Oolong tea
	Meizhan	Oolong tea
	Mingke 1	Selected from controlled pollinated progeny
	Mingke 2	Selected from controlled pollinated progeny
	Ruixiang	Field clone
	Tieguanyin	Oolong tea
	Xiapu Chunbolu	Field clone
	Yuemingxiang	Field clone
	Zhenghe Dabaicha	White tea, black tea
	Zimudan	Field clone
China (Guagnxi)	Guihong 3	Black tea
	Guihong 4	Black tea
China (Guangdong)	Baimao 2	Field clone
	Fenghuang Shuixian	Oolong tea
	Hongyan 1	Field clone
	Hongyan 12	Field clone
	Hongyan 7	Field clone
	Hongyan 9	Field clone

(continued)

Cultivation location	Cultivar	Important traits
	Lechang Baimaicha	Landrace
	Lingtou Dancong	Field clone
	Wulinghong	Field clone
	Xiuhong	Black tea
	Yunda Danlv	Field clone
China (Guangxi)	Guilv 1	Green tea
	Guixiang 18	Field clone
	Lingyun Baimaocha	Landrace
	Yaoshan Xiulu	Field clone
	Meitan Taicha	Landrace
	Qianmei 419	Black tea
	Qianmei 502	Black tea
	Qianmei 601	Green tea
	Qianmei 701	Green tea
China (Henan)	Hainan Daye	Landrace
China (Hainan)	Xingyang 10	Field clone
China (Hubei)	E'cha 1	Green tea
	E'cha 5	Field clone
	Yichang Dayecha	Landrace
	Yihongzao	Green tea
China (Hunan)	Baihaozao	Green tea
	Gaoyaqi	Green & black tea
	Jiebohuang 13	Green & black tea
	Yulu	Selected from controlled pollinated progeny
	Yuntaishanzhong	Landrace
	Zhuyeqi	Suitable for machine harvesting
	Zhuyeqi 12	Green & black tea
China (Jiangsu)	Xicha 11	Green tea
	Xicha 5	Green tea
	Yixingzhong	Landrace
	Damianbai	Green tea
	Gancha 2	Selected from open-pollinated progeny
	Ningzhou 2	Green & black tea
	Ningzhouzhong	Landrace
	Shangmeizhou Zhong	Green tea
China (Shanxi)	Ziyangzhong	Landrace
China (Sichuan)	Mingshan Baihao	Field clone
	Zaobaijie	Landrace
China (Yunnan)	Fengqing Dayecha	Black tea

(continued)

Cultivation location	Cultivar	Important traits
	Menghai Dayecha	Landrace
	Mengku Dayecha	Landrace
	Yunkang 10	Black tea, blister blight resistant
	Yunkang 14	Green, pu'er & black tea
China (Zhejiang)	Biyun	Green tea
	Chunyu 1	Field clone
	Chunyu 2	Field clone
	Cuifeng	Green tea
	Hanlv	Green tea
	Jingfeng	Black & green tea
	Jiukengzhong	Green tea
	Juhuachun	Selected from open pollinated progeny
	Longjing 43	Green tea, brown blight resistant
	Longjing Changye	Green tea
	Maolu	Field clone
	Qingfeng	Field clone
	Yingshuang	Green tea
	Zhenong 113	Green tea
	Zhenong 117	Green tea
	Zhenong 139	Green tea
	Zhenong 21	Field clone
	Zhenong12	Green tea
	Zhongcha 102	Green tea
	Zhongcha 108	Green tea, high quality, pest & disease resistant
	Zhongcha 302	Green tea
Indonesia	GMB 1	Black tea, high yield, blister blight resistant
	GMB 10	Black tea, high yield, blister blight resistant
	GMB 11	Black tea, high yield, blister blight resistant
	GMB 2	Black tea, high yield, blister blight resistant
	GMB 3	Black tea, high yield, blister blight resistant
	GMB 4	Black tea, high yield, blister blight resistant
	GMB 5	Black tea, high yield, blister blight resistant
	GMB 6	Black tea, high yield, blister blight resistant
	GMB 7	Black tea, high yield, blister blight resistant, drought resistant
	GMB 8	Black tea, high yield, blister blight resistant
	GMB 9	Black tea, high yield, blister blight resistant, high quality
Japan	Akane	Black tea
	Asagiri	Green tea
	Asahi	Powdered Green tea. high quality
	Asatsuyu	Green tea, High quality
	Benifuki	Black tea

(continued)

Cultivation location	Cultivar	Important traits
	Benihikari	Black tea
	Benihomrare	Black tea
	Benikaori	Black tea
	Benitachiwase	Black tea
	Binifuji	Black tea
	Fukumidori	Green tea
	Fushun	Green tea
	Harumidori	Green tea
	Harumoegi	Green tea
	Hatsumidori	Green tea
	Hatsumomiji	Black tea
	Himemidori	Green tea
	Hokumei	Green tea
	Indo	Black tea
	Izumi	Pan fried green tea
	Kanayamidori	Green tea, Unique aroma, high yield
	Koyanishi	Green tea
	Kyomidori	Powdered green tea
	Makinoharawase	Green tea
	Meiryoku	Green tea, High yield
	Meiryoku	Green tea
	Minamisayaka	Green tea
	Minekaori	Pan fried green tea
	Miyamakori	Green tea
	Miyoshi	Green tea
	Musashikaori	Green tea
	Nanmei	Green tea, peach scale & grey blight resistant, early budding, high quality
	Natsumidori	Black tea
	Okumidori	Green tea, high quality
	Okumusashi	Green tea
	Okuyutaka	Green tea
	Rokurou	Green tea
	Ryofu	Green tea
	Saemidori	Green tea, high quality, early budding and harvesting
	Sainomidori	Green tea
	Sakimidori	Green tea
	Samidori	Powdered green tea, high quality
	Satsumabeni	Black tea
	Sayamakaori	Green tea, high yield, peach scale resistant
	Sayamamidiri	Green tea
	Seimei	Green tea, early budding, cold resistant, high quality, processing of Kabuse-cha, Matcha, powdered tea

