Analysis of the Genetic Diversity in Citrus (*Citrus* spp.) Species Using SSR Markers

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Abstract
Determination of genetic diversity level is important in clarifying genetic relationships, characterizing germplasm and the registration of new cultivars. In this study, genetic variation among 56 accessions (G1~G56) of *Citrus* including several undefined local or native genotypes and some known varieties in Jiroft, Kerman province, Iran was investigated using SSR markers. In total, 12 SSR primers produced 54 alleles. The lowest number of alleles was observed on cAGG9 locus with 2 alleles and the highest number of alleles was observed in TAA41 locus with 8 alleles. Polymorphic Information Content (PIC) varied from 0.19 to 0.37 with mean of 0.28. The percentage of heterozygosity per marker detected in our samples ranged from 25% to 96% with an average of 67%. Grouping of the accessions using Jaccard similarity coefficient and based on the Neighbor-Joining method assigned the 56 accessions into four major clusters. The SSR data indicated a high relationship between G2 and G41 (unknown natural types) with grapefruits (*Citrus paradisi*) (G50 and G51), G17 (unknown natural type) with orange (*C. sinensis*) (G56) and G40 (unknown natural type) with pummelo (*C. grandis*) (G49). Unidentified genotype G43 in a single-accession cluster didn't show any close molecular similarity to control samples [mandarin (*C. reticulata*), pummelo, sweet orange, sour orange (*C. aurantium*), etc.]. Mandarin, pummelo and citron (*C. medica*) were clustered into three particular groups as major species of *Citrus*. Also, our results demonstrated that SSR markers can be useful in evaluating citrus genetic diversity and in classifying accessions to phylogenetic groups based on their genetic similarity values.

Key words: Citrus; Genetic diversity; Heterozygosity; Microsatellites

Introduction

Citrus is one of the world's important fruit crops which is widely grown in most areas with suitable climates between latitude 35°N~35°S. The area under citriculture was recently reported to be 8643501 ha with production of 123.755 million tons (FAO 2010). Also in Iran, citrus industry is of a paramount importance (Golein and Adouli 2011).

Most species in *Citrus* genera are diploid (2n=2x=18), with relatively small genomes; for instance, sweet orange (*Citrus sinensis*) has a genome of about 367 Mb, nearly three times the size of the 125 Mb Arabidopsis genome (Arumuganathan and Earle 1991; Xu *et al*. 2013).

Variation among *Citrus* species and varieties is common due to frequent bud mutation, interspecific and intergeneric hybridization, apomixis and long history of cultivation (Scora 1988). Determination of genetic diversity is the first step in proper utilization of plant resources (Graham *et al*. 1996). Assessment of genetic diversity and germplasm characterization using morphological markers alone have serious

limitations, especially in species of a complex genus like *Citrus*, whose taxonomy is otherwise in a chaotic state due to frequent incidences of hybridization, polyploidy and bud mutations (Weising *et al*. 2005).

Molecular markers have become very efficient and powerful tools in citrus in a wide range of applications including fingerprinting the accessions, evaluation of phylogenetic relationships among accessions and examine the level of genetic diversity. Many of these studies have targeted specific citrus groups or sampled a few individuals of each taxon. Breto *et al.* (2001) examined the variability of 24 Clementine (*C. clementina*) accessions by utilizing ISSR, RAPD and AFLP markers and found that only two varieties could be distinguished. Gulsen and Roose (2001) utilized ISSR, SSR and isozymes to assess diversity, phylogenetic relationships and parentage in lemon (*C. limon*) accessions and related taxa, finding little genetic variation among lemon accessions. Fang and Roose (1997) utilized ISSR markers to distinguish closely related *Citrus* cultivars, many of which had arisen by selection of spontaneous mutations. This study showed that ISSR markers could distinguish some (but not all) of these closely related accessions. Nicolosi *et al.* (2000) used RAPD, SCAR and cpDNA markers to elucidate phylogenetic relationships and genetic origins of hybrids in 36 accessions of citrus and one accession from each of four related genera. Shahsavar *et al.* (2007) utilized ISSR marker to study phylogenetic relationships among 33 citrus genotypes including several undefined local or native varieties as well as some known varieties in

the Fars Province, Iran, finding little genetic variation among local lime accessions.

