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Full Length Research Paper

Assessing genetic variability in male sterile and low fertile citrus cultivars utilizing simple sequence repeat markers (SSRs)

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Understanding phylogenetic relationships and genetic diversity in citrus are important in clarifying genetic relationships, characterizing germplasm and the registration of new cultivars. 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). Genetic similarities among accessions were calculated according to Jaccard similarity index and used to construct a dendrogram based on the unweighted pair group method using arithmetic averages (UPGMA) which put the 28 samples into four major groups (A, B, C and D). The cultivars of male sterile satsuma mandarin were clustered into group A; those of orange, grapefruit and Page (a complex hybrid) into group B; mandarin cultivars into group C; and lemon Lisbon into group D. Genetic analysis of sterile and low fertile citrus, provide useful information for further breeding programs, collection, preservation and utilization.

Key words: Male sterility, genetic diversity, SSR markers, citrus.

INTRODUCTION

Citrus is one of the most important fruit crops in the world. The genus *Citrus* L. (family Rutaceae; subfamily Aurantioideae) includes some of the principal fruit crops of the world such as the citrons (*C. medica* L.), lemons [*C. limon* (L.) Osbeck], limes [*C. aurantifolia* (Christm.) Swingle], mandarins (*C. reticulata* Blanco), sour oranges (*C. aurantium* L.), sweet oranges [*C. sinensis* (L.) Osbeck], grapefruits (*C. paradisi* Macf.) and pummelos [*C. maxima* (Burm.) Merr.]. Citrus fruits are well-known for their dietary, nutritional, medicinal and cosmetic properties and are also good sources of citric acid, flavonoids, phenolics, pectins, limonoids, ascorbic acid, etc. (Dugo and Di Giacomo, 2002).

A large amount of genetic variation exists within the true *Citrus* tree species (*Citrinae* subtribal group C). This variation results in different species, cultivars and clones having very different phenotypic appearance and agricultural performance, with many possessing especially desirable breeding characteristics. Such traits include cytoplasmic male sterility (example, *C. unshiu*), shortened juvenile periods (example, *C. aurantifolia*) and resistance to disease, including citrus tristeza virus, *phytophthora*, and burrowing nematode (example, *Poncirus trifoliata*) (Cameron and frost, 1968). Genetic variability in citrus is related to the high number of taxonomic units (species and hybrid), apomixis, widely sexual compatibility between *Citrus* and related genera, high frequency of bud mutations and the long history of cultivation and wide dispersion (Scora, 1975).

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Since 1930, through the mutant selection, seedling selection and breeding in other countries, Iran has received more than 50 seedless citrus varieties/ genotypes (Ebrahimi, 1979), most of them are sterile and male sterile types, with old scattered resources; Iran has obvious diversity of citrus materials.

It is well known that adequate genetic diversity is necessary in breeding program for the development of high yielding varieties. Tsegaye (2002) indicated that lack of knowledge about the genetic diversity of the enset crop complicated the conservation, improvement and utilization by farmers, conservationists and breeders. He also noted that knowledge about clonal diversity allows the selection of clones prioritized for conservation, by removing duplication and optimizing genetic diversity and hence optimizing cost benefit ratio in maintaining the crop germplasm. For this reason, the determination of genetic diversity is the first step in using plant resources (Graham el al., 1996).

Assessment of genetic diversity using morphological markers alone has 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, apomixis, polyploidy and bud mutations. Genetic diversity assessment in plants has now become far more simple, cost effective, reliable and reproducible; thanks to the advent of PCR-based DNA marker techniques such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), simple sequence repeats (SSR), directed amplification of minisatellite DNA (DAMD), etc. (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 examining the level of genetic diversity. Many of these studies have targeted specific citrus groups or sampled a few individuals of each taxon. For example, Breto et al. (2001) examined the variability of 24 Clementine (*C. reticulata* Blanco) accessions by utilizing ISSR, RAPD, and AFLP markers and found that only two varieties of 24 could be distinguished. Gulsen and Roose (2001a) utilized ISSR, SSR and isozymes to assess diversity, phylogenetic relationships and parentage in lemon [*C. limon* (L.) Burm. f.] accessions and related taxa, finding little genetic variation among lemon accessions. In another study, Fang et al. (1997) employed isozymes, RFLP, and ISSR markers to classify 48 trifoliate orange [*Poncirus trifoliata* (L.) Raf.] accessions into four groups. 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. Federici et al. (1998) examined the phylogenetic relations of 88 accessions representing 45 *Citrus* species and six related genera by utilizing RFLP and RAPD markers. 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 of Iran, finding little genetic variation among local lime accessions.