(continued)

Cultivation location	Cultivar	Important traits
	Shunmei	Green tea
	Sofu	Green tea
	Takachiho	Pan fried green tea
	Tamamidori	Pan fried green tea
	Toyoka	Green tea
	Unkai	Pan fried green tea
	Yabukita	Green tea, high quality, mid yield
	Yaeho	Green tea
	Yamanami	Pan fried green tea
	Yamatomidori	Green tea
	Yumekaori	Green tea
	Yumewakab	Green tea
	Yutakamidori	Green tea, high yield, early budding & harvesting
Kenya	TRFK 100/5	Black tea, high yield, pest & disease tolerance
	TRFK 108/82	Black tea, high yield
	TRFK 11/4	Black tea, fast fermenting, good nursery rooting
	TRFK 12/12	Black tea, fast fermenting, good nursery rooting
	TRFK 12/19	Black tea, fast fermenting, good nursery rooting
	TRFK 301/4	Black tea, high yield
	TRFK 301/5	Black tea, high yield, pest & disease resistant
	TRFK 303/1199	Black tea, high yield, high quality
	TRFK 303/152	Black tea, high yield, pest & disease resistant
	TRFK 303/156	Black tea, high yield, pest & disease resistant
	TRFK 303/178	Black tea, high yield, pest & disease resistant
	TRFK 303/179	Black tea, high yield
	TRFK 303/186	Black tea, high yield
	TRFK 303/199	Black tea, high yield, pest & disease resistant
	TRFK 303/216	Black tea, high yield
	TRFK 303/231	Black tea, high yield
	TRFK 303/259	Black tea, high yield
	TRFK 303/348	Black tea, high yield
	TRFK 303/35	Black tea, high yield, pest & disease resistant
	TRFK 303/352	Black tea, high yield, pest & disease resistant
	TRFK 303/366	Black tea, high yield
	TRFK 303/388	Black tea, high yield
	TRFK 303/577	Black tea, high yield, high quality
	TRFK 303/745	Black tea, high yield
	TRFK 303/791	Black tea, high yield
	TRFK 303/978	Black tea, high yield
	TRFK 303/999	Black tea, high yield, high yield
	TRFK 306	Purple tea, pest & disease resistant
	TRFK 31/11	Black tea, high quality
	TRFK 31/27	Black tea, high yield

(continued)

Cultivation location	Cultivar	Important traits
	TRFK 31/28	Black tea, high yield
	TRFK 31/29	Black tea, high yield
	TRFK 31/8	Black tea, fast fermenting, good nursery rooting
	TRFK 337/138	Black tea, high yield
	TRFK 337/3	Black tea, high yield, pest & disease resistant
	TRFK 338/13	Black tea, high yield
	TRFK 347/26	Black tea, high yield, pest & disease resistant
	TRFK 347/336	Black tea, high yield
	TRFK 347/573	Black tea, high yield, pest & disease resistant
	TRFK 371/3	Green tea, high yield, nematode & mite resistant
	TRFK 430/90	Black tea, high yield, suitable for mechanical harvesting, nematode & mite resistant
	TRFK 54/40	Black tea, high yield
	TRFK 55/55	Black tea, high yield
	TRFK 55/56	Black tea, high yield
	TRFK 56/89	Black tea, high yield
	TRFK 6/8	Black tea, fast fermenting, good nursery rooting
	TRFK 7/14	Black tea, fast fermenting, good nursery rooting
	TRFK 7/3	Black tea, fast fermenting, good nursery rooting
	TRFK 7/9	Black tea, high yield
	TRFK347/314	Black tea, high yield
North India	TV 1	Black tea
	TV 10	Black tea
	TV 11	Black tea
	TV 12	Black tea
	TV 13	Black tea
	TV 14	Black tea
	TV 15	Black tea
	TV 16	Black tea
	TV 17	Black tea
	TV 18	Black tea, high yield
	TV 19	Black tea, high yield
	TV 2	Black tea
	TV 20	Black tea
	TV 21	Black tea, average yield, high quality
	TV 22	Black tea, high yield
	TV 23	Black tea, high yield
	TV 25	Black tea, high yield
	TV 26	Black tea, high yield
	TV 27	Black tea
	TV 28	Black tea
	TV 29	Black tea, high yield
	TV 3	Black tea

(continued)

