Chapter 5
Citrullus

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5.1 Introduction

Watermelon is an important crop in the United States, whose farm value is estimated at $340 million (http://www.watermelon.org). Economic and nutraceutical importance of this crop is rapidly increasing throughout the world. During the last century, the importance of watermelon is steadily increased and accounts for 2% of the world area devoted to vegetable production (FAO 1995; Levi et al. 2001a, b). Enhancing disease and pest resistance of watermelon cultivars and improving their response to environmental stress can be accomplished by widening genetic diversity through hybridization with wild *Citrullus* accessions (Levi and Thomas 2005).

The family Cucurbitaceae consists of two well-defined subfamilies, eight tribes, 118 genera, and about 825 species (Robinson and Decker-Walters 1997; Jarret and Newman 2000). Cultivated watermelon and its wild relatives belong to the genus *Citrullus* of the subfamily Cucurbitoideae, tribe Benincaseae Ser., Subtribe Benincasinae (Ser.) C. Jeffrey (Robinson and Decker-Walters 1997). The name of *Citrullus* was first coined by Forskal in the year 1775 but H. Schrader was the first who classified the genus systematically, which was adopted by the Eighth International Botanical Congress, 1954 to be included in the *Nomina Conservanda* (Fursa 1972). Some of the morphological traits of taxonomic importance in various species of *Citrullus* genus are pollen structure, anatomy of fruits, seed structure, presence or absence of nectary flowers, characteristics of embryos, and variations in chromosome karyotypes. Genus *Citrullus* includes *C. lanatus* (var. *lanatus* (Thunb.) Matsum and Nakai., var. *citrides* (Bailey) Mansf.), *C. ecirrhosus* Cogn., *C. rehmii* De Winter., *C. colocynthis* (L.) Schrad, and *Acanthosicycos naudinianus* (Sond.) C. Jeffrey (Robinson and Decker-Walters 1997; Jarret and Newman 2000). Genus *Citrullus* in a wild state is distributed mostly in xerophytic habitats of the northern (*C. colocynthis*) and southern Africa (*C. lanatus*, *C. ecirrhosus*, *C. rehmii*, and *A. naudinianus*). *C. rehmii* and *C. lanatus* are monoecious annuals, whereas *C. colocynthis*, *C. ecirrhosus*, and *A. naudinianus* are perennials (Jarret and Newman 2000).

According to Meeuse (1962) and Pitrat et al. (1999), the species *Citrullus lanatus* ($n = 11$) originated in the Kalahari region of Namibia and Botswana (Bates and Robinson 1995; Robinson and Decker-Walters 1997; Ellul et al. 2007). Cultivated watermelon includes three subspecies: *C. lanatus* subsp. *lanatus* (Shrad. Ex Eckl. et Zeyh.), *C. lanatus* subsp. *vulgaris* (Shrad. Ex Eckl. et Zeyh.) Fursa, and *C. lanatus* subsp. *mucosospermus* Fursa (Levi et al. 2001a, b). On the other hand, now all these three species are under the var. group *lanatus* (Jeffrey 2001). Currently, the species *C. lanatus* (Thunb. Matsum and Nakai) includes two botanical varieties, namely var. *lanatus* (Bailey) and var. *citroides* (Mansf). Cultivated watermelons belong to var. *lanatus* and have endocarps in wide ranging colors. The var. *citroides* is cultivated in southern Africa, and also called "Tsamma" or "citron" melon, whose rind is used as preservative in pickles (Whitaker and Davis 1962; Fursa 1972; Whitaker and Bemis 1976; Burkill 1985; Jarret et al. 1997; Jeffrey 2001). The citron fruits have green- or white-colored flesh and their taste

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varies from bland to bitter. Seed production fields should be isolated from weedy citron types since these two botanical varieties cross readily (Wehner 2008). The species *Citrullus colocynthis* (Schrad) is a perennial herb known as bitter apple and is a desert species with a rich history as a medicinal plant (Dane et al. 2007). T.W. Whitaker considered *C. colocynthis* to be a likely ancestor of watermelon as it is morphologically similar to *C. lanatus*, and is freely intercrossable and produces fertile hybrids (Wehner 2007). Dane et al. (2007) reported divergent lineages of *C. colocynthis* that are from tropical Asia and Africa, now widely distributed in the Saharo-Arabian phylogeographic region of Africa and in the Mediterranean region. In earlier reports, isozyme and random amplified polymorphic DNA (RAPD) markers were used extensively in molecular diversity and phylogenetic analyses in *Citrullus* spp. (Zamir et al. 1984; Navot and Zamir 1986; Biles et al. 1989; Levi et al. 2001a, b).

