Genetic Resources of Citrus and Related Genera

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Abstract

Genetic resource is the part of the organisms that can reproduce identical copies of the organism with identical genomic profiles. Generally, seeds, pollen, bud wood are considered as genetic resources of *Citrus* and its related genera. Citrus have three basic taxa, pummelos (*Citrus maxima* (L.) Osb.), citrons (*C. medica* L.) and mandarins (*C. reticulata* Blanco). *Citrus* species, cultivar, landraces have evolved by series of interspecific hybridizations, consequently, a huge diversity gained in citrus genus. Recently, Australian limes, Mangshanyegan, Kumquats and *Clymenia* spp. are included in the citrus genus. Due to high economic value of *Citrus*, researchers collect citrus germplasm and maintain them in the gene bank at major citrus producing countries worldwide. Significant progress has been made on the citrus germplasm collection, management and conservation. In this chapter we summarize the present status of the citrus germplasm collection and management, as well as discuss the future path to protect the valuable citrus genetic resources for future use.

3.1 Introduction

In general genetic resource is any part of the living organisms that can reproduce identical copy of the organisms comprised of identical genomic profiles. It is the functional unit of heredity and the most essential basic raw materials to convene the current and future requirements of crop improvement programmes. Normally seeds, pollen, bud wood are considered as genetic resources of citrus and its related genera. In addition, some cases leaves and other vegetative organs of citrus and its related genera were also considered as genetic resources (Khan 2007). Genetic resources collection, proper maintenance, identification and characterization are prerequisites for efficient and sustainable breeding programme. A wide genetic base broadens the horizon of breeding to achieve ultimate goal of the breeders with improved traits of new cultivars. Hence, genetic resources institutes or centers collects and conserves genetic

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resources in a systematic manner as large numbers as possible. In fact, most of the countries established their own national germplasm institute or center, whose main responsibility was to collect and maintain the local genetic resources, most cases important crop plants. Citrus is one of the most common, well known, highly consumed fruit crop worldwide. Accordingly, this fruit species gained lot of attention to plant breeders and scientists worldwide, as a result genetic resources of citrus collected and conserved in a wide range of countries of its major producing regions. A global citrus germplasm network was established in 1997 to monitor the world wide citrus germplasm collection, proper maintenance and ensure their sustainable use in citrus breeding programme and protect citrus genomic resources erosion (Roose et al. 2015).

Citrus belongs to the plant family Rutaceae and it has large diversity. The citrus genera composed with uncertain number of species, landrace or cultivars (Wu et al. 2018). The taxonomy and ancestry relationships among the wild and domesticated species of the citrus genus are complex, even in some of the state very much confusing or unclear. The assessment of the ancestry of modern *Citrus* spp. is relatively complicated due to the apomixis nature and highly cross-compatibility among the related species. Citrus apomixis is sporophytic type, therefore, embryos develop from somatic nucellar cells (Wang et al. 2017). This phenomenon is typically known as 'polyembryony' and it is very stable among modern commercial cultivars like mandarins, sweet oranges, grapefruits and lemons. Polyembryony nature of citrus has both positive and negative impacts on citrus breeding, for example, polyembryony was widely used to propagate large numbers of uniform rootstocks from seeds, while it hampers the cross-breeding programme. The polyembryony remains genetic materials unchanged from generation to generation in citrus species, as a consequence the genetic erosion may reduce.

It is widely believed that citrus genera have three basic taxa viz. pummelos (*Citrus maxima* (L.) Osb.), citrons (*C. medica* L.) and mandarins (*C. reticulata* Blanco) (Nicolosi et al. 2000), from which modern citrus species like, sweet oranges (*C. sinensis* (L.) Osb.), clementines (*C. clementina* Hort. ex Tan.), satsumas (*C. unshiu* (Mak.) Marc.), lemons (*C. limon* (L.) Burm *f.*), limes (*C. aurantifolia* (Christm.) Swing.) and grapefruits (*C. paradisi* Macf.) are evolved by series of interspecific hybridization, consequently, a huge diversity is gained in citrus genus. Apart from these core citrus species, recently discovered some of the species, viz Australian limes, Mangshanyegan, Kumquats and *Clymenia* sp. are included in the citrus genus (Garcia Lor 2013). The commercial rootstock Trifoliate orange (*Poncirus trifoliata*) is the closest relatives of citrus, often they are categorized as citrus. Bayer et al. (2009) suggested that *Oxanthera* sp. from New Caledonia should be transferred to the citrus genus.