Cultivation location	Cultivar	Important traits
	TV 30	Black tea, high yield
	TV 31	Black tea
	TV 4	Black tea
	TV 5	Black tea
	TV 6	Black tea
	TV 7	Black tea
	TV 8	Black tea
	TV 9	Black tea
	TV24	Black tea
South India	TRF 1	Black tea, high yield
	TRF 3	Black tea
	TRF 4	Black tea, high yield, high quality, suitable for machine harvesting
	TRF2	Black tea
	UPASI 1	Black tea
	UPASI 10	Black tea
	UPASI 11	Black tea
	UPASI 12	Black tea
	UPASI 13	Black tea
	UPASI 14	Black tea
	UPASI 15	Black tea
	UPASI 16	Black tea
	UPASI 17	Black tea, high yield
	UPASI 18	Black tea
	UPASI 19	Black tea
	UPASI 2	Black tea, drought tolerant
	UPASI 20	Black tea, drought tolerant
	UPASI 21	Black tea
	UPASI 22	Black tea
	UPASI 23	Black tea
	UPASI 24	Black tea
	UPASI 25	Black tea, high yield
	UPASI 26	Black tea, drought tolerant
	UPASI 27	Black tea, high yield
	UPASI 28	Black tea, high yield
	UPASI 3	Black tea, high yield
	UPASI 4	Black tea
	UPASI 5	Black tea
	UPASI 6	Black tea, drought tolerant
	UPASI 7	Black tea
	UPASI 8	Black tea, high yield
	UPASI 9	Black tea, high yield, drought tolerant
Sri Lanka	CH 13	Black tea, high yield, blister blight tolerant

(continued)

Cultivation location	Cultivar	Important traits
	CY 9	Black tea, high yield, drought tolerant
	DG 39	Black tea, high yield, drought tolerant
	DG 7	Black tea, high yield, drought tolerant
	DN	Black tea, high yield, shot hole borer tolerant
	DT 1	Black tea, high yield, high quality
	H 1/58	Black tea, high yield, high quality
	K 145	Black tea, high yield, drought tolerant
	KEN 16/3	Black tea, high yield, low country live wood termite tolerant
	KP 204	Black tea, high yield, low country live wood termite tolerant
	N2	Black tea, high yield, high quality, blister blight tolerant
	NAY 3	Black tea, high yield, blister blight tolerant
	PK 2	Black tea, high yield, high quality, blister blight tolerant
	S 106	Black tea, high yield, drought tolerant
	TRI 2022	Black tea, high yield, stem canker tolerant
	TRI 2023	Black tea, high yield, shot hole borer tolerant
	TRI 2024	Black tea, high yield, nematode tolerant
	TRI 2025	Black tea, high yield, drought tolerant
	TRI 2026	Black tea, high yield, blister blight tolerant
	TRI 2027	Black tea, high yield, nematode & canker tolerant
	TRI 2043	Black tea, "silver tip" production, high yield, blister blight tolerant
	TRI 3013	Black tea, high yield, stem canker tolerant
	TRI 3014	Black tea, high yield, drought tolerant
	TRI 3015	Black tea, high yield
	TRI 3016	Black tea, high yield, nematode tolerant
	TRI 3017	Black tea, high yield, nematode tolerant
	TRI 3018	Black tea, high yield
	TRI 3019	Black tea, high yield
	TRI 3020	Black tea, high yield, nematode tolerant
	TRI 3022	Black tea, high yield, drought tolerant
	TRI 3025	Black tea, high yield, low country live wood termite tolerant
	TRI 3035	Black tea, high yield, drought tolerant
	TRI 3047	Black tea, high yield, low country live wood termite tolerant
	TRI 3051	Black tea, high yield, drought tolerant
	TRI 3052	Black tea, high yield, drought tolerant
	TRI 3055	Black tea, high yield, stem canker tolerant
	TRI 3069	Black tea, high yield, low country live wood termite tolerant
	TRI 3072	Black tea, high yield, blister blight tolerant
	TRI 3073	Black tea, high yield, blister blight tolerant

(continued)

Cultivation location	Cultivar	Important traits
	TRI 4004	Black tea, high yield, shot hole borer tolerant
	TRI 4006	Black tea, high yield, nematode tolerant
	TRI 4014	Black tea, high yield, drought tolerant
	TRI 4024	Black tea, high yield, shot hole borer tolerant
	TRI 4034	Black tea, high yield, shot hole borer tolerant
	TRI 4042	Black tea, high yield, low country live wood termite tolerant
	TRI 4043	Black tea, high yield, shot hole borer tolerant
	TRI 4046	Black tea, high yield, drought tolerant
	TRI 4047	Black tea, high yield, stem canker tolerant
	TRI 4049	Black tea, high yield, low country live wood termite tolerant
	TRI 4052	Black tea, high yield, blister blight tolerant
	TRI 4053	Black tea, high yield, stem canker tolerant
	TRI 4054	Black tea, high yield, shot hole borer tolerant
	TRI 4055	Black tea, high yield, drought tolerant
	TRI 4059	Black tea, high yield, shot hole borer tolerant
	TRI 4061	Black tea, high yield, stem canker tolerant
	TRI 4067	Black tea, high yield, high quality
	TRI 4071	Black tea, high yield, drought tolerant
	TRI 4078	Black tea, high yield, low country live wood termite tolerant
	TRI 4079	Black tea, high yield, high quality
	TRI 4085	Black tea, high yield, shot hole borer tolerant
	TRI 62/5	Black tea, high yield, nematode tolerant
	TRI 62/6	Black tea, high yield, shot hole borer tolerant
	TRI 62/9	Black tea, high yield, high quality
	TRI 777	Black tea, high yield, high quality
Vietnam	LDP 1	Black & Green tea, high yield, pests & drought resistant
	LDP 2	Black tea, pests & drought resistant
	PH 10	Oolong tea, high quality
	PH 11	Black tea, high yield, Green hoppers & bauxite resistant
	PH 8	Oolong tea, high yield
	PH 9	Green tea, high yield
	Shan Chat Tien	Black tea, pest resistant
	Shan Tham Ve	Green tea, pest resistant