Among these markers, simple sequence repeats (SSR) or microsatellites are especially functional for characterization of germplasm collections because they are highly polymorphic and with heterozygous conserved sequences which can be used as co-dominant markers (Zane *et al*. 2002; Barkely *et al*. 2006), but they have not been widely used in citrus and only in few studies have utilized this marker (Gulsen and Roose 2001; Corazza-Nunes *et al*. 2002; Pang *et al*. 2003; Noveli *et al*. 2006; Barkley *et al*. 2006; Rohi Ghorbaei *et al*. 2010; Golein *et al*. 2012).

Because of above mentioned points, there are also some unidentified *Citrus* accessions in citrus collections of Iran which have been characterized or labeled solely based on their morphological characteristics. Jiroft Collection is located in Kerman Province and contains several accessions of *Citrus* and related taxa, many with unknown background. Due to existence of indigenous citrus genetic resources in country, it is necessary to characterize and analyze native citrus genotypes. The aim of this study was to assess the usefulness of SSR markers in characterizing the unidentified citrus accessions from Jiroft collection and inferring their relationships with known citrus cultivars.

Materials and Methods

Plant materials: Fifty-six *Citrus* accessions including undefined local or native varieties and some known varieties were collected in Jiroft Agricultural Research Center for SSR analysis. List of the accessions is presented in Table 1.

Table 1. Plant material used in this study for SSR analysis

No.	Plant code	Genotype name	Cultivar or common name	Location
1~45	G1~G45	*Citrus* sp.	Unknown	Jiroft
46	G46	*C. limon* (L.) Burm. f	Lisbon lemon	Jiroft
47	G47	*C. medica* L.	Citron	Jiroft
48	G48	*C. aurantium* L.	Sour orange	Jiroft
49	G49	*C. grandis* (L.) Osbeck.	Pummelo	Jiroft
50	G50	*C. paradisi* Macf.	Marsh grapefruit	Jiroft
51	G51	*C. paradisi* Macf.	Redblush grapefruit	Jiroft
52	G52	*C. unshiu*	Satsuma mandarin	Ramsar
53	G53	*C. reticulata* Blanco	Local mandarin	Jiroft
54	G54	*C. reticulata* Blanco	Dancy mandarin	Ramsar
55	G55	*C. sinensis* (L.) Osbeck.	Olinda valencia	Jiroft
56	G56	*C. sinensis* (L.) Osbeck.	Local orange	Jiroft

DNA extraction: From each accession, four young leaves were taken and total genomic DNA was extracted according to Murray and Thompson (1980). DNA concentration and quality were measured spectrophotometrically (Nano Drop 1000) at 260 nm and confirmed using 0.8% agarose gel electrophoresis against known concentrations of unrestricted lambda DNA. DNA templates were diluted to 12.5 ng/µl.

PCR amplification: For DNA amplification, 17 SSR primers were initially screened and finally 12 primers that produced scorable polymorphic bands were used for further analyses (Table 2). DNA amplification was carried out in 10 µl reactions containing 50 ng of template DNA, 0.2 mM dNTPs, 0.5 µM each of forward and reverse primers, 1.0 µl of 10X PCR buffer (Cinnagen, Iran), 1.5 mM of magnesium chloride, 1.55 µl double distilled water and 1 unit of *Taq* DNA polymerase (Cinnagen, Iran). Cycling conditions consisted of 95°C for 5 min; 38 cycles of 95°C for 1 min, 45-55°C for 30 s, and 72°C for 1 min; and one final cycle of 72°C for 7 min. PCR products

were run on a 6% denaturing polyacrylamide gel and visualized by silver staining.