The aim of this chapter is to make clear the importance of citrus genetic resources in breeding as well as their world wide present scenario, major challenges, future direction to overcome the hurdle for their better uses.

3.2 Present Status of the Citrus Genetic Resources Around the World

Citrus germplasm is the building block of its varietal improvements, consequently, the citrus research community prefers to collect the citrus germplasm and maintain them in the gene bank. Citrus germplasm collection usually includes primitive landraces, wild species, developed varieties and breeding lines. Major citrus producing countries have huge collection of citrus germplasm in their gene bank. Here briefly we illustrate worldwide present citrus germplasm collection scenario.

Australia: Several citrus related genera like *Eremocitrus* and *Microcitrus* were originated in Australia (Krueger and Navarro 2007). Citrus germplasm collection is maintained in Australia by the State Government, Departments of Primary Industries and the Commonwealth Scientific and Industrial Research Organization (CSIRO). Apart from these, there are several other citrus germplasm banks (Royal Botanic Garden, Sydney; Brisbane Botanic Gardens; Waite Research Institute, University of Adelaide). Australia mainly follows *ex situ* conservation for wild and cultivated citrus and related genera.

Brazil: Brazil is the major citrus producer in south America (FAO 2018). Brazil initiated citrus germplasm collection in the year 1948. Initially, about 470 citrus accessions were collected from Riverside California, USA and maintained in the Brazilian citrus gene bank; new local accessions were then added to the gene bank and now the total collection reached about 1546 accessions. These collections include nucellar clones, newly introduced cultivars, and new hybrid collections for breeding and rootstocks. Some duplicate accessions have been identified in the Brazilian citrus germplasm collection. Among them some accessions have the same clone with different names, and some have same name but they are from different clones. One plant per accession in the gene bank was maintained under the screen house (Roose et al. 2015).

China: Exploration and collection of Chinese indigenous citrus varieties was initiated in the 1950s; unfortunately the survey was interrupted by the Cultural Revolution of 1967–1972, but was restarted by the Chinese government in the 1970s and 1980s. Conservation of citrus germplasm in PR China is mostly *ex situ* conservation. The national citrus germplasm repository of China was established in 2007 at Beibei, Chongqing, Sichuan province. Beside the national citrus gene bank there are many other sites found in China where wild citrus germplasm has been maintained. Among them Huazhong Agricultural University (HZAU), Huangyan, Zhejiang province; Guiling, Guangxi province; Changsha, Hunan province; Guangzhou, Guangdong province; Jiangjin, Chongqing municipality; Wuzhung province and Hubei province are notable (Krueger and Navarro 2007). HZAU has its own citrus germplasm collection center where more than 400 accessions are maintained, along with 100 embryo callus lines. The HZAU collection includes trifoliate orange, tangerines, kumquat, lemon, lime, pummelo, sweet oranges, papeda, citron, and grapefruit.

France: There are two institutes involved in the citrus germplasm collection and maintenance in France, INRA and CIRAD, both the institutes are located at San Giuliano, Corsica, France. A total of 928 genotypes have been maintained here in the field; among these 340 were mandarins and about 220 sweet oranges (Roose et al. 2015). The origins of these collections are quite diverse, with the highest contribution from California and Florida, and the other from other citrus producing countries around the globe. 60% of the France collection was introduced by budwoods and remaining 40% from seeds. There are no restrictions to import plant seeds in France but live plant materials must go through quarantine check. Therefore, imported citrus budwoods are sent to another location for quarantine check prior to admission into citrus gene bank. Budwood is tested for viruses, viroids and diseases every 1–3 years. France uses cryopreservation of the citrus seeds collection for security backup. The collections are often evaluate by molecular markers (SSR, SNP, Indel markers) and phenotyping. Phenotyping is performed according to IPGRI descriptors. Biochemical traits and abiotic stress tolerance are also evaluated. French collection is available for commercial and scientific uses and no material transfer agreements (MTA) are required for this exchange. Every year in INRA and CIRAD about 25,000 citrus buds are distributed in 22 locations in France and in other Mediterranean countries.