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Index

A
Abiotic stress, 11, 38, 87, 88, 240, 255, 273–275, 277, 285, 286, 341, 359, 382, 406, 416, 431, 432, 436, 437, 440, 442, 452, 493, 502, 523, 536, 538, 541, 543
Adaptation, v, 7, 11, 12, 22, 32, 33, 66, 88, 116, 122, 137, 139, 158, 161, 169, 171, 195–197, 212, 240, 314, 376, 447, 502, 544
Aflatoxin, 60, 264, 275, 282, 363
Agrobacterium, 84, 88, 240, 271–273, 342, 417, 442, 447, 494–496, 538
Agrobacterium-mediated genetic transformations, 271, 342
Agrobacterium rhizogenes, 84, 276, 343
Agrobacterium tumefaciens, 88, 271, 434, 442, 494, 496, 538
Agronomic traits, 15, 17–18, 20–22, 199, 206, 261, 262, 267, 426, 437, 478, 492, 534, 536
Allopolyploidy, 259
Allotetraploid, 267
Almond, vi, 4, 164, 235, 356
Alternate bearing, 208, 357, 359, 362, 382, 385, 387, 389
Amazon nuts, 58
Amazon region, 58, 60, 65, 66
Amplified polymorphic DNA (AFLP), 261
Anacardiaceae, 78, 79, 354
Analysis of genetic structure, 338–340, 342
Analysis of molecular variance (AMOVA), 45, 432
Angiospermae, 58
Anisogramma anomala, 163, 169, 175, 178, 195
Anther culture, 418, 524, 527
Anthropomorphic actions, 32
Argan, vi, 32
Argania spinosa, 32–49
Arganiculture, 49
Asian chestnut gall wasp, 109, 112, 120, 124, 125
Association mapping, 183, 200, 201, 421, 423, 431, 432, 534, 545
Auxins, 37, 443, 489, 524, 525

B
Bertholletia excelsa, 58–71
Betulaceae, 158, 183, 184
Biodiversity, 32, 33, 38–44, 61, 65–67, 142, 178–186, 231–233, 262, 263, 365, 367–372, 405, 410, 412, 417, 418, 528, 529, 532, 533
Bioinformatics, 88, 186, 234, 236, 237, 255, 270, 419, 423, 430, 437, 438, 447
Biolistics transformation, 271–273
Bioreactors, 374, 376, 490, 527
Biotechnological approaches, 489–496
Biotechnology, v, vi, 36, 81, 98, 137–140, 142, 144, 197, 244, 255, 261, 262, 264, 287, 342–345, 367, 417, 418, 427, 450, 476, 503, 504, 523, 546
Biotic and abiotic stress, v, vi, 11, 87, 88, 142, 255, 260, 272, 273, 285, 286, 365, 382, 431, 436–438, 442, 452, 502, 523, 536, 538, 541, 543
Botany and taxonomy, 370
Brazil nut, vi, 58–71
Breeding cycles, 88, 240, 308, 310, 325, 338, 342, 345, 346, 360, 365, 372, 417, 431, 433

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Breeding process, 302, 305, 310, 344, 488, 497, 502
Breeding programs, vi, 4, 33, 81, 106, 158, 228, 259, 305, 362, 404, 482, 533
Breeding strategy, 305, 338, 414, 447, 521
Bud grafting, 318

C
Cashew nut, vi, 78–98
Castanea, 106, 158
Castanea crenata, 106–109, 111, 115–119, 127, 133–136
Castanea dentata, 106–109, 112, 114, 115, 120–123, 127, 128, 130–134, 137–141
Castanea henryi, 106–109, 117
Castanea mollissima, 106–109, 111, 114–119, 122, 124–128, 130–136, 144
Castanea pumila var. *alabamensis*, 107
Castanea pumila var. *alnifolia*, 107
Castanea pumila var. *floridana*, 107
Castanea pumila var. *ozarkensis*, 107–109, 114, 115, 120
Castanea pumila var. *pumila*, 106–109, 114
Castanea sativa, 106–112, 115, 118, 119, 124, 133, 138–140, 144
Castanea seguinii, 106–109, 117
Chandler, 405–408, 410, 414, 418, 423–426, 428, 429, 431, 432, 435, 439, 440, 456, 458
Chestnut, vi, 58, 106, 158, 237, 356
  blight, 106, 107, 110, 112, 117, 121, 124, 127, 129, 130, 138, 139, 143
  cultivars, 109, 111, 118–120, 125, 136
Chinquapins, 106, 120, 121, 123, 137
Cisgenesis, 383
Climate change, 7, 22, 32, 35, 49, 70, 88, 141, 176, 206, 207, 233, 287, 358, 364, 406, 430, 447, 479, 518, 522, 544, 545
Clonal breeding, 316–319, 325
Clonal propagation, 82, 115, 170, 184, 205, 229, 240, 243, 378, 380, 441
Clonal selection research, 328, 335, 338
Coffea arabica, 476
Coffea canephora, 476, 478–484, 486–490, 492, 494–498, 501
Coffee, vi, 228, 476, 518
  breeding, 497
  tissue culture, 490
Cold resistance, 169, 437
Collection of hardwood scion, 316
Conservation, 7, 32, 59, 78, 112, 184, 231, 265, 302, 380, 405, 476, 518
Controlled hybridization, 41, 521, 542, 543
Conventional breeding, 82, 84–89, 273, 285, 310, 312–319, 321–332, 334–342, 356, 358–360, 362–366, 381, 419, 476, 488, 489, 496–502, 527
Core collections, 45, 46, 264, 285, 531, 532
Correlation analysis of traits, 325
Correlations, 8, 41, 44, 45, 47, 86, 90, 96, 225, 226, 228, 243, 311, 324–333, 335–338, 340
Corylus, 158–207, 304
Crop improvement, 81, 88, 98, 267, 382, 383, 518, 521–523, 527, 529, 533, 537, 542, 544
Crossbreeding, 47, 65, 306, 308, 312–314, 347
Cross-pollinating, 63, 84, 89
Cryopreservation, 89, 186, 307, 347, 380, 476, 480–482, 524, 532
Cryopreservation of coffee, 482
Cryphonectria, 109, 112, 120, 121, 134
Cultivar characterization, 178–186, 232, 410, 412, 418
Cultivars, v, 6, 42, 64, 88, 107, 158, 224, 260, 346, 356, 405, 476, 518
Cultivation, 5, 33, 61, 78, 106, 158, 224, 255, 303, 354, 403, 478, 518
Cultivation and production, 78
Cyanogenesis, 235
Cytogenetics, 127–129, 186, 265, 266, 370, 533
Cytokinins, 37, 83, 274, 374, 489, 525

