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Study of Genetic Variability in Citrus Fruit Crop by Molecular Markers - A Review

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ABSTRACT

Markers have been used over the years for the classification of plants. Markers are any trait of an organism that can be identified with confidence and relative easy, and can be followed in a mapping population on another hand markers be defined as heritable entities associated with the economically important trait under the control of polygenes. Morphological markers can be detected with naked eye (naked eye polymorphism) or as difference in physical or chemical properties of the macromolecules. In other words, there are two types of genetic markers viz. morphological markers or naked eye polymorphism and non-morphological markers or molecular markers.

Application of molecular markers, have now been increasingly adopted to address the problems in Citrus taxonomy. Compared to morphological data, molecular tools provide abundant information, highly efficient and are insensitive to environmental factors. Molecular markers has provided an ideal means for identifying genotypes, estimation of relatedness between different accessions and following inheritance of economically important characters. In Citrus, a wide variety of DNA based markers has been used in order to study their genetic variation as well as phylogenic and taxonomic relationship among different genera. RAPD markers provide a fast and easy approach for taxonomic classification and cultivar typing of Citrus fruits. SSR have proven to be the marker of choice in Citrus breeding research, because of their variability, ease of use, accessibility of detection and reproducibility. ISSR, SRAP, CAPSSNP, AFLP are also used to study the genetic diversity of Citrus throughout the world. In addition, cpDNA is especially useful in phylogenetic analyses due to its evolutionary conservatism, relative abundance in plant tissue, small size and pre dominant uniparental inheritance.

Key words: *Citrus, Genomics, Molecular characterization, Microsatellites, Molecular markers, AFLP, ISSR, RAPD, SSR, Polymorphism, Genetic diversity*

INTRODUCTION

Citrus is one of the most important and widely grown of the fruit crops, with total production in India is reported to be 1077.73 thousand hectare area and 11147.06 thousand metric ton production in 2013-2014[75]. Citrus fruit is produced throughout the tropical and subtropical regions of the world, where the winter temperatures are adequate for tree survival and avoidance of freeze devastation, and where there is sufficient water and suitable soils to support tree growth and fruit production. It is widely grown in most areas with suitable climates tropical, subtropical, and borderline subtropical/temperate[56].

The genus Citrus L. belongs to the subtribe Citrineae, the tribe Citreae within the subfamily Aurantioideae of the Rutaceae family[111]. The Aurantioideae is one of seven subfamilies of Rutaceae which consists of two tribes and 33 genera. Each of tribes Clauseneae and Citreae is composed of three subtribes. Clauseneae includes Micromelinae, Clauseninae and Merrillinae, and Citreae has Triphasiinae, Citrinae and Balsamocitrinae. The Citrinae is distinct from all the other subtribes in the subfamily by having pulp vesicles in the fruit. This subtribe contains three groups; primitive citrus fruit, near citrus fruit, and true citrus fruit trees. True citrus fruits have six genera: Clymenia, Eremocitrus, Microcitrus, Poncirus, Fortunella and Citrus[101].

Most of genus including Citrus belongs to subfamily Aurantioideae originated from Monsoon regions and expand from West Pakistan to China, India islands, Northwest Australia, New Guinea. In this subfamily, four of 33 genus (Afraegle, Aeglopsis, Balsamocitrus and Citropsis) native to tropical Africa an one genus (Clausena) native to Monsoon and tropical Africa. Besides, Microcitrus and Eremocitrus originated from Australia[104].

Despite its manifold economic importance and increasing demands in the global Citrus industry, Citrus taxonomy and phylogeny are controversial and confusing mainly due to the sexual compatibility between Citrus and its related genera, high frequency of bud mutations, long history of cultivation, and wide dispersion[73]. Many of Citrus cultivars are very closely related, apparently having diverged by mutations that alter specific horticultural traits. In addition, many Citrus cultivars produce apomictic seedlings and nucellar seedlings that differ in horticultural traits. Similarly, the level of difference in relation to species status in Citrus is uncertain. Consequently, there has been no consensus among the taxonomists as to the actual number of species that constitute the genus Citrus. In addition, taxonomic characterization leading to unambiguous identification of Citrus species and their genetic resources are essential requisites for Citrus breeding. To this end, molecular markers based on DNA sequences are being widely used in studying polymorphism between species or in populations. The application largely depends on the type of markers employed, distribution of markers in the genome, type of loci they amplify, level of polymorphism and reproducibility of products[31,109].