Overall, these previous studies demonstrate that molecular markers are powerful tools for elucidating genetic diversity, determining parentage, and revealing phylogenetic relationships among various *Citrus* species; however, accessions arising from spontaneous mutation are often difficult to distinguish.

Of the many molecular techniques available to researchers, SSRs or microsatellites is becoming increasingly widespread because it is co-dominant, highly polymorphic, frequently and evenly distributed throughout the genome and it was regarded to be the most reliable marker. It has been used in the genetic diversity studies of many plants such as citrus (Barkley et al., 2006), apple (Guilford et al., 1997) and grape (Thomas and Scott, 1993). Hence, this experiment was designed to cluster 28 genotypes of citrus including male sterile, sterile, low fertile and fertile cultivars using SSR markers into different diversity classes and thereby estimating the extent of genetic distance between clusters, and to choose and recommend genetically divergent parent for hybridization and other utilizations.

MATERIALS AND METHODS

Plant materials

Leaf samples were collected for SSR analysis from 28 citrus accessions including male sterile, sterile, low fertile and fertile cultivars from Iran Citrus Research Institute, which is located in the west of Sari, Mazandaran Province, Iran. List of the accessions is shown in Table 1.

DNA extraction

From each accession, four young leaves were taken and total genomic DNA was extracted according to Murray and Thompson (1980) with some modifications. The leaves were grounded to a fine powder in liquid nitrogen and resuspended in cetyl trimethylammonium bromide (CTAB) extraction buffer (1% CTAB, 100mM Tris-HCl pH 7.5, 10 mM EDTA, 0.7 M NaCl, 2% sarcosyl and 140 mM 2-mercaptoethanol). The supernatant was extracted with chloroform–isoamyl alcohol (24:1), precipitated in absolute ethanol and pellet resuspended in TE containing 10 mg/ml RNAse. DNA concentration was measured spectrophotometrically (Nano Drop 1000, USA) at 260 nm and DNA templates were diluted to 12.5 ng/µl.

PCR amplification

For DNA amplification, ten SSR primers were initially screened and finally eight primers that produced scorable polymorphic bands

Table 1. Plant materials used in this study.

Plant code	Scientific name	General name	Type of fertility
G1	*Citrus. unshiu*	Clausellina Wase satsuma	Male sterile
G2	*C. unshiu*	Hashimoto Wase satsuma	Male sterile
G3	*C. unshiu*	Owari satsuma	Male sterile
G4	*C. unshiu*	Wase satsuma	Male sterile
G5	*C. unshiu*	Sugiyama Wase satsuma	Male sterile
G6	*C. unshiu*	Okitsu Wase satsuma	Male sterile
G7	*C. unshiu*	Miyagawa Wase satsuma	Male sterile
G8	*C. sinensis*	Fukumoto navel orange	Sterile
G9	*C. sinensis*	Navelate navel orange	Sterile
G10	*C. sinensis*	Delta seedless orange	Sterile
G11	*C. sinensis*	Navelina navel orange	Sterile
G12	*C. sinensis*	Spring navel orange	Sterile
G13	*C. sinensis*	Newhall navel orange	Sterile
G14	*C. sinensis*	Frost navel orange	Sterile
G15	*C. paradisi*	Marsh grapefruit	Low fertile
G16	*C. paradisi*	Thompson grapefruit	Low fertile
G17	*C. sinensis*	Hamlin orange	Low fertile
G18	*C. sinensis*	Valencia orange	Low fertile
G19	*C. sinensis*	Gross sanguine orange	Low fertile
G20	*C. limon*	Eureka lemon	Low fertile
G21	*C. clementina*	Clementine mandarin	Low fertile
G22	*C. reticulata*	Ponkan mandarin	Fertile
G23	*C. reticulata*	Atabaki mandarin	Fertile
G24	*C. reticulata*	Local mandarin	Fertile
G25	*C. reticulata*	Bami mandarin	Fertile
G26	*C. clementina* × (*C. paradisi* × *C. reticulata*)	Page	Fertile
G27	*C. sinensis*	Siavaraz local orange	Fertile
G28	*C. reticulata*	Dancy mandarin	Fertile

were selected for further analyses. The primer sequences were obtained from http://www.plantbiology.ucr.edu/documents/Wles_of_Roose/rooselink2.html (Table 2), and were synthesized by Cinnagen Co-Ltd (Iran). 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 10 × PCR buffer (Cinnagen, Iran), 1.5 mM of magnesium chloride, 1.55 µl double distilled water and 1 unit of Taq polymerase (Cinnagen, Iran). Cycling conditions consisted of 95°C for 5 min; 38 cycles of: 95°C for 1 min, 45 to 55°C for 30 s (annealing temperature was optimized for each primer) (Table 2), and 72°C for 1 min; and one final cycle of 72°C for 7 min (MJ research, PTC-200).