D
Descriptors, 97, 178, 180, 419, 529
Design of new cultivars, 8, 23
Dioecious, 324, 354, 360, 371
Disease resistance, 15, 112, 118, 126, 127, 129, 130, 133, 137, 140, 145, 197, 198, 240, 261, 262, 275, 278, 285, 341, 343, 406, 408, 416, 447, 450, 452, 521, 540
Distribution, 32, 45, 49, 58–59, 65, 69, 78, 79, 86, 107–109, 118, 121, 122, 124, 145, 158–164, 178, 222, 225, 231, 232, 241, 255–258, 302–305, 322, 325, 339, 340, 363, 403, 425, 476–478, 534
Diversity analysis, 78, 89–97, 268, 483–487
DNA markers, 15–18, 33, 94, 96, 130, 145, 179, 198, 206, 367, 369, 371, 380, 420, 424, 445, 536
Domestication, 544
Domestication and natural evolution, 259
Drought and heat tolerance, 193, 359

Drought resistance, 4, 13
Drought stress, 269, 274, 275, 277, 415, 417, 438, 534
Drought tolerance, 88, 137, 193, 261, 279, 409, 417, 433, 447, 450, 497, 501, 534
Dryocosmus kuriphilus, 109, 112, 116–118, 124, 133, 135

E
Early harvest, 313, 406, 408, 414–416, 447, 458, 459
Eastern filbert blight (EFB), 163, 164, 169, 172, 175, 178, 179, 182, 187, 191, 195, 198–200, 206, 208–212
Easy-peel, 116, 135, 136
Edible pine nut tree, 301–346
Electroporation, 272, 493
Embryo culture, 81, 139, 418, 527, 528
Embryo rescue, 527, 528
Epigenetics, 20, 145
Epigenetics marks, 4, 20–22
Ex situ, 38, 46, 89, 114, 144, 178, 186, 233, 242, 418, 479–481, 528, 529
Expressed sequence tags (ESTs), 8, 129, 184, 237, 268, 269, 285, 372, 422, 425, 429, 435, 436, 438, 492, 538

F
Fatty acids, 14, 18, 32, 33, 41, 235, 258, 270, 280, 304, 325, 328, 329, 336, 404, 537
Field evaluation, 240
Filbert, 158, 168
Flowering, 10, 32, 79, 123, 161, 222, 257, 306, 358, 434, 523
Fruit production, 42, 52, 59, 62, 64, 65, 67, 69, 70, 225, 231
Fruit trees, 8, 11, 13, 20, 21, 49, 135, 144, 226, 233, 356, 365, 369, 372, 378, 381, 419, 432, 434, 438, 445, 546
Functional genomics, 201–204, 234, 235, 269, 270, 287, 383, 419, 435–437, 489, 502, 504, 518, 523, 536, 537, 544
Functional properties, 4, 14