History of Molecular Markers

The concept of genetic markers is not a new one. Gregor Mendel used phenotype-based genetic markers in his experiment in the nineteenth century. The phenotype based genetic markers for Drosophila led to the establishment of the theory of genetic linkage[3]. The limitations of phenotype based genetic markers led to the development of DNA based markers. The publication of Botstein *et al*[13]., about the construction of genetic maps using RFLP was the first reported molecular marker technique[3]. Thereafter, the advent of the Polymerase Chain Reaction (PCR) and the use of arbitrarily designed primers that did not require any knowledge of the DNA sequence of the species have been made. These so called random amplified polymorphic DNA (RAPD) markers[112] were easy to produce for a negligible cost in terms of labour and investment, and thus quickly became popular. They were soon paired with another kind of marker produced with the use of arbitrarily designed oligonucleotides; Amplified

Fragment Length Polymorphism (AFLP) markers[110]. Microsatellites or SSRs (simple sequence repeats) are PCR-amplified as single loci in diploid genomes; they are co-dominant and therefore all alleles are displayed; they are highly polymorphic, often with dozens of alleles. SSR-based fingerprinting is becoming more and more popular in many horticultural species. Single Nucleotide Polymorphism (SNP) is another class of markers that can be identified by comparing DNA sequences[84].

Markers

A molecular marker is defined as a particular segment of DNA that is representative of the differences at the genome level. An ideal marker should be polymorphic, independent, and reliable, providing sufficient resolution relatively easily, quickly and with fairly low costs[88]. Development and utilization of molecular markers to detect differences in the DNA of individual plants has many applications in crop improvement in fruits. These differences are known as molecular markers because they are often associated with specific genes and act as 'signposts' to those genes[42]. In addition, markers and comparative mapping of various species have been very helpful in enhancing our understanding of genome structure and function.

Use of molecular markers has more advantages than that of morphologically based phenotypic characterization, because molecular markers are generally unaffected by external impact. It is possible to compare accessions of a collection at any time of year using molecular markers, while phenotypic characteristics can be influenced by environmental or cultural affects (The Citrus and Date Crop Germplasm Committee, USA, CDCGC, 2004). Regarding to germplasm management molecular characterization has a number of applications such as relationships between accessions, characterizing newly acquired germplasm, monitoring shifts in population genetic structure in heterogeneous germplasm, exploiting associations among traits of interest and genetic markers and genetic enhancement.

The markers have been used over the years for the classification of plants. Markers are any trait of an organism that can be identified with confidence and relative ease, and can be followed in a mapping population with other words; they can be defined as heritable entities associated with the economically important trait under the control of polygenes[10]. Morphological markers can be detected with naked eye (naked eye polymorphism) or as difference in physical or chemical properties of the macromolecules. Therefore, there are two types of genetic markers, respectively: morphological markers or naked eye polymorphism and non-morphological markers or molecular markers.

Molecular markers are now widely used to track loci and genome regions in several crop-breeding programmes, as molecular markers tightly linked with a large number of agronomic and disease resistance traits are available in major crop species[48,51,87,108]. These molecular markers include: (i) hybridization-based markers such as restriction fragment length polymorphism (RFLP), (ii) PCR-based markers: random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellite or simple sequence repeat (SSR), and (iii) sequence-based markers: single nucleotide polymorphism (SNP). The majority of these molecular markers has been developed either from genomic DNA libraries (e.g. RFLPs and SSRs) or from random PCR amplification of genomic DNA (e.g. RAPDs) or both (e.g. AFLPs). These DNA markers can be generated in large numbers and can prove to be very useful for a variety of purposes relevant to crop improvement. For instance, these markers have been utilized extensively for the preparation of saturated molecular maps (genetical and physical). Their association with genes/QTLs controlling the traits of economic importance has also been utilized in some cases for indirect marker-assisted selection (MAS)[60,61]. Other uses of molecular markers include gene introgression through backcrossing, germplasm characterization,

genetic diagnostics, characterization of transformants, study of genome organization and phylogenetic analysis[51]. For plant breeding applications, SSR markers, among different classes of the existing markers, have been proven and recommended as markers of choice[47]. RFLP is not readily adapted to high sample throughput and RAPD assays are not sufficiently reproducible or transferable between laboratories. While both SSRs and AFLPs are efficient in identifying polymorphisms, SSRs are more readily automated[96]. Although AFLPs can in principle be converted into simple PCR assays (e.g. STSs), this conversion can become cumbersome and complicated as individual bands are often composed of multiple fragments[95], particularly in large genome templates.