PCR products were denatured at 95°C for 5 min and separated on a 6% denaturing polyacrylamide gel containing 7 M urea and 1 × TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA) at 85 W for 60 min (Sequi-Gen electrophoresis, Bio-Rad). For DNA detection, silver staining was performed according to the protocol of Bassam et al. (1991) following these steps: fixation (10% acetic acid, 20 min), rinsing (H_2O, 2 min, 3 times), staining (0.1% silver nitrate ($AgNo_3$), 1.5 ml 37% formaldehyde liter -1, 30 min), rinsing (H_2O, 20 s), developing (3% sodium carbonate (Na_2Co_3), 1.5 ml 37% formaldehyde liter -1, 2 mg sodium thiosulfate ($Na_2S_2O_3.5H_2O$) liter -1, 2 to 5 min), stopping (10% acetic acid, 5 min), rinsing (H_2O, 2 min).

Data analysis

Each band was scored as present (1) or absent (0) and data were analyzed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) software package version 2.02 (Rohlf, 2005). Cluster analysis was done by unweighted pair groups' method arithmetic average (UPGMA) with Jaccard similar coefficient. Polymorphic information content (PIC) values were calculated according to Smith et al. (1997), using the following algorithm for all primers, where f_i^2 is the frequency of the *i*th allele. $PIC = 1-\Sigma f_i^2$.

RESULTS AND DISCUSSION

SSR amplification

Genetic diversity in citrus was evaluated using 28 accessions with SSR primers. From ten pair primers

Table 2. Forward and reverse primer sequences and annealing temperature for the eight SSR markers.

Marker	Forward sequence (5′→3′)	Reverse sequence (5′→3′)	Repeat motif	Annealing temperature (°C)
AG14	AAAGGGAAAGCCCTAATCTCA	CTTCCTCTTGGAGTGTTG	AG	50
ATC09	TTCCTTATGTAATTGCTCTTTG	TGTGAGTGTTTGTGCGTGTG	ATC	47
CCSM18	GTGATTGCTGGTGTCGTT	AACAGTTGATGAAGAGGAAG	AG	55
CTT01	TCAGAACATTGAGTTGCTTGCTCG	TAACCACTTAGGCTTCGGCA	CTT	47
GT03	GCCTTCTTGATTTACCGGAC	TGCTCCGAATTCATCATTG	GT	50
TAA1	GACAACATCAACAACAGCAAGAGC	AAGAAGAAGAGCCCCCATTAGC	TAA	55
TAA15	GAAAGGGTTACTTGACCAGGC	CTTCCCAGCTGCACAAGC	TAA	55
TAA27	GGATGAAAAATGCTCAAAATG	TAGTACCCACAGGGAAGAGAGC	TAA	45