G
Gamma radiation, 541
GenBank®, 198, 269, 383, 425, 428, 435
Gene flow, 45, 46, 66, 69, 70, 115, 123, 143, 183, 231, 236
Gene pyramiding, 286
Genetic differentiation of natural populations, 339, 340
Genetic diversity, v, 13, 32, 66, 81, 112, 178, 231, 257, 305, 368, 413, 478, 532
Genetic engineering, 21, 22, 88, 137, 139–140, 197, 204, 237–240, 270, 272–278, 287, 342, 343, 346, 377, 378, 381, 383, 405, 439–444, 447, 538
Genetic fidelity, 378, 380, 491, 492, 525
Genetic improvement, 64, 65, 67–69, 71, 169, 187, 206, 225, 233, 255, 275, 285, 305, 314, 324, 340, 342, 356, 373, 377, 381, 404, 406, 407, 413, 415, 419, 424–426, 429, 431–434, 437, 438, 441, 476, 488, 489, 496–498, 502
Genetic linkage maps, 130, 135, 237, 267, 285, 344, 372, 426, 518, 533, 535, 544, 545
Genetic markers, 47, 233, 234, 269, 338, 369, 419, 423, 425, 483
Genetic patrimony, 49
Genetic resources, vi, 24–25, 35, 47, 52, 71, 81, 89, 98–99, 112, 133–135, 140, 144, 145, 158, 169, 178, 186, 233, 244, 264, 265, 286, 288–291, 305, 306, 308–310, 312, 338, 348, 385, 417, 418, 424, 434, 451, 476–502, 518, 528, 529, 531, 532, 545, 548
Genetic structures, 15, 45–47, 69, 70, 118, 143, 178, 338–340, 342, 369, 431, 437
Genetic trait variation, 324–333, 335–338
Genetic transformation, 21, 88, 262, 270–273, 275, 286, 342, 345, 367, 378, 381, 489, 492–495, 525, 538, 544
Genetic variability, 8, 10, 33, 47, 63, 65, 67, 69, 86, 145, 178, 184, 206, 234, 259, 267, 320, 324, 419, 420, 422, 486, 492
Genome, 15, 45, 68, 88, 117, 179, 231, 259, 342, 354, 403, 487, 533
  analysis, 425, 431, 446, 487
  sequencing, 197, 201, 231, 372, 427, 428, 430, 447, 492
  wide association, 136, 233, 367, 383, 419, 424, 430, 432, 434
Genome wide association studies (GWAS), 136, 233, 237, 243, 367, 383, 419, 423, 424, 430–434, 447
Genomics, v, 22, 90, 144, 203, 234, 269, 344, 382, 417, 488, 523
Genomic selection (GS), 129, 130, 136, 233, 234, 237, 243, 342, 367, 383, 419, 423, 424, 431–434, 447, 536

Genotypes, 8, 33, 64, 80, 114, 171, 228, 260, 305, 357, 406, 480, 523
Genotyping by sequencing (GBS), 131, 424, 434
Germplasm, 8, 33, 67, 81, 107, 168, 231, 257, 307, 361, 405, 479, 523
  biodiversity, 38–44, 65–67, 178–186, 231–233, 262, 263, 365, 367–372, 405, 410, 412, 417, 418, 528, 529, 532, 533
  collections, 8, 67, 89, 168, 178, 207–208, 264, 278, 362, 371, 431, 480, 528, 529, 545
Grafting, 8, 36, 37, 49, 62–64, 81, 82, 107, 118, 171, 224, 228, 243, 280, 306, 312, 313, 316–319, 373, 376, 405, 406, 416, 418, 439, 501

H
Haploid, 284, 439, 444–447, 527
Hard-branch grafting, 306, 317, 319
Hardwood scion, 316
Hazelnut, vi, 158, 237, 304
Health properties, 14, 223
Heat tolerance, 193, 359
Heritability, 41, 85, 131, 178, 179, 226, 231, 234, 314, 315, 325, 328, 335, 360, 405, 433
Heterosis, 81, 85, 87, 314, 497
Heterozygosity, 88, 187, 232, 341, 342, 381, 383, 419, 431
Husk, 159, 162–164, 175, 177, 179, 189, 191, 194, 195, 223, 228, 234, 244
Hybrid cultivars, 120, 127, 163, 241, 242
Hybridization, 41, 81, 112, 158, 241, 261, 309, 383, 405, 488, 523

I
Ink disease, 106, 109, 112, 140
In planta transformation, 271, 276
Insect pests, 109, 112, 124, 260, 262, 273, 276, 290, 362, 497, 501
In situ, 35, 46, 47, 49, 89, 112, 127, 144, 178, 231, 233, 264, 339, 479, 480
Integrifolia, 222–224, 231, 232, 235, 236, 239–242
Inter simple sequence repeats (ISSR), 8, 33, 44–46, 48, 90, 93–97, 179, 267, 344, 367, 368, 372, 380, 421, 485–487, 533
Interspecific hybridization, 112, 123, 143, 191, 195, 241, 261, 283, 284, 314, 496, 527
Intragenesis, 383
In vitro, 21, 37, 63, 82, 139, 235, 307, 373, 436, 489, 523
  propagation, 63, 140, 237, 373, 439, 443
  regeneration, 37, 63, 239, 240, 262, 270, 490

J
Jansenii, 222, 231, 241, 242
Juglans regia, 402–447
Juvenile period, 8, 22, 225, 226, 234, 243, 360, 371, 381–383, 419, 431, 433, 434, 483
Juvenility, 21, 64, 197, 381

K
Kalle-Ghoochi, 358, 362, 382
Karyotype, 266, 533
Kerman, 359, 362–364, 368, 372, 373, 387, 390, 402, 415
Kernel, 4, 32, 66, 78, 118, 160, 200, 222, 275, 315, 359, 404
  color, 404–408, 410, 418, 431, 433, 447, 454–459
  recovery, 226, 245
  weight, 16, 17, 41, 42, 85–87, 179, 200, 324–328, 333, 335–338, 410

L
Late leafing, 172, 208, 209, 211, 405–408, 414–416, 418, 447, 450, 453–458
Lateral bearing, 405–408, 414–416, 425, 432, 433, 447, 450, 452, 454–458
Lecythidaceae, 58
Linkage mapping, 86, 135, 423, 425, 426
Linoleic acid, 16, 44, 304

M
Macadamia, vi, 222
Magnoliopsida, 58
Marker assisted selection (MAS), 11, 15, 18, 22, 86, 129, 130, 135, 136, 184, 187, 190, 197, 206, 233, 234, 237, 243, 261, 269, 286, 341, 367, 371, 383, 419, 422, 424, 426, 431, 447, 483, 500, 518, 527, 533, 534, 536, 544, 545
Market value and utilization, 304
Mating system, 10, 112, 284, 341, 497
Microarray, 269
Micrografting, 83, 375, 380
Micropropagation, 37, 63, 81–84, 140, 145, 171, 204, 240, 372, 374–376, 378, 380, 405, 425, 439, 440, 450, 476, 489, 491, 492, 503, 523, 524, 527