5.2 Phylogenetic Relationships

Phenetic relationships among the main *Citrullus* species and subspecies were examined by using isozymes (Zamir et al. 1984; Navot and Zamir 1987) and nuclear DNA markers (Jarret et al. 1997; Levi et al. 2000). Dane et al. (2004) summarized domestication of cultivated watermelon to be ancient and its cultivation dates back to pre-historic times. It was grown by the ancient Egyptians (Robinson and Decker-Walters 1997). Dane and Lang (2004) further reviewed about introduction of watermelons to Europe by the Moors during their invasion of Spain and to the Americas in the seventeenth century on slave ships and have been cultivated ever since in the western hemisphere. The domesticated watermelon is classified as *C. lanatus* var. *lanatus*, whereas wild citron, which is common in central Africa, is classified as var. *citroides* (Bailey) Mansf. Citron is a preserving melon as its rind is used to make pickles (Dane et al. 2004). Wehner (2008) reported that the fruits of *citroides* are used for food for livestock in Africa. In West Africa, especially Nigeria, Egusi-type watermelons with bitter fruit (*citroides*) are cultivated for their seeds as they have high edible oil content. Dane et al. (2004) reported cpDNA variation after studying 55 *C. lanatus* types and 15 *C. colocynthis* that are from diverse geographical areas. This study revealed insertion–deletion sites (Indels) at *ndh*A, *trn*S–*trn*fM, and *trn*C–*trn*D regions of cpDNA along with single nucleotide polymorphisms (SNPs) to separate *lanatus* and *colocynthis* species. Dane and Lang (2004) also reported diagnostic SNPs at *ndh*F and *trn*C–*trn*D of chloroplast to distinguish between the var. *lanatus* and var. *citroides*, respectively. In this study, several indels at *ndh*A, *trn*S–*trnf*M and *trn*C–*trn*D regions, and several substitutions at restriction sites were characterized between *colocynthis* and *lanatus*. Dane and Liu (2007) critically studied var. *lanatus* and var. *citroides* using PCR-based restriction fragment length polymorphism (RFLP) such as cleaved amplified polymorphic sequence (CAPS) of chloroplast regions to conclude that they had a common ancestor and resolved subspecies-specific haplotype fixation. This study identified that the ancient *citroides*-type haplotype originated in Swaziland and South Africa and followed colonization routes from these areas to all over the world. Levi and Thomas (2005) used 20 cpDNA and 10 mitochondrial DNA probes for RFLP analysis for phylogenetic analysis. A combined analysis of large data sets (3,089 AFLPs and 127 SSR alleles) by Nimmakayala et al. (2010) provided strong evidence of phylogenetic signal that clearly resolved a tree with three clusters of *lanatus*, *citroides*, and *colocynthis* supported by significant bootstrap values. In this study, tree topologies inferred by Neighbor-Joining analysis have resolved the phylogenic relationships among the species with special reference to established taxonomic classification. Further, boundaries of various taxa belonging to *citroids*, *lanatus*, and *colocynthis* could be drawn. Clustering pattern of principal coordinate analysis (PCA) with the shared polymorphisms using the subsets of data between any two taxa combinations helped to elucidate the introgression and interrelationships among the species. This research resolved two major groups of *lanatus* taxa, one of which has undergone wide introgressions with the taxa of *citroids* and *colocynthis*.