India: Several citrus species are native to India and this country is rich of citrus genetic resources. To protect citrus diversity, NBPGR (National Bureau of Plant Genetic Resources) of ICAR (Indian Council of Agricultural Research) established the 'Citrus Gene Sanctuary' in the Garo hills of Meghalaya in 1981. Around 1500 indigenous and exotic citrus accessions are conserved and maintained in 20 field gene bank at different locations in India. Among them, a large collection of about 600 accessions including *Citrus* spp., *Poncirus trifoliata, Severinia* species are maintained at National Research Center (NRC) Citrus, Nagpur. For long term preservation, Indian collections are also maintained using cryopreservation (Singh et al. 2001; Sharma et al. 2004).

Spain: Spain is one of the major citrus producing countries. The Spanish Citrus Germplasm gene bank is located at Instituto Valenciano de Investigaciones Agrarias (IVIA). Spanish citrus gene bank collects and maintains germplasm from *Citrus* and its related genera of *Aurantioideae* subfamily. Till to date 620 accessions have been introduced in Spanish Citrus gene bank, including 425 accessions from 51 *Citrus* species, 53 from 20 *Citrus* relatives, and 142 intra- and inter-specific hybrids. Spanish germplasm collection is maintained at three states, (a) fields, (b) screenhouses and (c) cryopreservation. All the genotypes are phenotyped according to IPGRI (IPGRI 1999) and UPOV (http://www.upov.int/portal/index.html.en) descriptors and genotyped by 36 SSRs markers. After genotyping and phenotyping duplicate entities are removed from the gene banks and only healthy and pathogen-free accessions are maintained. In vitro shoot-tip grafting technique is also applied to obtained pathogen-free Citrus accessions.

USA: The University of California, Riverside (UCR) first initiated Citrus variety collection in USA in 1910. UCR established Citrus Experiment Station to conserve and maintain Citrus and its relatives. UCR has the most extensive collection of citrus diversity in North America and till to date over 1,000 accessions have been introduced in UCR gene banks. UCR collection includes commercial citrus varieties, breeding line, other citrus germplasm and wild relatives, particularly important for research and extension activities. Apart from UCR collection, USDA ARS National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) has been established in 1987 for collection, evaluation, maintenance, and preservation of citrus and date palms genetic resources. Currently about 400 accessions pathogen-free are available for free distribution on request for research purpose.

3.3 Significance of Citrus Genetic Resources for Breeding

Genetic resources and plant breeding are intimately linked to each other. Genetic resources are the fuel for breeding, without it breeding programme cannot run. The fundamental goal of any plant breeding programme is developing cultivars with high yielding, high quality and quantity, disease resistance/tolerance, pests/nematode resistance/tolerance, increasing adaptation ability to diverse climate and soil conditions. Genetic variation is required to achieve the breeding goals, therefore plant breeders always hunt for the diverse genetic materials for improving the cultivars through breeding. Consequently, plant genetic resources are considered as the treasures in which key genes (traits) are kept stored. For example, wild species are disease resistant but low yielding while cultivated varieties are high yielding but disease susceptible. In that circumstances plant breeders select wild and cultivated species as parent for breeding and subsequently, make a series of crosses between selected parents until getting the most desirable combination of genetic traits. Citrus breeders also follow a similar strategy for high yielding disease resistant cultivar development. Citrus breeders around the world initiated breeding programme in the nineteenth century and the process is still continuing for improving the citrus cultivars to meet the consumption demands (Cooper et al. 1962).