Microsatellites, 33, 44, 68–70, 86, 107, 136, 179, 181–183, 268, 338, 368, 422–424, 431, 483, 484
Microshoots, 83, 84, 375, 381, 439, 524, 525
Mini-core collections, 264, 285
Molecular breeding, v, vi, 33, 44–49, 68–70, 88, 98, 129–137, 142, 197–204, 233–237, 267–270, 342–346, 365, 367–372, 381, 405, 408, 415, 419–438, 447, 448, 453, 533, 534, 537–540
Molecular characterization, 65
Molecular markers, v, 10–12, 15, 16, 22, 33, 38, 44, 45, 47, 68, 70, 88, 112, 129, 179, 183, 198–203, 233, 234, 237, 241, 243, 261, 267–269, 338, 341, 344, 367–369, 380, 414, 419–421, 424, 426, 432, 435, 436, 482–487, 491, 518, 523, 533, 534, 544
Molecular tools, v, vi
Monitoring process, 18
Morocco, 6, 7, 13, 24, 32, 34, 40, 44, 45, 49–51, 451
Morphological characteristics, 47, 64, 65, 302, 303, 369
Morphology, 4, 19, 32–33, 38, 39, 41, 47, 64, 65, 79, 86, 89, 90, 96, 97, 114, 117, 118, 131, 132, 137, 143, 158, 161, 164, 178, 201, 233, 241, 259, 263, 265, 267, 302, 303, 314, 325, 327, 367–369, 371, 419, 421, 446, 479, 482, 483, 489, 492, 529, 532
Morphotypes, 38–42, 44
Mound layerage, 170
Mutation, v, 12, 46, 69, 88, 138, 168, 197, 204, 205, 239, 241, 269, 278–282, 284, 286, 369, 405, 425, 444, 447, 496, 518, 521, 541, 542, 544
Mutation breeding, v, 12, 88, 138, 205, 241, 278–282, 284, 405, 444, 447, 518, 521, 541, 542
Myrtaceae, 58

N

Natural evolution, 259
Natural geographic distribution, 66
Natural selection, 34–35, 65
Nematodes, 262, 264, 343, 359, 365, 391, 409, 417, 434, 436, 442, 447, 497, 501, 505, 554, 556, 557
Next generation sequencing (NGS), 8, 20, 234, 237, 381, 419, 422, 424, 430, 434, 438, 488, 493, 502, 533, 538
NGS efforts in coffee, 493
Non-suckering, 163, 164, 168, 174, 191, 193, 195
Nut, vi, 4, 58, 78, 106, 158, 222, 254, 302, 354, 402
Nut weight, 85–88, 90, 91, 98, 136, 179, 196, 410, 447
Nutraceutical values, 4, 14–15, 23, 177
Nutrient management, 260
Nutritional value, 59, 80, 304, 492

O

Oleic acid, 14, 16, 32, 44, 205, 279–281
Origin, 5, 41, 64, 81, 120, 161, 232, 257, 354, 404, 476, 519
Origins and distribution, 476, 478
Oxalate oxidase (OxO), 135, 139, 278

P

Pará nuts, 58
Particle bombardment, 270–273, 342, 345, 494
Peanut, vi, 139, 254
PeanutMap, 270
Persian walnut, 403, 404, 406, 414, 416, 420, 425, 431, 438, 440, 441, 446, 453
Pest and disease resistance, 240, 521
Phenotype, 9, 15, 18, 22, 33, 38, 64, 81, 82, 86, 88–92, 112, 113, 127, 128, 130, 131, 134, 136, 138, 170, 183, 184, 187, 201, 228, 234, 243, 261, 268, 269, 280, 320, 325, 328, 331, 332, 335, 338, 340, 371, 380, 383, 405, 419, 425, 426, 429, 431, 433, 438, 447, 502, 531, 532, 540
Phylogenetic, 107, 121, 233, 344, 370, 424, 426, 428
Phytophthora cambivora, 109, 112
Phytophthora cinnamomi, 109, 112, 133, 134
Pine nuts, vi, 301–346
Pistachio, vi, 164, 237, 354–383
Pistacia, 354–383
Plant breeding, v, 82, 88, 140, 241, 261, 314, 367, 405, 414, 421, 424, 438, 444–446, 478, 503
Plant growth regulators (PGR), 83, 373, 374, 377, 378, 525
Pollen, 11, 47, 70, 85, 116, 161, 222, 282, 314, 355, 445, 525
Polycross, 87
Polyploids, 266, 370, 447, 521, 528, 533
Polyploidy, 266, 519, 544
Plant breeding, v, vi, 82, 88, 140, 241, 261, 314, 367, 405, 414, 421, 424, 438, 444–446, 478, 503

Principal component analysis (PCA), 41, 86, 120, 335
Processing, 6, 60, 78, 118, 166, 228, 258, 357, 425, 503, 518
Production, v, 4, 59, 78, 106, 158, 223, 255, 301, 356, 404, 476, 524
Progenies, 18, 49, 64, 67, 69, 85, 87, 88, 98, 112, 114, 127, 130–134, 138–140, 186, 187, 190, 198, 229, 234, 241, 278, 279, 306, 320, 341, 344, 363, 371, 380, 422, 425, 426, 431–434, 488, 492, 494, 497, 521, 535, 542, 549–551
Propagation, 33, 63, 78, 107, 170, 229, 262, 306, 373, 406, 491, 520
Protoplast fusion, 284, 543
Prunus, 4–23, 130, 203, 235, 304, 381