Morphological Markers

Morphological markers are those traits that are scored visually, or morphological markers are those genetic markers whose inheritance can be followed with the naked eye. The traits included in this group are plant height, disease response, photoperiod, sensitivity, shape or color of flowers, fruits or seeds etc. Although they are generally scored quickly, simply and without laboratory equipments, such markers are not put too much use, because of the following reasons: genotypes can be ascertained generally at whole plant or plant organ level and frequently the mature plant is used. Such markers frequently cause major alternations in the phenotype which is undesirable in breeding programs. Dominant, recessive interactions frequently prevent distinguishing all genotypes associated with morphological traits. Morphological markers masks the effect of linked minor gene, making it nearly impossible to identify desirable linkages for select and are limited in number, influenced by environment and also specific stage of the analysis. Non-morphological markers or molecular markers until recently virtually all progress in both breeding and modern genetics have relied on the phenotypic or morphological assay. But with the advent of molecular markers a new generation of markers was introduced over the last two decades that have become an important tool in the genetic improvement of crop species and has changed the entire scenario of biological sciences. Molecular markers are any kind of molecule indicating the existence of a chemical or a physical process. Molecular markers include biochemical constituents (e.g. secondary metabolites in plants) and macromolecules (e.g. proteins and deoxyribonucleic acid)[54]. These macromolecules show easily detectable differences among different strains of a species or among different species. Strauss *et al*[100]., distinguished the molecular markers into two classes. Biochemical molecular markers derived from the chemical products of gene expression i.e. protein based markers and molecular genetic markers derived from direct analysis of polymorphism in DNA sequences i.e. DNA based markers presented in (Table.1)

Table 1. Comparison between morphological, isozyme and DNA markers

Feature	Morphological Markers	Biochemical molecular markers	DNA based markers
Feature of the organism scored	Phenotype	Protein	DNA base sequence
Biological meaning of the markers	Consequences of gene action	Genes that are expressed	DNA sequences, may or may not represent genes
Plant material required for detection	Intact plant or plant organ	Little amount of tissue	Little to medium amount of tissue and no matter what tissue is used
Efforts required for detection	Simple	Moderate	Moderate to difficult
Ease of use	Very easy	Moderately difficult	Moderately difficult to difficult
Reproducibility	High	High	Moderate to high
Dominance/ Codominance	Generally dominant	Codominant	Dominant (RAPD, AFLP) Codominant (RFLP, SSR)

Biochemical molecular markers

The first biochemical molecular markers used were the protein based markers. Proteins are attractive for direct genetic study because they are the primary products of structural genes. Changes in coding base sequence will under many circumstances, resulting in corresponding changes in the primary structure of proteins. Even single amino acid substitutions, deletions or additions can have marked effects on the migration of proteins under an electric field during electrophoresis. One of the earliest protein based markers to be used was Isozyme. Market and Moller[71] coined the term to describe the multiple molecular forms of the same enzyme with the same substrate specificity. Isozymes are different forms of an enzyme exhibiting the same catalytic activity but differing in charge and electrophoretic mobility. In Isozyme analysis, crude plant extracts are subjected to electrophoresis using starch or polyacrylamide gels. Following electrophoresis, the enzymes of interest are detected by treating the gels with specific activity stains. Variation in bending patterns obtained between individual samples can be used to sort out genetically the varieties tested.

DNA based markers

DNA contains individual genetic blue print. The sequence of nucleotides in DNA of an individual is unique and thus determines its identity. The ultimate difference between individuals lies in the nucleotide sequence of their DNA. The detection of such differences employing different molecular biological techniques led to the development of DNA markers. On plants DNA markers were first developed in 1985-86 by two groups of researchers working independently at native plants incorporated, USA and Cornell University Ithaca USA. DNA markers should not be considered as normal genes, as they usually do not have any biological effect and instead can be thought of as constant landmark in the genome. DNA markers are the identifiable DNA sequences found at specific locations on the chromosomes and transmitted by the standard laws of inheritance from one generation to the next one. They rely on DNA assay in contrast to morphological markers based on visible traits and biochemical molecular markers based on protein products by gene. So DNA is an ideal molecule for studying polymorphism. DNA markers can be used to diagnose the presence of the gene without having to wait for gene effect to be seen[86].

Use of Molecular Markers in Citrus

Molecular techniques such as RAPD, RFLP[2,53], AFLP and microsatellite markers[36] have been used to identify citrus species with high accuracy. SSRs have been recognized as good sources of genetic markers in many plants including citrus[59]. The existence of microsatellite sequences was first reported in citrus in 1995[58]. Many differences among mandarin cultivars had been reported by Fang and Roose[28].