Data Analysis: Each band was scored as present (1) or absent (0) and also weighed (a, b, ...). Data were analyzed with the GGT, version 2.0 (Van Berloo 1999) and Popgene, version 1.31 softwares. Cluster analysis was carried out using Neighbor Joining (NJ) algorithm with Jaccard similarity coefficient. The resulting clusters were represented as a dendrogram. Polymorphic information content (PIC) values were calculated according to Naghavi *et al.* (2005), using PIC= $1-\Sigma f_i^2 - \Sigma \, 2\, f_i^2 f_j^2$.

Results and Discussion

SSR amplification: In total, 12 SSR primers produced 54 alleles ranging from 100 to 300 bp (Table 2). The lowest number of alleles was observed in cAGG9 locus with 2 alleles and the highest number of alleles was observed in TAA41 locus with 8 alleles. The PIC values for the 12 markers ranged from 0.19 (cAGG9) to 0.37 (TAA1 and CAC15) with mean of 0.28. The

observed heterozygosity was calculated for each individual marker as a measure of marker diversity. The percentage of heterozygotes per marker detected in our citrus accessions ranged from 25% in marker cAGG9 to 96% in markers TAA45 and CAC33. The mean observed heterozygosity for all markers was 67%. Many of the SSR primers amplified more than one band per genotype, indicating residual heterogeneity within genotypes.

Table 2. Diversity statistics for 12 SSR markers studied in 56 *Citrus* accessions

SSR loci	Allele size (bp)	Alleles observed	PIC value	H_{obs}
TAA52	119-210	4	0.33	0.46
cAGG9	100-150	2	0.18	0.25
TAA1	150-210	4	0.37	0.91
TAA15	100-150	6	0.32	0.80
GT03	160-190	6	0.30	0.70
CAC15	200-300	7	0.37	0.95
TAA33	150-210	3	0.23	0.39
TAA27	110-190	4	0.19	0.50
CAC33	100-150	3	0.23	0.96
TAA45	100-150	4	0.20	0.96
TAA41	150-220	8	0.31	0.77
CAC19	140-180	3	0.27	0.42
Mean	-	4.2	0.28	67%

Phylogenetic relationships among genotypes: The genetic distance (D) among the different genotypes studied using the 12 SSRs was reproduced in the neighbor joining (NJ) dendrogram (Figure 2), according to the original data obtained in the similarity matrix. The dendrogram generated from the NJ cluster analysis with Jaccard/ coefficient (r = 0.92) showed four main groups (1, 2, 3 and 4).

Group 1 with two subgroups A and B, contains 13 accessions. The first subgroup A was further divided into two minor subgroups C and D. The minor subgroup C included G1, G33, G32, Local orange (*Citrus sinensis* (L.) Osbeck) (G56) and G17. Five unknown genotypes G15, G21, G14, G26 and G20 were clustered into the second minor subgroup D. Subgroup B contained Olinda Valencia orange (G55) and G36 which separated out from Satsuma (*C. unshiu* Marc.) (G52) with

genetic distance of 0.38. Those genotypes which were nested in sweet oranges (G55 and G56) group, probably are natural hybrids that separated out from each other and oranges. The morphological, phytochemical and molecular data support the hypothesis that sweet orange originated from a cross between pummelo (*C. maxima* Merill) and mandarin (*C. reticulata* Blanco) (Barrett and Rhodes 1976; Green *et al*. 1986; Yamamoto *et al*. 1993), suggesting that sweet orange has a majority of its genetic structure from mandarin and only a small part from pummelo, which is in agreement with the result of present study, since genetic distances between sweet orange with mandarin and pummelo were 0.38 and 0.54, respectively.