Q
qPCR, 19, 20
QTL mapping, 15, 86, 129, 135, 136, 419, 426, 432–434
Quality, v, 6, 34, 60, 86, 110, 158, 223, 258, 302, 357, 404, 482, 521
Quantitative trait loci (QTL), 15, 16, 86, 98, 129, 130, 132, 133, 136, 198–201, 234, 262, 269, 282, 344, 345, 383, 419, 425–427, 432–434, 483, 533, 534, 536

R
Rafsanjan, 365, 368, 369
Random-amplified polymorphic DNA (RAPD), 44, 68, 90, 129, 179, 261, 338, 367, 420, 483, 533
Recombinant inbred line (RIL), 269
Regeneration, 21, 32, 63, 82, 145, 205, 239, 262, 343, 377, 482, 541
Resistance, 4, 88, 112, 158, 228, 261, 406, 482, 529
Resource distribution, 302, 303
Resource evaluation, 478
Restriction fragment length polymorphism (RFLP), 15, 18, 33, 44, 130, 259, 261, 267, 285, 338, 367, 369, 420, 421, 426, 483, 484, 486
RNA markers, 18–19
RNA sequencing (RNA-seq), 20, 203, 237, 268, 422, 428, 438, 538
Rootstock, 11, 37, 81, 112, 163, 313, 356, 405, 501
Rootstock breeding, 359, 365, 371, 416, 417, 434, 450, 453
Rust, 13, 143, 260, 278, 282, 288–290, 343, 488, 497, 499, 500, 504, 506

S
Salinity stress, 277, 382
Sapotaceae, 32
Seed orchard management, 449, 452
Seed orchard planting scheme, 320, 321
Seed quality, 279, 306, 311, 321, 324, 340, 347
Seed resources, 306, 320–324
Selection, 7, 33, 64, 81, 106, 158, 224, 255, 306, 362, 404, 483, 518
Selection of genetic resource, 310, 312
Self-compatibility, 8, 10, 11, 177, 188
Self-incompatibility, 11, 21, 22, 64, 161, 173, 178, 187, 188, 445, 523
Sequence, 8, 33, 68, 90, 117, 179, 234, 261, 343, 367, 419, 483, 533
Sequence characterized amplified region (SCAR), 33, 44, 261, 367, 371, 421, 534
Shelling percentage, 87, 90, 98, 99, 269
Simple sequence repeat (SSR), 8, 33, 68, 96, 118, 179, 236, 261, 338, 367, 420, 483, 532
S-locus, 179, 188, 199
Somaclonal variations, 272, 280, 282, 284, 380
Somatic cell hybridization, 241, 284, 383, 543
Somatic embryogenesis, 63, 82, 197, 205, 206, 262, 306, 347, 377, 378, 380, 381, 441, 442, 489–492, 496, 524–526
Somatic embryos, 139, 273, 309, 378, 381, 420, 439, 441–444, 446, 482, 490, 491, 493, 494, 525, 526, 538
Somatic hybridization, 81, 543
Species, v, 4, 32, 58, 79, 106, 158, 222, 256, 302, 354, 402, 476, 519
Species distribution, 107–109, 303
Stress managements, 255, 260
Suckers, 159, 161, 170, 171, 174, 199

T
Targeting induced local lesions in genomes (TILLING), 270, 446
Taxonomy, 370
Tea, vi, 87, 135, 228, 518
  breeding, 518–545
  cultivars, 518, 521–525, 534, 536, 537, 541–545

genome, 536, 545
micropropagation, 524
quality, 531, 534
Ternifolia, 222, 231, 232, 235, 241, 242, 245
Tetraphylla, 222, 223, 231, 232, 235, 239, 241, 242
Tissue culture, 63, 137, 204, 237–240, 377, 380, 381, 418, 439, 440, 450, 490–492, 503, 523, 524, 527, 528
Traditional breeding, v, vi, 21, 60–65, 78, 110–112, 187–197, 224–231, 259, 261, 262, 285, 381, 383, 405–408, 414–417, 447, 488, 521–528
Trait improvement, 22, 23, 206, 261, 267, 445
Transcriptomes, 18, 20, 117, 133, 179, 198, 201, 203, 235, 237, 268, 422, 424, 429, 434–436, 438
Transcriptomics, 16, 22, 129, 135, 203, 344, 419, 422–424, 436, 437, 447, 536, 538–540
Transformants, 88, 343, 495
Transgenic, 21, 88, 139, 262, 342, 378, 434, 476, 518
Twig grafting, 312, 317–319

U
UCB1 hybrid, 375
UCB1 rootstock, 365, 366
Unweighted pair group method with arithmetic mean (UPGMA), 46, 48, 181, 367, 368

V
Variety breeding, 311
Vegetative propagation, 36, 49, 63, 78, 81, 82, 264, 316, 373, 520, 523

W
Walnut, vi, 158, 164, 237, 304, 356, 373, 378, 381, 402–447
breeding, 402–447
genetic improvement, 406, 407, 413, 415, 419, 424, 425, 429, 432–434
Wild germplasm, 187, 225, 264
World production, 78, 158, 224

Y
Yield, 6, 32, 80, 110, 158, 223, 259, 305, 358, 404, 492, 521