ISSR were analyzed by Fang *et al*[29]., to study phylogenetic relationships among 46 citrus L. accessions representing 35 species. RAPD markers were used by Abkenar and Issikis[1] to evaluate genetic similarity and inter-relationship among 31 acid citrus species and cultivars, including sour oranges (six accessions), 'Yuzu' (four accessions) and its relatives. Studies on development and characterization of microsetallite markers in citrus were conducted by Ahmad *et al*[4]. They concluded that microsatellite markers were able to identify cultivars at species level but individual cultivars within each species, believed to be evolved from mutation, were undistinguishable. Molecular polymorphisms among 370 mostly sexually derived citrus accessions from the collection of citrus germplasm maintained at the University of California, Riverside was detected by utilizing 24 SSR markers by Barkley *et al*[8]. Twenty four microsatellite loci on 12 genotypes of Citrus, Poncirus, and an intergeneric hybrid were evaluated by Yaly *et al*[114]. SRAP markers were studied by Uzun *et al*[107]., to evaluate genetic diversity among 27 grapefruit (C. paradisi Macf.), 5 pummelo (C. maxima (Burm.) Merr.) accessions and 4 pummelo hybrids.

Molecular differentiation in 24 accessions representing 19 taxa of Indian citrus through sequence analysis of ITS region of nrDNA (nuclear ribosomal DNA) were studied by Kumar *et al*[62]. First genome, based exclusively on Sanger sequencing, is from a haploid plant derived from 'Clementine' mandarin, to serve as the reference genome for citrus[38]. The genetic control of apomixis was studied by Garcia *et al*[34]., in a 50-tree progeny derived from the cross C. volkameriana and Poncirus trifoliata using 69 molecular markers and bulked segregant analysis. They reported that one of the markers associated to apomixis (Apo2) is also associated to embryony type. They further revealed that the genetic control of apomictic reproduction found in citrus (nucellar embryony) s quite complex compared to what has been reported for gametophytic apomixis. Molecular markers linked to QTLs governing apomixis will be useful to assist selection of future apomictic rootstocks for citrus varieties. Characterization was done in 65 mandarin accessions by using simple sequence repeat (SSR-14) and sequence-related amplified polymorphism (SRAP-21) based molecular approaches by Kacar *et al*[55].

The characterization of 'Daisy', a hybrid between 'Fortune' and 'Fremont' mandarins was studied by Nicotra[79]. The combination of visual selection of leaf apex morphology and SSR analysis for the identification of hybrids derived from the cross of polyembryonic was studied by Carlos *et al*[16]. ISSR marker for identification of zygotic and nucellar seedlings in citrus interploid crosses were examined by Tusa *et al*[103]. RAPD and Expressed Sequence Tag (EST)-SSR markers were used to characterize the zygotic and nucellar seedlings after introgression crosses of mandarin (C. reticulata) and pummelo (C.maxima) by Rao *et al*[90].

Zygotic and nucellar seedlings in citrus interspecific hybridizations were identified by utilizing inter simple sequence repeat markers by Golein *et al*[41]. They concluded that ISSR analyses are very efficient and reliable for identification of hybrids in polyembryonic citrus cultivars. Golein *et al*[40]., investigated phylogenic relationships among 'Bakraee' and some commercially important citrus varieties through SSR and PCR-RFLP molecular markers.

Assessing genetic variability in male sterile and low fertile citrus cultivars utilizing simple sequence repeat markers (SSRs)[40]. In this study, the genetic diversity of 28 accessions of citrus including male sterile, sterile, low fertile and fertile cultivars were investigated using eight pairs of simple sequence repeat markers (SSR) markers, which in total, 54 polymorphic alleles with an average of 4.2 alleles per primer were detected. The lowest number of alleles was observed in TAA27, CTT01, CCSM18 and ATC09 loci with only three alleles and the highest number of alleles was observed in TAA15 locus with eight alleles. Polymorphic information content (PIC) values changed from 0.34 (AG14) to 0.90 (CCSM18).

Knowledge of genetic variation and genetic relationship among genotypes is an important consideration for classification, utilization of germplasm resources and breeding. The genetic diversity and structure of plant populations reflect the interaction of many factors, including the long-term evolutionary history of the species (e.g. shifts in distribution patterns, habitat fragmentation, and population isolation), mutation, genetic drift, mating system, gene flow and selection[92,98]. All of these factors can lead to complex genetic structuring within populations, and losses of genetic diversity, with severe potential consequences since genetic variation at the intra specific level is a prerequisite for future adaptive change or evolution[93]. Thus, understanding the genetic variation within and among populations is essential for the establishment of effective and efficient conservation programs for rare plants[32].