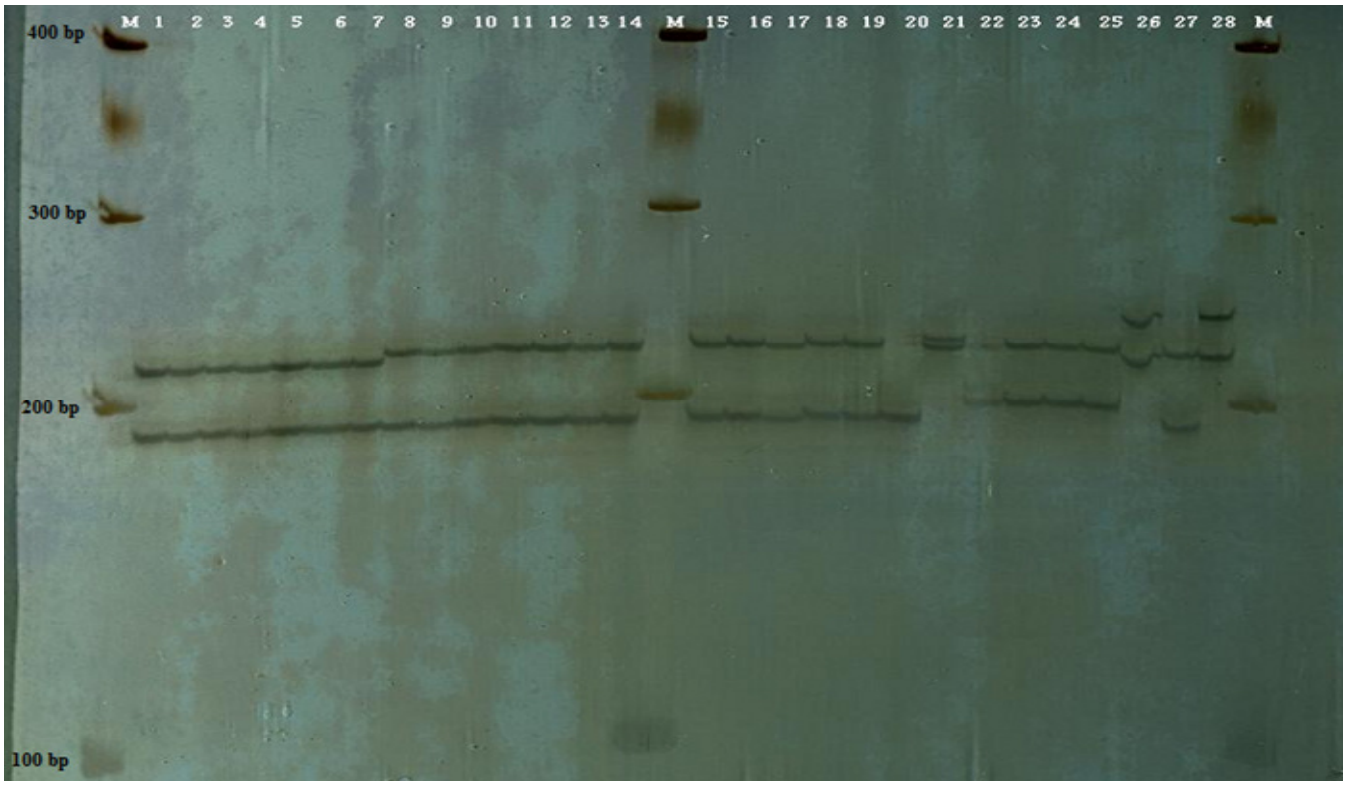

Figure 1. SSRs amplified with the primer TAA15 using DNAs of different citrus genotypes (Table 1). M, 100 bp ladder; lanes 1-28 are SSR products of the citrus accessions.

used, eight polymorphic SSR primers that produced clear and scorable bands were analyzed for this study. Representative banding patterns observed with locus TAA15, are shown in Figure 1. Table 3 summarizes the number of alleles observed and PIC values. The amplified fragment sizes ranged from 119 to 380 bp. The number of alleles detected among the eight studied markers varied from 3 to 8. The lowest number of alleles was observed in ATC09, CCSM18, CCT01 and TAA27, and the highest was in TAA15. A total of 54 alleles were detected with a mean number of alleles per locus of 4.2 and used for analysis on NTSYS software version 2.2 (Rohlf, 2005). 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 population ranged from 21.4% in marker AG14 to 100% in markers TAA27. The mean observed heterozygosity for all markers was 68.4%. The PIC values from the eight markers ranged from 0.34 (AG14) to 0.9 (CCSM18). The average PIC value for SSR markers was 0.59. Many of the SSR primers amplified more than one band per genotype, indicating residual heterogeneity within genotypes. The UPGMA dendrogram based on SSR marker data clearly

Table 3. Diversity statistics for the eight SSR markers studied in 28 citrus accessions

SSR loci	Allele sizes (bp)	Alleles observed	PIC value	H_{obs}
AG14	119-163	5	0.34	0.214
ATC09	169-202	3	0.55	0.75
CCSM18	380-150	3	0.90	0.375
CTT01	134-164	3	0.47	0.92
GT03	149-197	4	0.59	0.963
TAA1	147-190	5	0.46	0.285
TAA15	190-240	8	0.88	0.964
TAA27	197-242	3	0.59	1
Mean	-	4.2	0.59	0.684

discriminated among accessions (Figure 2). The cluster analysis separated all the accessions at similarity index 0.56 into four main clusters.