**Figure 1. SSRs amplified with the primer GT03. M: 100 bp ladder; Lanes 1-45
are SSR products of the *Citrus* accessions.**

Group 2 contained two subgroups E and F. The subgroup E was split into two minor subgroups G and H. The first minor subgroup G consisted of G-1 and G-2. The minor subgroup G-1 included citron (*C. medica* L.) (G47), G3, G30, G37 and G8. Genotype G3 with genetic distance value of 0.39 showed more relationship to citron among G30, G37 and G8. Citron is one of the citrus types that Barrett and Rhodes (1976) considered as true species. The minor subgroup G-1 also contained G12, G45, G24, G25 and G13 that were grouped with Lisbon lemon (*C. limon* (L.) Burm. f.) (G46), probably are natural hybrids of lemon. Barrett and Rhodes (1976) speculated that lemons are a complex hybrid similar to limes (*C. auranifolia* (Chrism.) Swingle) but carrying a greater proportion of citron genes. Molecular data indicate that lemon should have originated from citron and sour orange (*C. aurantium* L.) with sour orange being the maternal parent (Nicolosi *et al*. 2000), which was consistent with this study because lemon had 0.48 and 0.70 genetic distance values to citron and sour orange, respectively.

The second minor subgroup G-2 included G23 and G7 that were closed with sour orange (G48). There is a high intraspecific affinity among common sour oranges which is believed to be maintained by facultative apomixes (Moore 2001). Sour orange showed genetic distance values of 0.65 and 0.71 to pummelo and mandarin, respectively. The sour oranges are considered to be natural hybrids of a mandarin and a pummelo (Scora 1975; Barrett and Rhodes 1976) which was later supported by SCAR and RAPD analyses (Nicolosi *et al*. 2000). The results of this study support this idea and sour orange accessions clustered with pummelo in subgroup E.

In the minor subgroup H, the accessions of G35, G42, G40 and G19 were nested with pummelo (G49), that may be hybrids of pummelo. Genotype G40 showed high similarity to pummelo among other accessions. Pummelo is thought to be a true *Citrus* species which has given rise to sour oranges through hybridization (Scora 1975). Barrett and Rhodes (1976)
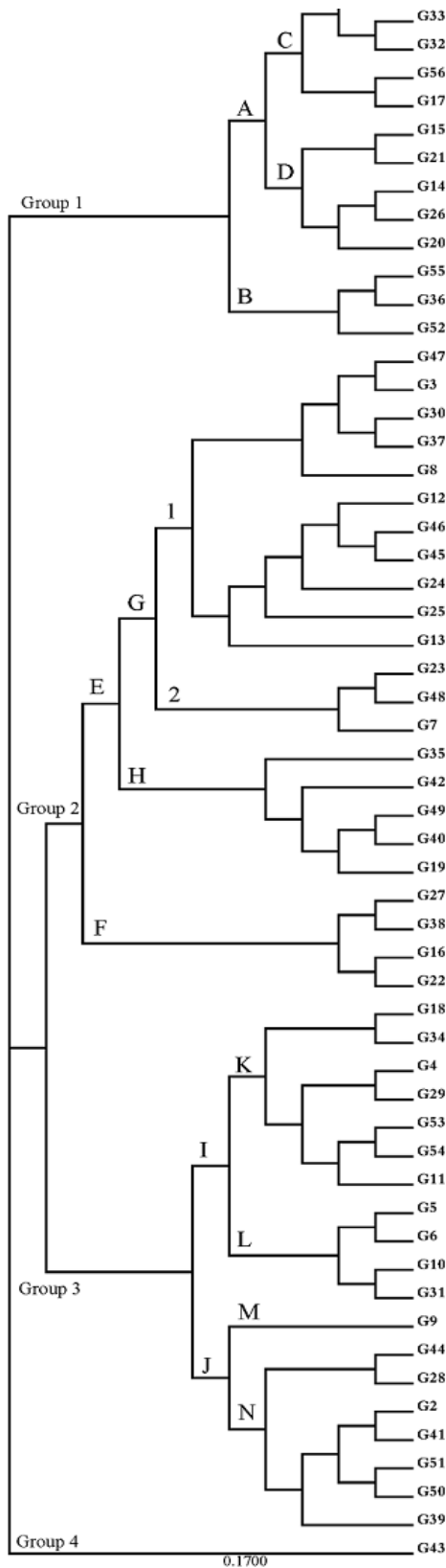
Figure 1. Neighbor Joining tree generated for 56 accessions of *Citrus* based on SSR data. Name of the accessions are shown in Table 1.

found that intraspecific affinity to be high in pummelos and this idea was supported by isozyme data which pummelo was homozygous at all 10 loci evaluated (Torres *et al*. 1982).