Use of morphological traits may be helpful but often inadequate in differentiation of closely related cultivars. On the other hand, certain morphologically different variants may

be phylogeneticly closely related. In addition morphological traits are highly influenced by the environment[29]. Thus, using morphological traits, it can be difficult to distinguish between many Citrus cultivars[28]. Since morphological characters are only of limited use and cytogenetical parameters are time consuming, alternate approaches, including application of appropriate molecular markers, have now been increasingly adopted to address the problems in Citrus taxonomy[62]. Compared to morphological data, molecular tools provide abundant information, are highly efficient and are insensitive to environmental factors. Molecular markers has provided an ideal means for identifying genotypes, estimation of relatedness between different accessions and following inheritance of economically important characters. These techniques allow the analysis of variation at the genomic level and permit detection of genetic variation at the genomic level. Therefore, information obtained from the molecular level could be used to assess genetic relationships among the major germplasm groups. A better understanding of the effectiveness of the different molecular markers is considered a priority step towards germplasm classification and characterization, and a prerequisite for more effective breeding programs[11]. They represent one of the most powerful tools for the analysis of genomes and enable the association of heritable traits with underlying genomic variation[27]. Consequently, it is used widely in a range of applications including cultivar identification[12,72], phylogenetics studies[82], zygotic and nucellar seedlings identification[81] in Citrus.

In Citrus, a wide variety of DNA based markers has been used in order to study their genetic variation aswell as phylogenic and taxonomic relationship among different genera, and some of the important examples are: Microsatellite[58,82], RAPD[30,78], RFLP[30], ISSR[29,44], organelle genome analysis[115], PCR–RFLP analysis of non-coding regions of chloroplast DNA (cpDNA)[44,45,78] and sequence analysis of cpDNA region, RAPD and PCR–RFLP[2,30], RAPD, SCAR and PCR–RFLP[53,78],

AFLP[64,83], SSR[8], ISSR[29,94] and sequence data analysis of ITS region of nrDNA[63,85,113] and non-coding cpDNA regions[7,18,66,74]. These molecular studies have provided some insight to Citrus phylogeny and three species concept was generally supported.

The most prominent finding from these studies was clonal variation within the major Citrus groups such as lemon, sweet orange and grapefruit. However, accessions arising from spontaneous mutation are often difficult to distinguish[8]. The most important advance was that molecular evidence supported the hybrid origin of many so-called species (i.e. sweet orange, grapefruit, and lemon) and identified their putative parental species[45,78,83]. To date, molecular markers have significantly clarified genome structure of the genus Citrus.

RAPD markers have been used for analysis of genetic diversity in Citrus[20,22], characterization of Citrus hybrids[9], cultivar identification[25,70] and for phylogenetic analysis[68,78]. RAPD analysis has also been used in Citrus to build genomic maps[14], to identify markers linked to relevant agronomic traits[19,37] and for taxonomy studies[67]. RAPDs have been employed most widely in Citrus, since this technique is more simple and less expensive than RFLPs[26].

Genetic diversity analysis of Citrus now became simple, easy with the help of RAPD markers. In Citrus, a number of examples are there where RAPD markers have also been used for genetic diversity analysis[1,15,70,80,94] and phylogenetic analysis[78]. Moreover, in Citrus several traits of horticultural importance, including resistance to Citrus tristeza virus[37], nematode resistance[65] and dwarfing[19] have been tagged with RAPD markers. In addition, most of these markers could be converted into reliable sequence specific PCR-based markers or sequence characterized amplified region (SCARs)[24,28]. The converted SCARs are highly reliable and can be easily manipulated. Thus, they are valuable in marker-assisted selection (MAS) and map-based gene cloning.

Pessina *et al*[85]., investigated the fingerprinting and phenotyping of 54 distinct accessions, including 43 genotypes of the Citrus species (18 species or supposed species) and 11 genotypes of the Poncirus genus by using RAPD markers. The results of the multidisciplinary analyses confirmed a remarkable differentiation between Poncirus and Citrus genera and highlighted a close relationship among the three investigated Citrus species but a distinct difference between these three species and other species in the Citrus genus. RAPD fingerprints pointed out a variation gradient between C. limon and C. medica, with C. limonimedica as a possible intermediate species.