Phylogenetic relationships analysis

Considering the dendrogram (Figure 2), Group A contains male sterile citrus varieties (Satsuma strains). SSR markers did not detect the difference between the mutant varieties, indicating that these mutant varieties were found with relatively uniform genetic background. Filho et al. (1998) used RAPD markers to evaluate genetic similarity among mandarin accessions which indicated a high genetic similarity among them. Satsuma mandarins are horticulturally similar but differing from each other mainly in the time of harvest and fruit size. Since, they are coming through the breeding of mutant selection, so genetic background is relatively narrow.

Group B is a major group including 14 accessions containing the sweet oranges, Page and grapefruits. This cluster contains two subgroups (E and F); group E included Page and sweet oranges and two cultivars of grapefruits were in the cluster F.

All accessions subgenotyped E were separated into two groups, G (sweet oranges) and H (Page is a synthetic hybrid and fertile genotype), which diverged at a similarity index of 0.68. In group G, the accessions of G8, G9, G10, G11, G12, G13 and G14 from sterile navel orange genotypes showed absolute similarity. It might be concluded that these genotypes are somatic mutants which have not been distinguishable with SSR markers utilized in this study. Previous studies have suggested that sweet orange cultivars are monophyletic and are derived from a single ancestor through mutation and selection of desirable clones (Fang and Roose, 1997; Luro et al., 1995). Luro et al. (1995) found no differences among the ten cultivars of sweet oranges when they used microsatellite probes.

In group G, G27 (fertile Siavaraz local orange) was distinguished from sterile and low fertile orange genotypes with similarity coefficient of 0.69. Also, low fertile sweet oranges (G17, G18 and G19) were separated from sterile ones.

In subgroup F, the accessions of G15 and G16 from grapefruit genotypes were identical (similarity coefficient of 1). Studies using RAPD and SCAR markers have indicated that grapefruit has been derived from a backcross between sweet orange and pummelo (Gmitter, 1995; Nicolosi et al., 2000) which was confirmed in our study and grapefruit subgroup separated from sweet oranges subgroup with similarity coefficient of 0.64.

Group C, consisted of two subgroups with similarity coefficient of 0.55 (subgroup I and subgroup J). The first subgroup was further divided into two subgroups. The first minor subgroup included Clementine (G21) while the second minor subgroup included mandarins G22, G23, G24 and G25 which showed absolute similarity.

The second subgroup J consisted of Dancy mandarin (G28). In cluster C, all accessions except G21 were fertile. The dendrogram (Figure 2) showed that all accessions of fertile mandarins were closely clustered and well separated from the other low fertile and male sterile mandarins. Although fertile and low fertile mandarins in group C have been clustered into different subgroups, the genetic similarity among them was relatively high, suggesting that the genetic base of domesticated mandarin germplasm is quiet narrow. Federici et al. (1998) also found that the mandarin group did not form a unified clade when hybrid and non-hybrid accessions were analyzed. Mandarins are considered to be a true *Citrus* species.Group D included only lemon lisbon (G20), one of the species of *Citrus* genus. In this research, low genetic relationship (18%) was obtained between fertile lemon lisbon and other fertile mandarin and orange cultivars. Lemons are thought to be natural hybrids of a citron and a lime (Scora, 1975; Barrett and Rhodes, 1976) or a hybrid of citron and sour orange (Gulsen and Roose, 2001b; Nicolosi et al., 2000) which confirm the results of this research.