Dane et al. (2007) characterized phylogeography of the species *C. colocynthis*, a non-hardy species, which is predominantly drought-resistant perennial herbaceous vine, now widely distributed in the Sahar-Arabian region in Africa and also in the Mediterranean region. This species was characterized by angular stems, lobed leaves, solitary pale yellow

flowers, and can produce up to 15–30 fruits (Dane et al. 2007). Seeds are small, smooth, and brownish in color (Robinson and Decker-Walters 1997). Dane et al. (2007) further summarized that these species were known since Biblical times as bitter apple and were used to extract deadly poison. Fruits are widely used medicinally as laxative because of colocynthin content. The seeds are edible and used to make bread as well as extract oil (17–19% oil with 80–85% unsaturated fatty acids) (Dane et al. 2007). The oil is edible and also useful for candle light, medicinal, and industrial purposes (Zohary and Hopf 2000). *C. colocynthis* primarily accumulates citrulline under drought conditions, which contributes to oxidative stress tolerance (Yokota et al. 2002). Dane et al. (2007) characterized several polymorphic intergenic cpDNA and a relatively large intron (0.6 kb) of G3*pdh* to resolve geographical structure among the world collections of *C. colocynthis*. The study revealed the migration of the species from Africa into Middle East and Far East. This study also revealed divergent haplotypes in *C. colocynthis* population based on differential patterns of adaptation.

Jarret and Newman (2000) amplified internal transcribed spacer regions (ITS1 and ITS2) of the 18S–25S nuclear ribosomal DNA on: *C. lanatus* (var. *lanatus* (Thunb.) Matsum and Nakai., var. *citrides* (Bailey) Mansf.), *C. ecirrhosus* Cogn., *C. rehmii* De Winter., *C. colocynthis* (L.) Schrad, and *A. naudinianus* (Sond.). Cladistic and phenetic analysis in this study resulted in robust tree placing the species *C. rehmii* closure to the clade of *C. lanatus*. This study confirmed the species status to *C. rehmii* and indicated its closeness to the cultivated watermelon than to the species *C. colocynthis*. Phenetic analysis in this study resolved the branch separating *C. rehmii* and *C. lanatus* from the species *C. ecirrhosus* with high bootstrap values. The terminal placement of annual species *C. rehmii* and *C. lanatus*, relative to the xerophytic perennials *C. ecirrhosus* and *C. colocynthis* in this investigation, supported the argument of Jobst et al. (1998) concerning the derivation of annual species from perennial forms. Leaves of *C. rehmii* more closely resembled to those of *C. lanatus* rather than to those of *C. ecirrhosus*, in which the leaves were distinctly rigid with strongly recurved margins (Meeuse 1962). The fruits of *C. ecirrhosus* are hard and bitter with ellipsoid shape and maturity duration of 60 days after anthesis (Meeuse 1962). The fruits of *C. rehmii* resembled more to *C. lanatus*, in which the fruits are not hard and bitter but globose in shape and mature within 30 days post-anthesis (Jarret and Newman 2000). Martyn and Netzer (1991) reported that the species *C. ecirrhosus* harbors several important genes for disease resistance.

5.3 Genetic and Genomic Resources

5.3.1 Molecular Markers, ESTs, and Unigenes

A large number of fruit-related expressed sequence tags (ESTs) were developed by Ok et al. (2000) and Levi et al. (2009). Several genomic and EST-specific simple sequence repeat (SSR) markers were developed in *Citrullus* species. Very interestingly, the SSRs developed for watermelon will amplify as well as show polymorphism indicating their transportability across the other species/genera of cucurbits including melon, cucumber, squash, and pumpkin (Figs. 5.1 and 5.2). The gel picture presented in Fig. 5.1 represents amplification pattern of a fruit-specific EST SSR across various cucurbit genera. Figure 5.2 is from the summary of transportability of 124 SSRs, showing amplifications and polymorphic levels across the genera.

Nimmakayala et al. (2010) amplified 127 alleles using a set of 42 SSRs in 31 watermelon accessions. A range of 2–15 alleles were amplified per SSR. The number of specific alleles within the group was 20, 13, and 7 specific to var. *lanatus*, var. *citroides*, and