Webber and Swingle hypothesized that the selection of individual from seedlings obtained from crosses may give better results than seedling grove selection. In order to improve citrus varieties they initiated their cross-breeding programme in the spring of 1893. They used sweet orange, grapefruit and mandarin varieties as parents in their hybridization programme (Webber 1894; Swingle 1905). Afterward, a series of cross-breeding and selection programme has been incited by USDA to improve the citrus variety. *P. trifoliata* has been hybridized with other *Citrus* species to produce cold-hardy varieties (Webber 1900). During 1908–14 Webber and Swingle produced 2,500 hybrids from crosses of the *P. trifoliata* with varieties of mandarin, lemon, lime, grapefruit, sour orange, and kumquat. In 1942 Gardner and Bellows made a series of crosses among different citrus genotypes viz. tangors, tangelos, mandarins, and sweet oranges. One of the crosses, Clementine x

Orlando, produced many promising new hybrids; most of them are early-maturing, large in size, sweet taste and have an orange-red rind; but unfortunately they are rather seedy and some of them have prominent navels.

There were several citrus genotypes widely cultivated and maintained in Japan, such as satsuma mandarin, natsudaidai (*C. natsudaidai* Hayata), kunenbo (*C. nobilis* Lour.), kinokuni (*C. kinokuni* Hort. ex Tanaka), iyo (*C. iyo* Hort. ex Tanaka), hyuga-natsu (*C. tamurana* Hort. ex Tanaka), hassaku (*C. hassaku* Hort. ex Tanaka) and yuzu (*C. junos* Sieb. ex Tanaka) (Omura and Shimada 2016). Japanese citrus breeders use these genetic resources to improve cultivated citrus varieties through cross-hybridization and selection. A large scale citrus breeding programme was initiated in Japan in 1937, therefore, several promising citrus varieties have been released and among them Shiranuhi and Kiyomi are very popular for the commercial cultivation (Nesumi and Matsumoto 2000). The first Japanese citrus hybrid was Tanigawa-buntan, which was released in the Taisho period (Omura and Shimada 2016).

China has more than 4,000 years old history of citriculture. Han Yen-Chih, in 1178, firstly recorded and described citrus variety found in Wenzhou, Zhejiang province. Chinese citrus breeders initiated citrus breeding programme to produce seedless citrus variety in the mid twentieth century (Liu and Deng 2007). Their main strategy was bud sport mutant selection to acquire seedless varieties. As a result, until now 150 clones of seedless or less seedy clones were identified. Among them Qianyang, a seedless red sweet orange (Chen et al. 1992), seedless Shatian pummelo (Zhuang et al. 1994), seedless Shatangju (Zi-xing et al. 2006), seedless Ponkan and Xuegan sweet orange (Chen et al. 1997) are the most representative. Similarly, Chinese citrus breeders selected several late-ripening bud mutants, e.g. Yanxi wanlu Ponkan, founded in Fujian, China, whose harvest time is delayed of two months, from late November to February (Zhong and Chen 1994). Another late-ripening citrus variety, Mingliu Tianju, derived from a bud mutation of Chun Tianju tangerine was obtained in Guangdong province (Min 2006). Recently, citrus scientist from the HZAU discover two late-repining bud mutants, Fengjiewancheng and Zaohong navel orange from the Three Gorge Citrus Natural Germplasm Collection center (Li et al. 2006). The result of the citrus breeding programmes over the few centuries demonstrated that the citrus genotypes (germplasm) played a crucial role in the success of the cross-breeding programme.

3.4 Major Challenges for Citrus Genetic Resources

Germplasm represents the genomic treasure that is transmitted down through generations by an unbroken chain (Brown et al. 1989). Genetic erosion is one of the major cases for breakdown of this chain. Many of the plant genetic resources including citrus species facing the challenge for survival. There are several factors that cause the genetic resource erosion, among them population growth, global warming, pollution, deforestation, destroy of the natural habitat of the plants, mono culture, selection and domestication pressure, natural disasters, diseases, etc.

Natural habitat or ecosystem is very important for survival of wild species, destruction or alteration of habitat or ecosystem is the cause for loss of certain gene pools forever. In general, center of origin is the natural habitat of the wild plant species, for example north-eastern Himalayan region and foothills of the central and western Himalayan tracts (Ghosh 1977; Govind and Yadav 1999; Malik et al. 2006) are considered as the natural habitat of the wild citrus species. Citrus species including mandarin and orange (Tanaka 1958; Singh and Chadha 1993; Chadha 1995) originated from this region and their wild progenitors grew in this region from millions of years. Unfortunately, many natural habitats of citrus of this region have been destroyed by urbanization. Consequently, many of the ancient progenitors of citrus and their relatives were extinct and the rest of them are endangered.