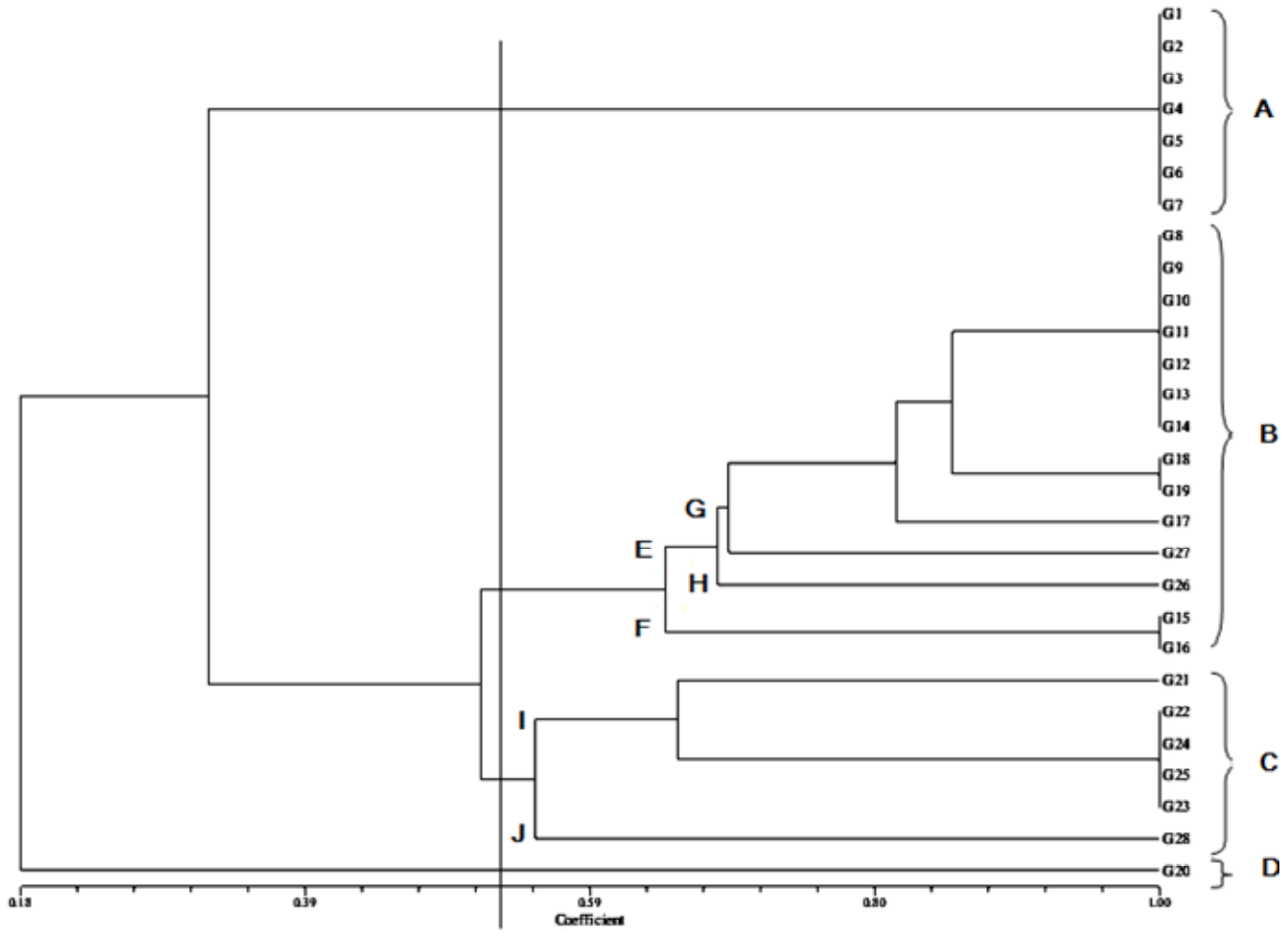

Figure 2. Dendrogram analysis of the 28 citrus accessions by UPGMA method with jaccard similarity coefficient. Name of the accessions are shown in Table 1.

Conclusion

In conclusion, the eight SSR markers were fairly successful for the identification of sterile, male sterile, fertile and low fertile citrus accessions and their phylogenetic relationships among them in spite of the fact that these SSR markers could not distinguish clear phylogenetic relationships in a few accessions. There is an extremely high rate of bud and limb mutations in *Citrus* genus (Moore, 2001), and SSR markers which develop through sexual reproduction and recombination are disabled in detecting such mutations in asexually propagated species. Therefore, it seems that in plants with asexual propagation system, inclusion of dominant markers such as PCR-RFLP and ISSR in mutation detection power does not mean that meiosis would increase the resolution of varietal identification. However, the results of this study confirms that SSR markers are useful for characterization of germplasm collections as were mentioned in previous studies (Barkley et al., 2006; Brown et al., 1996; Hokanson, 1998).

Low fertile, sterile and male sterile types are valuable sources in establishing the citrus orchard, because consumers prefer low seed or seedless fruits. Also, the use of male sterile species in the traditional cross-breeding and protoplast fusion technology to create new sterile verities are useful. We suggest that collection of accessions from male sterile, sterile and low fertile cultivars would likely increase the genetic variation available to plant breeders. Therefore, their collection, preservation and molecular discrimination seem to be of great necessity.

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