Gouri Sankar *et al*[43]., used RAPD markers to evaluate genetic similarity and inter relationship among twelve sweet orange varieties. They reported that Jaffa and Kodur Sathgudi were genetically closer with value 0.84 followed by Himakuntla Sweet orange and Kodur Sathgudi (0.80). Sathgudi Tirupati and Ankalamma Gudur Sathgudi formed one cluster and remaining varieties formed another cluster which in turn divided into two sub-clusters were Nadimpalli Sathgudi and Valentia formed first sub-cluster and Mosambi and Red blood Malta formed second subcluster; Jaffa and Kodur Sathgudi formed as one group and Ananthapur Sathgudi, Himakuntla Sweet orange, Valentia late and Hamlin Sweet orange did not resemble any other variety.

Simple sequence repeats (SSRs) or microsatellites are short sequence elements composed of tandem repeat units one to seven base pairs (bp) in length[102]. SSRs are becoming increasingly widespread because it is co-dominant, multi allelic, highly polymorphic genetic markers and appropriate for genetic diversity studies, evenly distributed throughout the genome and regarded to be the most reliable marker[39,52]. SSRs have proven to be the marker of choice in Citrus breeding research, because of their variability, ease of use, accessibility of detection and reproducibility[6,21,33,50,52,57,116].

Amar *et al*[6]., studied the genetic diversity among 24 Citrus and its relative species by using 61SSRmarkers to evaluate the level of polymorphism and discriminating capacity. In their study, a total of 596 polymorphic amplicons were observed in SSR markers with average polymorphism information content (PIC) of 0.97. High levels of polymorphism were recorded for SSR. The highest correlations (r = 0.930) were obtained between SSR and SRAP markers, whereas SSR and CAPS-SNP were poorly correlated (r = 0.833). Cluster analysis was performed to construct dendrograms using unweighted pair group method arithmetic average (UPGMA). The dendrogram from SSR data was most congruent with the general dendrogram.

Shrestha *et al*[97]., studied the genetic diversity of 62 acid lime landraces, using SSR markers. Twelve SSR primer pairs were used to assess the genetic diversity of acid lime. The average genetic similarity level among the 62 accessions was 0.77, ranging from 0.54 to 1.0 and separated five major cluster groups. Total of 33 alleles were detected by eleven primer pairs and size of alleles ranged from 50 to 225. Average polymorphic information content (PIC) value was 0.50, whereas highest 0.75 and lowest 0.18 was observed in CAT01 and GT03 loci respectively.

Al-Mouei and Choumane[5] studied the genetic variability with 14 samples representing four groups of Citrus genus using SSR markers and their results revealed that the lemon group (15 accessions of the five cultivars) had the highest number of different alleles (32 alleles) with the highest value of heterozygosity (0.728), while the pummelo possessed the lowest allele number and the lowest values of heterozygosity (26 and 0.31). The highest value of genetic diversity was detected in the mandarin group (GD = 0.53) and the 12 cultivars were represented by 12 different patterns. All the studied cultivars grouped in the same cluster except Ortanique, which is considered as a hybrid between C. sinensis and C. reticulata, was closer to the orange group.

In Citrus, ISSR markers are well distributed over linkage groups[91] and there is little tendency of linkage between markers amplified with a single degenerate primer. For example, 93 %of those marker pairs amplified with the same primer mapped to different linkage groups. Therefore, use of a few ISSR primers that amplify many polymorphic markers should cover much of the genome and provide an accurate assessment of genetic identity of seedlings. ISSR markers have successfully been used in Citrus to identify closely related varieties[28], to determine genetic diversity, characterization, assess phylogenetic relationships among the Citrus and related genera[29,44,45,69,94,105] and to fingerprint and group trifoliate accessions[28] ISSR has been previously used to fingerprint trifoliate orange germplasm accessions[28] and other closely related Citrus cultivars[28].

De Pasquale *et al*[23]., characterized 5 sour orange clones using ISSR markers by using 11 primers and reported clearly distinct patterns among the clones. The high grade of polymorphism was showed from AACNR 32 clone. It fits very well with the particular morpho-physiologic character shown by this plant and confirms its supposed natural hybrids.

Uzun *et al*[106]., distinguished 29 grapefruit (Citrus paradisi Macf.), 5 pummelo (Citrus maxima (Burm.) Merr.) and 1 Citrus hassaku Hort. ex Tanaka accessions by using ISSR markers. Twelve ISSR primers produced a total of 100 fragments and 62 of them were polymorphic. The number of average polymorphic fragments per primer was 5.2. The mean PIC was 0.37. The UPGMA analysis demonstrated that the accessions had a similarity range from 0.79 to 1.00. The accessions were separated into two main clusters; group A with five pummelos and group B with grapefruits. There was a low level of variation in the grapefruits due to their mutation origin.