Environmental pollution and global warming simultaneously change the global weather vastly, as a result frequently happening cyclone, flooding coastal region, huge rainfall, unexpected draught in the year round. These weather changes have devastating effects on the natural habitats, which may be the cause of citrus germplasm loss. For instance, natural habitat of the citrus are in untraced hill slopes, this kind of landscape practically has not adopted any soil conservation measures, therefore, heavy rainfall washes away the nutrient-rich surface that leads exposure of comparatively less fertile and extremely acidic subsurface and this may increase the risk of genetic erosion of the citrus germplasm. Furthermore, loss of soil fertility, prolonged high rainfall and high humid environment increase the chances of citrus pathogens break out and this could be threatening for citrus germplasm.

Various ancient citrus varieties in the north-east region of India generally have been found in home gardens or backyard gardens since very ancient times. Due to less commercial importance of these ancient citrus species, they are not properly maintained or propagated, consequently, they are at high risk of extinction. Due to consumer demand and economic benefits, citrus growers prefer to grow commercial cultivars like mandarins, sweet oranges, lemon, navel oranges, etc. As a result, they replaced wild and ancient types of citrus varieties from their orchards and replanted commercial cultivars leading to the loss of many citrus genotypes permanently.

3.5 Present Activity and Future Challenges

The genetic diversity of citrus and its relatives are shrinking at alarming rate due to population growth, global warming, pollution, large scale deforestation, selection pressure, diffusion of newly introduced cash crops. Therefore this is the high time for the researchers to explore all the rare and endangered resources of citrus and develop sustainable management systems to prevent their extinction. In this section we briefly discuss the present scenario and the additional needs in order to meet the upcoming challenges of citrus genetic resources conservation.

a. **Genetic resources collection and conservation**

Plant genetic resources collection and their proper conservation is the first step to prevent the extinction of genetic resources. In order to prevent the extinction of citrus genetic resources and maximizing their utility in the breeding programme, citrus germplasm has been collected and conserved in major citrus producing countries.

The north-east part of India is one of the richest areas of citrus genetic diversity and it is the center of origin of many citrus species. The collection of citrus germplasm from this region intensively began in the early twentieth centuries by Tanaka (1928, 1937) and Bhattacharya and Dutta (1956). During this time, several new citrus species were identified, collected and documented. The comparison of recent survey reports with previous surveys of these regions, shows that some of the citrus species are extinct and some are endangered. For instance, Tanaka (1928) described *Citrus indica* and in the year 1937 he reported this species as one of the wild species found in Now gong district, Khasi hills and Manipur in Assam. Later, the survey report of Singh (1981) demonstrated that this wild *Citrus indica* only found in Naga hills of Nagaland, Kaziranga Reserve Forest in Assam and Garo hills of Meghalaya. Nevertheless, recent survey reports indicated the presence of this species only in the Tura ranges of Garo hills of Meghalaya (Upadhay and Sundriyal 1998; Singh 2003). In order to protect genetic erosion of citrus germplasm, Nokrek Biosphere Reserve has been nominated as Citrus Gene Sanctuary (Singh 1981) and here many of the citrus wild species are conserved. China initiated citrus germplasm conservation establishing the central citrus germplasm repository in 2007 at the Beibei, Chongqing, Sichuan province. Beside this Huazhong Agricultural University established citrus germplasm bank in where more than 400 accessions were collected and maintained. All these genotypes were collected from the different parts of China as well as around the globe. Similarly Brazil, USA, France, Spain, Italy collected citrus germplasm from the wild, semi-wild and cultivated species from the major citrus growing areas as well as from the center of origin of citrus.