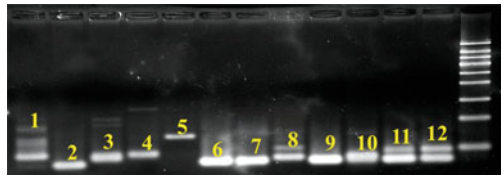

Fig. 5.1 Gel showing resolution of a microsatellite across various genera Cucurbitaceae family. 1. *Citrullus colocynthis* (PI386016), 2. *Citrullus lanatus* var. *citroides* (PI482252), 3. *Citrullus lanatus* var. *lanatus* (PI 248178), 4. *Lagenaria siceraria,* 5. *Momordica charantia,* 6. *Cucumis melo* var. *aestivalis,* 7. *Cucumis melo* var. *europius*, 8. *Cucurbita moschata,* 9. *Cucurbita pepo,* 10. *C. pepo* ssp. *Texanana,* 11. *C. pepo* ssp. 12. *Fraterna*, and 13. *C. pepo* ssp. *Ozarkana*

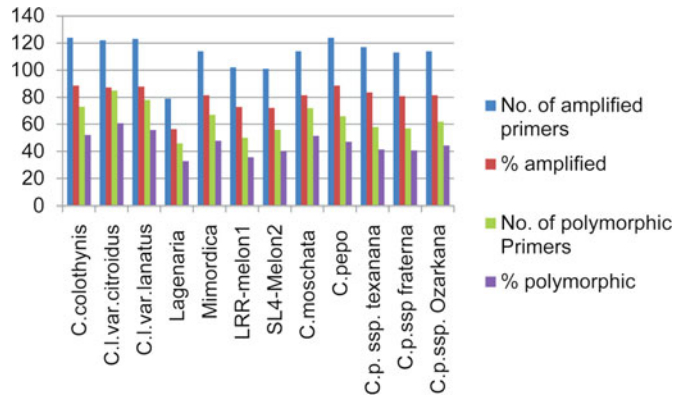

Fig. 5.2 Polymorphic levels of various microsatellites used across various *Citrullus* species and the other cucurbit genera

C. colocynthis, respectively. The shared alleles between *lanatus* and *citroides* were 38, *citroides* and *colocynthis* were 17 and *colocynthis* and *lanatus* were 25. SSRs are simple-to-use, multiallelic, and codominant marker systems that are sequence-based and produce highly repeatable amplifications. The SSRs in this study generated important diagnostic markers that are species-specific and can be of immense use for resolving species conflicts that are reported to exist between *lanatus* and *citroides*. A large set of fruit-specific ESTs and assembled unigene resources for various cucurbit crops are available at http://www.icugi.org. However as stated, the SSRs mined from these ESTs (about 315 can be accessed at http://www.icugi.org) are usable and when tested on reference accessions of *Citrullus* spp., all the SSRs amplified single products and a large number of them were polymorphic (U. Reddy unpublished data). This resource will help to develop syntenic maps across the cucurbit species and also will aid to identify heterologous locations for important horticultural and disease-resistant traits. In addition, Dane et al. (2004) identified diagnostic markers using cpDNA haplotypes for *lanatus* and *citroides* types and used them to track lineages with *C. rehmii* and *C. ecirrhosus*. Jarret et al. (1997) developed seven SSRs and used them to amplify 32 watermelon genotypes; they found that SSR-derived polymorphisms are very efficient in discriminating among various species. Guerra-Sanz (2002) identified 19 SSRs from cDNA sequence data.

We recently isolated SSR markers in large scale from a single run of watermelon genomic DNA using 454 Life Sciences sequencing technology. We have characterized a total of 2,143 contigs that contain a total of 2,727 SSRs from a pool of 13,176 contigs of 454 sequencing reads. We identified 1,025 SSR motifs that could be used as potential molecular markers based on their longer repeat lengths and quality of flanking sequence for primer design (U. Reddy unpublished data). Out of 2,727 total isolated microsatellite regions, 1,346 were dinucleotide repeats (DNRs), 980 trinucleotide repeats (TNRs), 287 tetranucleotide repeats (TTNRs), 83 pentanucleotide repeats (PNRs), and 24 SSRs with hexanucleotide repeats. Dinucleotide repeats constituted 49.36% of the total identified repeats. The most common motif type of DNRs was TA/AT (67.68% of DNRs) followed by AG/CT (24.45% of DNRs) and AC/GT (7.88% of DNRs) in watermelon genome.