Uzun *et al*[105]., used SRAP markers to detect molecular marker polmorphisms among 86 Citrus and their relatives in Aurantioideae. 21 SRAP primer combinations produced a total of 376 polymorphic fragments with an average of 17.9 per primer combination and an average PIC of 0.86. The UPGMA analysis demonstrated that the accessions had a similarity range from 0.28 to 1.00 with a mean of 0.64.

In another study, Amar *et al*[6]., assessed the genetic diversity among 24 Citrus and its relative species by using 33 SRAP markers and evaluated the level of polymorphism and discriminating capacity. In their study, a total of 656 polymorphic amplicons were observed in SRAP markers with average PIC of 0.98. High levels of polymorphism were recorded for SRAP. The highest correlations (r = 0.930) were obtained between SSR and SRAP markers.

Polat *et al*[89]., studied the genetic relationships and diversity of 51 accessions of sour orange (Citrus aurantium) and their relatives using SSR and SRAP markers. Twenty one SRAP primer combinations were tested on these accessions and relatives, producing 167 polymorphic fragments, with a mean of 8.0 and a mean PIC value of 0.47. Seventeen SSR primers also produced 30 polymorphic fragments, with a mean of 1.4 per primer and a mean PIC value of 0.39. The unweighted pair-group method with arithmetic average analysis using combined SSR and SRAP data showed a similarity range from 0.71 to 1.00 among the accessions. In the cluster analysis, sour orange relatives were indicated as a separate group from sour orange. 'Macrophylla' and 'Mexican lime' were the accessions most distinct (0.71) from the others.

A wide variety of methods has been developed to detect SNPs, and many of which use automated high throughput systems[49]. Among the simple SNP genotyping methods, the cleaved amplified polymorphic sequences (CAPS) and the derived CAPS (dCAPS) are widely applied[35,76]. CAPS marker is a PCR-based marker in which a restriction site is present in only one of two amplified sequences. This difference can be due to SNP marker[77]. Thus, CAPS proves useful for genotyping, positional or map based cloning

and molecular identification studies[99]. Consequently, study combining different marker systems across many Citrus species and its relatives may give better genome coverage especially for the closely related taxa[46,108].

Amar *et al*[6]., studied the genetic diversity among 24 Citrus and its relative species by using 24 CAPS-SNP markers and they evaluate the level of polymorphism and discriminating capacity. A total of 135 polymorphic amplicons were observed in CAPSSNP markers with average PIC of 0.89. The levels of polymorphism were recorded for CAPS-SNP markers was not very high.

Chao *et al*[17]., identified different Satsuma mandarin cultivars in California using AFLP markers including fourteen Japanese Satsuma mandarin cultivars, four Chinese Satsuma mandarin cultivars, and three Satuma mandarin cultivars of unknown origin. They separated twenty Satsuma mandarin cultivars into five subgroups based on the unweighted pairgroup method. Similarly, Pang *et al*[83]., investigated the phylogenetic relationships among Citrus and its relatives, including 29 genotypes belonging to Citrus, Poncirus, Fortunella, Microcitrus, Eremocitrus, Atalantia and Severinia using AFLP and their results demonstrated that Poncirus, Microcitrus and Eremocitrus are distant from Citrus. A strong affiliation exists between C. halimii B. C. Stone and Fortunella and the results did not support C. halimii B. C. Stone as the fourth basic species. C. ichangesis Swingle is a distinct species very different from other Citrus genotypes. C. reticulata Blanco and Citrus maxima (Burm.) Merr. (pummelo). were separated into three distinct clusters.

Uzun *et al*[106]., distinguishing Grapefruit and Pummelo Accessions using ISSR Markers by using a total of 12 ISSR primers were screened and a total of 100 bands with high intensity were scored. The number of bands scored per primer combination ranged from 4 (HVH(CA)7T) to 14 (GA)8YG), with a mean of 8.3. The polymorphic fragment number varied between 2 (HVH(CA)7T;(CAC)6) and 9 (GA)8YG),

with a mean of 5.2, 62 in total. Corazza-Nunes et al. (2002) obtained 4.6 polymorphic fragments per primer for grapefruit and pummelos according to their RAPD data. On the other hand, they found lover polymorphism (49%) than was found in our study. The PIC values for the 12 primers ranged from 0.06 (CAC) 6 to 0.53 (TAA) 8, with an average of 0.37.