Subgroup F consisted of G27, G38, G16 and G22 that showed low molecular similarity to known cultivars in this study. *Citrus* species are known to hybridize among themselves (Iwamasa *et al*. 1988) and several natural hybrids have been reported (Swingle and Reece 1967). Barkley *et al.* (2006) suggested that sexually derived accessions should be more genetically diverse than accessions arising apomictically or from controlled pollinations; therefore, their ancestral relationships are less well defined.

Figure 2 explains that group 3 included two subgroups I and J. Subgroup I diverged into two minor subgroups, one including G18, G34, G4, G29, G11 and mandarins (G53 and G54) and another with G5, G6, G10 and G31, which probably are mandarin hybrids. Mandarins are one of the three citrus types that Barrett and Rhodes (1976) proposed as true species. Filho *et al.* (1998) used RAPD markers to evaluate genetic similarity among mandarin accessions which indicated a high genetic similarity among them. Satsuma and other mandarins (Local and Dancy) were classified into two distinct groups. Federici *et al*. (1998) and Barkley *et al.* (2006) found that mandarins did not form a unified group when hybrid and non- hybrid accessions were analyzed which corroborate our data. Also, Webber (1943) classified the most important mandarins in four taxonomic groups: King, Satsuma, Mandarin, and Tangerine. It seems that mandarin accessions

consist of two groups of these four taxonomic groups.

Considering the dendrogram (Figure 2), subgroup J, could be divided into two minor subgroups M and N separated out from each other (GD= 0.28). Genotype G9 into minor subgroup M and genotypes G44, G28, G2, G41, Redblush grapefruit (*C. paradisi* Macf.) (G50), Marsh grapefruit (G51) and G39 into minor subgroup N were placed. Redblush and Marsh grapefruits were nearly identical (GD= 0.05). Selected somatic mutations that occur in buds or branches were the only methods used to develop new grapefruit varieties (Corazza-Nunes *et al*. 2002). The other genotypes in the subgroup J may be grapefruit hybrids.

Group 4 consisted of only genotype G43 which didn't show any close molecular similarity to control samples (mandarin, pummelo, sweet orange, sour orange, etc.).

Conclusion

SSR markers showed high levels of genetic polymorphism. The high level of polymorphism associated with SSR markers may be a function of the unique replication slippage mechanism responsible for generating SSR allelic diversity (Pejic *et al*. 1998). TAA15 and CAC15 primers considered a valuable markers because they were the most informative for polymorphic information content index calculated. On the whole, all of the primers applied in this study revealed high levels of polymorphism, and similar levels were observed among all of the primers tested, confirming that high genetic diversity exists within the citrus genome.

Cluster analysis, supported the hypothesis that there are only a few naturally occurring forms of *Citrus* (*C. medica*, *C. maxima*, *C. reticulata*) as previously suggested by Scora (1975) and Barrett and Rhodes (1976). Additionally, it confirm the idea that most other *Citrus* "species" are hybrids derived from these taxa and provide further support of their previously suspected ancestry.

Our study indicated that SSR markers expressed a high rate of polymorphism allowing the identification of *Citrus* accessions and their phylogenetic relationships to known cultivars in spite of the fact that these 12 SSR markers could not distinguish some accessions clearly. Previous studies also have found few molecular polymorphisms within groups like sweet oranges, Clementines (*C. clementina* Hort. ex Tanaka) and

Satsumas, consisting of cultivars developed by spontaneous mutation (Fang and Roose 1997; Barkley *et al.* 2006; Rohi Ghorabaie *et al.* 2010; Golein *et al.* 2012). However, the results of this study confirmed that SSR markers are useful for characterization of germplasm collections as were mentioned in the previous studies (Hokanson *et al.* 1998; Barkley *et al.* 2006; Rohi *et al.* 2010). Further study is needed with more SSR markers and even other markers to obtain the precise output.

Acknowledgments

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