5.3.2 Genetic Maps and QTLs

Densely-saturated genetic maps are very important in breeding programs of crop plants. They are useful for locating genes or quantitative trait loci (QTL) of various traits (Lee 1995). Extensive linkage maps have been constructed for such cucurbits as melon (Baudracco-Arnas and Pitrat 1996; Wang et al. 1997; Brotman et al. 2000; Oliver et al. 2000, 2001; Perin et al. 2002) and cucumber (Park et al. 2000; Staub and Serquen 2000). However in watermelon, only a few linkage maps with minimal coverage have been reported (Navot and Zamir 1986; Navot et al. 1990; Hashizume et al. 1996; Xu et al. 2000; Hawkins et al. 2001).

The first genetic map of watermelon was constructed by Navot and Zamir (1986) in a segregating population of *C. lanatus* × *C. colocynthis*, and later

extended by Navot et al. (1990) into seven linkage groups covering a length of 354 cM. Hashizume et al. (1996) constructed a linkage map of 11 linkage groups spanning only 524 cM with 58 random amplified polymorphic DNA (RAPD), one isozyme, one restriction fragment length polymorphism (RFLP), and two morphological markers. In 2003, Hashizume et al. constructed another linkage map using an F_2 population with 477 RAPD, 53 RFLP, 23 intersimple sequence repeat (ISSR), and one isozyme markers that covered 2,384 cM. Levi et al. (2001a, b) constructed a linkage map of 17 linkage groups using 155 RAPD markers and a sequenced characterized amplified region (SCAR) marker covering 1,295 cM in a backcross population [PI 296341 (*C. lanatus* var. *citroides*) × New Hampshire Midget (NHM, *C. lanatus* var. *lanatus*)] × NHM. Another linkage map was constructed by Levi et al. (2002) using a testcross population [Griffin 14113 (*C. lanatus* var. *citroides*) × NHM] × PI 386015 (*C. colocynthis*) with 141 RAPD, a SCAR, and 27 ISSR markers segregating in 25 linkage groups covering a total distance of 1,166 cM. Lately, they added another 114 amplified fragment length polymorphism (AFLP) markers to this map (Levi et al. 2006). However, a significant part of the genome (watermelon genome size 425 Mb; Arumuganathan and Earle 1991) has not been saturated yet, and a good number of markers therefore are still needed for construction of a high-density map.

Many economically important traits of crop plants are inherited as quantitative traits. The phenotypes appear to be conditioned by several loci with strong environmental effects. Quantitative traits were usually analyzed using biometrical models before the discovery of mapping techniques, and the biometrical approach cannot completely explain the effects of individual QTL controlling a trait. In recent years, the availability of DNA markers coupled with biometric methods has helped to make considerable progress in QTL mapping. The joint analysis of marker segregation and phenotypic values of individuals or lines in QTL mapping enables the scientists to detect and locate the loci governing quantitative traits (Asins 2002). QTL analysis not only provides DNA markers for efficient marker-assisted selection (MAS) in plant breeding, but also resolves the interacting environmental effects on important yield-related traits.

So far the detection and mapping of QTL for interesting agronomic traits are hindered by the scarcity of molecular markers (Danin-Poleg et al. 2002). Unfortunately, there are very few markers identified so far in watermelon which are linked to important agronomic traits. A molecular marker linked to resistance to *Fusarium* (Xu et al. 2000) and two other QTLs controlling fruit traits, viz. rind hardness and Brix of flesh juice (Hashizume et al. 2003), have been reported. Special efforts are currently underway to identify QTLs for important traits as well as to develop recombinant inbred lines that will facilitate extensive phenotypic evaluation at multiple locations (U. Reddy unpublished data).

5.4 Fluorescent In Situ Hybridization and Chromosome Organization in var. *lanatus* and var. *citroides*

Hereunder is presented the first attempt of fluorescent in situ hybridization (FISH) in var. *lanatus* (PI 270306) and its wild counterpart var. *citroides* (PI 244018), using 18S–28S rDNA and 5S rDNA probes. Well-separated somatic chromosomes were prepared from root meristems, using enzyme digestion technique for hybridization following the standard techniques (Islam-Faridi et al. 2007). Chromosome spreads that are presented below present very interesting chromosome organization between cultivated watermelon (Figs. 5.3 and 5.4) and its wild counterpart *C. lanatus*