Both in situ and *ex situ* germplasm conservation methods have been adopted for citrus. Among *ex situ* conservation methods, callus culture, cryopreservation, in vitro plant growth, can facilitate the citrus germplasm storage for long time. Tissue culture has been used to preserve disease-free citrus shoots. Also callus from citrus leaf tissue have been maintained on slow growth media for long term preservation. The most important activities for *ex situ* conservation of citrus germplasm have been conducted at the Citrus Research and Education Center, University of Florida, at Huazhong agricultural university, China and at Instituto Valenciano de Investigaciones Agrarias, Spain.

b. **Germplasm phenotyping and genotyping**

Proper phenotyping and genotyping of the germplasm is required for effective plant breeding programmes. Therefore plant breeders and geneticists used markers (both molecular and phenotypic traits) for characterization of the collected germplasm prior to utilizing them in breeding programme. Phenotyping and genotyping provide the opportunity to select best parental combination for the improvement of the cultivars. In addition the information on genetic background of any genotype is required for its proper management as well for its use in research and breeding. During the last few decades citrus breeders and researchers made efforts for citrus germplasm characterization, established phylogenetic relationship among the wild relatives and cultivated species and estimated genetic diversity among the citrus population. These basic information helps to overcome many citrus breeding obstacles. For proper genotyping citrus research community vastly uses molecular markers like SSR (Biswas et al. 2014), ITS, ISSR and SNP. These aspects will be further discussed in Chap. 7.

Although molecular marker-based citrus genotyping techniques are well established, a specific set of molecular marker that can be used for any citrus genotype identification is still missing. Recently, whole genome sequences of the main citrus species have been released (Xu et al. 2013; Wu et al. 2018). The whole genome sequence data could be useful to develop citrus species-specific markers and once developed, those markers could be very useful for the rapid citrus germplasm characterization, identification and their genetic relationships estimation. Eventually, citrus species-specific molecular markers could also accelerate the citrus breeding programmes.

Although many citrus genotypes are well characterized, most of them are rather unknown to young researchers and farmers due to their different name and lack of precise information. So it would be very useful if each unique citrus genotype could be encoded with a universal identifier name. This unique identification of the citrus genotypes may be helpful for a more precise germplasm management.

c. **Strategies for citrus genetic resources management**

Collected germplasm should be maintained and managed in proper way for its efficient use. The richest citrus germplasm collections have been held at national level by the most important citrus countries. A precise plan should be developed for monitoring, supporting, and conserving these collections. Global citrus germplasm network was established in 1997 with the aim of developing a global citrus germplasm management strategy; more than 60 delegates from the major citrus growing countries participate to this network and established a convention. According to this convention citrus germplasm curators and gene bank managers around the globe have been asked for the health and status of their collection using a specific questionnaire, which was developed and approved by the global citrus germplasm network. The survey report should be submitted to the collaborator at the University of California, Riverside and at the USDA ARS, Riverside. These strategies were implemented by the Global Crop Diversity Trust in collaboration with the International Society for Horticultural Science and several members of the international citrus research community (Roose et al. 2015).

d. **Free accessible online information on citrus germplasm**

Appropriate information about the germplasm including genetic background, cross compatibility, disease resistance, growing condition, origin, phylogenetic relationship with other members of the taxa, yield performances, agronomic traits etc., are useful for further use. These information can help breeders to take quick decision to design their experiment. In order to easily access information about genetic resources a few online genomic resource database have been developed for plant species. Among them, those concerning banana (https://www.crop-diversity.org/mgis/), tomato (https://tgrc.ucdavis.edu/index.aspx), lily (http://210.110.86.160/Lidb/Lilidb_Genotype.html), rice (http://www.ricediversity.org/proj/germplasm/repositories.cfm) are notable. Banana genetic resources database can be considered a model database for germplasm information for any plant species, since from this database users easily can access the present status of collected banana germplasm, as well as researchers can order genetic materials through the web site for research purposes. In addition this database is very rich of information on the collected banana germplasm and this information accelerate the banana germplasm conservation, management and breeding programme. Unfortunately, such kind of database is lacking for citrus species; therefore, it would be useful to develop a freely accessible online-based citrus genetic resources database in order to speed up the breeding process and to protect germplasm from genetic erosion.

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