Identification of Zygotic and Nucellar Individuals was done by using Simple sequence repeat (SSR) markers employed to eliminate nucellar individuals from a hybrid population produced by crossing. The crosses included 'Fremont' and 'Robinson' mandarins as the female parents and 'Midknight Valencia', 'Rhode Red Valencia', and 'Valencia Late' oranges and 'Rio Red' grapefruit cultivars as the male parents. Seedlings with the same banding patterns as the female parent were identified as nucellar seedlings by 11 SSR primers. Primers AG14 and TAA03 were found to be more effective at identifying zygotic individuals than other primers. 'Fremont' and 'Robinson' mandarins produced 36.91% and 31.09% of nucellar seedlings, respectively. As a pollen parent, 'Rio Red' grapefruit had a higher ratio of zygotic seedlings compared to 'Midknight Valencia', and can be recommended in breeding programs.

The seedlings of each parental combination were assessed using SSR primers. Whereas some individuals were tested to be either either zygotic or nucellar using only one SSR primer, others were tested using more than one SSR marker. In combinations where the 'Fremont' mandarin was used as the female parent, the TAA41 primer was able to identify more zygotic individuals (47) than the other primers used.

Also, in combinations where the 'Robinson' mandarin was used as the female parent, AG14 and TAA03 primers determined 59 and 58 zygotic individuals, respectively. In contrast, the CAT01, TAA01 and TAA45 primers (when 'Fremont' mandarin was used as female parent) and TAA45 and TAA52 (when 'Robinson' mandarin was used as

female parent) were not useful in determining the identity of zygotic seedlings. When the whole F1 population was considered, the AG14 and TAA03 primers were found to be more effective than other primers at distinguishing zygotic individuals, whilst the TAA45 primer was not able to identify zygotic seedlings. When a population is derived from a cross, all of the zygotic individuals show a genotype different from that of the mother at any discriminating locus, provided that the father has alleles different than those of the mother. Of the 24 seedlings coming from the combination of 'Robinson' mandarin as the female parent and 'Midknight Valencia' orange as male parent, five seedlings showed a banding pattern different from that of the mother plant when the TAA03 primer was used.

Future Directions and Conclusion

Knowledge of the levels and distribution of genetic diversity are important for designing conservation strategies for threatened and endangered species. Preservation of the genetic diversity represented in all the plant ecosystems throughout the world has become a major issue of international concern. The loss of increasingly large numbers of plant species through habitat destruction threatens the availability of a diverse plant germplasm base which will be needed to feed future generations. Advances in biotechnology, especially in the area of in vitro culture techniques and molecular biology provide some important tools for improved conservation and management of plant genetic resources.

The present study highlights the usage of different marker system for studying genetic diversity across DNA level in the genus Citrus. All the marker techniques provided useful information on the level of polymorphism and genetic diversity in Citrus, showing their utility in the characterization of germplasm accession. RAPD markers, as a fast and simple technique, can detect enough polymorphism to differentiate between different Citrus species and cultivars and to understand their inter-relationships. RAPD markers are useful for the construction of a linkage map because a sufficient number of markers can be generated and used for construction in a relatively short period of time. RAPD markers are more stable since more than 50 % of the polymorphisms observed showed linkage and mapped to a specific linkage group. Although, RAPD provides better results in some contexts as compared to other types, but RAPD had problem of reproducibility and transferability among the laboratories. On the other hand, ISSR markers as a powerful tool can differentiate closely related individuals of Citrus. The relationships of unknown genotypes of Citrus with known varieties can also be clarified. SRAP markers could be more advantageous over SSR markers due to occasional loss of amplification sites of SSR primers in distant Citrus relatives and its relative simplicity. The SSR may be more useful for segregation studies and genome mapping in Citrus. All these kind of markers have potential use in studies of diversity, linkage mapping, cultivar identification, and germplasm organization. As a result the use of retrotransposon based markers can be a valuable tool for Citrus breeders. In summary, the combination of different kinds of molecular markers proved to be a powerful tool in carrying out a more complete analysis of Citrus phylogeny and origin.

Sampling of wild and semi wild species of Citrus, which are not yet studied would clarify the probable speciation and sequence of founding events that gave rise to this species, and possibly other relatives. This could be achieved by a combined approach of phylogeography and classical population genetics using other molecular markers, and perhaps DNA sequencing data, which could be used to identify the population phylogenetic histories. The second promising line of investigation would be to determine how crossing may have affected the population structure of this species. It would also be interesting to determine how utilization by the local people has influenced the structure of genetic diversity of this species. The last important line of research would be to study

the mechanisms of pollination, seed and clone dispersal, as well as the variation in breeding system for this species. An integrated research program that combines genetic analyses with studies of reproductive biology may provide further valuable data that would greatly extend the present conclusions.

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