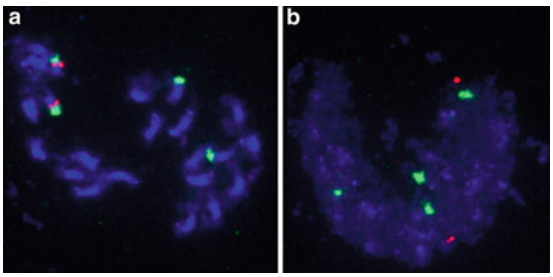

Fig. 5.3 FISH with 18S–28S rDNA (*green signals*) and 5S rDNA (*red signals*) on watermelon (var. *lanatus*, accession # PI 270306) chromosome spread. (**a**) Late prophase chromosome spread cell, and (**b**) interphase cell of var. *lanatus*

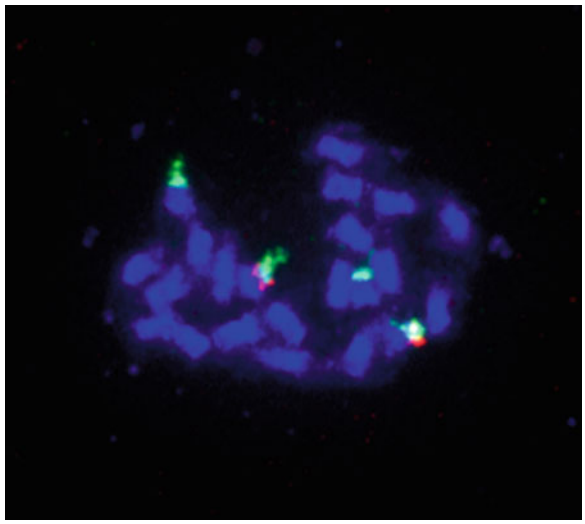

Fig. 5.4 A prometaphase stage in var. *lanatus*

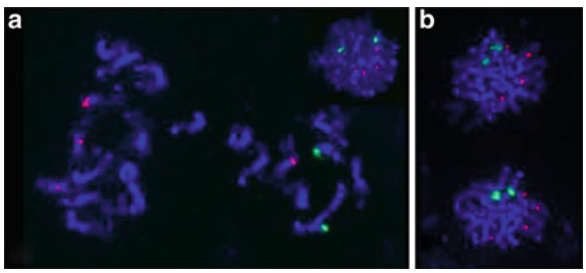

Fig. 5.5 FISH with 18S–28S rDNA (*green signals*) and 5S rDNA (*red signals*) probes on *citroides* chromosome spreads. (**a**) Mid prophase chromosome spread and showing two major 18S–28S rDNA signals, four 5S rDNA signals, and an interphase nucleus also showing two green signals from 18S–28S rDNA probe, (**b**) A late anaphase chromosome spread with the same kind of hybridization pattern

var. *citroides*. In *lanatus* spread, we noticed that there are two 18S–28S rDNA sites and one 5S rDNA site (Figs. 5.3a, b and 5.4). The 5S rDNA site is located interstitially and appears to be syntenic to one of the 18S–28S rDNA sites. As revealed by the interphase FISH (Fig. 5.3b), the sites of 18S–28S rDNA and 5S rDNA site are not linked. In contrary, there were three different sites of rDNA in *Citroides* accession (PI 244018) (Fig. 5.5a, b), one was for 18S–28S rDNA and two were for 5S rDNA, and all were on three different chromosomes. These results clearly indicate that there are major structural differences between these subspecies.

5.5 Conclusions

Enhancing disease and pest resistance in watermelon cultivars to improve their response to drought and other biotic resistance is possible through hybridization with wild *Citrullus* accessions as all the cultivars are freely crossable and produce fertile hybrids. Since the EST, unigene, and SSRs are highly conserved across the *Citrullus* genera, it is possible to develop dense maps with the positions of map locations of important traits. Generating extensive molecular cytogenetics resources, such as FISH, will allow integrating the mapping information, BACs/physical maps, and other probes that allow precise karyotype analysis of various species. Mapping endeavors such as high-throughput genotyping, developing reciprocal recombinant inbred populations, multiple environment evaluation, and QTL localization will speed up the introgression process as well as launch watermelon breeding in the new era that would impact the productivity and quality of watermelon cultivars.

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