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Assessment of Genetic Variability in Some Iranian Sweet Oranges (*Citrus sinensis* [L.] Osbeck) and Mandarins (*Citrus reticulata* Blanco) Using SSR Markers

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ABSTRACT

The genetic variability of eight sweet oranges (*Citrus sinensis* [L.] Osbeck) and six mandarins (*Citrus reticulata* Blanco) accessions was evaluated using simple sequence repeats (SSRs) analysis. In total, 52 putative alleles were detected using seven primer pairs. The number of putative alleles per primer pair ranged from 3 to 10 with an average of 7.42. Polymorphic information content (PIC) value changed from 0.505 to 0.950. Microsatellite markers discriminated variation within mandarins, but low variation observed between sweet oranges. A UPGMA phenetic tree was constructed and one main sweet orange group consisting of three sub-groups and four main mandarin groups were identified. The majority of sweet orange accessions showed a narrow genetic base suggesting that the observed morphological polymorphism within the group must be associated with somatic mutations, which were not exactly detected by these molecular markers.

Key Words: *Citrus sinensis; Citrus reticulate;* Molecular markers; SSR; Polymorphism

INTRODUCTION

Citrus is one of the most important fruit crops in the world. 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 shortened juvenile periods (e.g., *Citrus aurantifolia*) and resistance to disease, including citrus tristeza virus, phytophthora, and burrowing nematode (e.g., *Poncirus trifoliata*) (Cameron & 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, the high frequency of bud mutations and the long history of cultivation and wide dispersion (Scora, 1988). The sweet orange, *C. sinensis* (L.) Osbeck, is the main evergreen fruit–crop species, responsible for 75% of citrus production used both as fresh fruit and processed juice (Spiegel–Roy & Goldschmidt, 1996). Sweet oranges are vegetatively propagated and new cultivars are obtained after careful selection of spontaneous somatic mutations. Unlike the sweet orange group in which nearly all cultivars have arisen by somatic mutation, the genetic variation in the mandarin group (*C. reticulata* Blanco) is associated with sexual hybridization among a great number of species and intraspecific hybrids (Cameron & Frost, 1968). Genetic improvement of citrus species through conventional breeding methods has been hampered by the long juvenile period, high heterozygosity, large plant size and nucellar embryony (Gmitter *et al*., 1992).

Genetic markers which are stable, detectable in all tissues, and independent of environmental conditions or production practices, have become very efficient and powerful tools in *Citrus* in a wide range of applications including cultivar identification (Fang & Roose, 1997; Filho *et al*., 1998; Novelli *et al*., 2000), phylogenetics (Pang *et al*., 2003), zygotic and nucellar seedlings identification (Oliveira *et al*., 2002; Ruiz *et al*., 2000) and the construction of linkage maps (Kijas *et al*., 1997; Sanker & Moore, 2001) for marker assisted breeding and map–based cloning of genes. Of the many molecular techniques available to researchers, simple sequence repeats (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 (Nunes *et al*., 2002), apple (Guilford *et al*., 1997) and Grape (Tomas & Scott, 1993). The present study used SSR markers to evaluate genetic polymorphism and similarity among accessions of sweet oranges and mandarins.

MATERIALS AND METHODS

Plant material. All commercially cultivated accessions of sweet oranges and mandarins with some their morphological characterizations are represented in this study (Table I). The plant materials were collected from

citrus research institute of Iran.

DNA extraction. Total DNA was extracted from young leaves according to the modified method of Murray and Thompson (1980). The leaves were ground to a fine powder in liquid nitrogen and resuspended in 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), and precipitated in absolute ethanol and pellet resuspended in TE containing 10mg/ml RNAse. The quality and quantity of DNA were determined as described by Sambrook *et al.* (1989) and DNA templates were diluted to 12.5 ng/µl.

SSR Assay

PCR amplification. A total of seven primer pairs were used (Table II). The primer pairs were synthesized by Geneworks (Australia) according to published sequences of Kijas *et al.* (1997). The PCR amplifications were conducted in a total volume of 20 µl (2.0 µl of 10 × buffer, 50 ng of genomic DNA, 0.2 mM of each dNTP, 0.1 mM of each forward and reverse primer, 0.5 unit of DNA Taq polymerase (Sigma). The amplifications were performed according to Kijas *et al.* (1997) with a first denatureation at 94°C for 5 min followed by 32 cycles of 1 min at 94°C, 30 sec at 55°C, and 1 min at 72°C, with a final extension at 72°C for 4 min.

Detection of the SSR products. The PCR products were run in 6% polacrylamid gels in 1× TBE buffer (45 mM Tris-Boric, 1mM EDTA pH 8.0) at 120 V for 1 hour. Gels were stained in 0.5 µg/ml ethidium bromide, viewed and photographed under U.V. illumination.

Data analysis. Fragments amplified by microsatellite primers were scored as present (1) or absent (0). Genetic distances were estimated according to Nei and Li (1979). Cluster analysis was performed with the syntax 5.02 package based on average linkage (UPGMA, un weighted pair-group method with arithmetic average) and diversity level of loci were evaluated with the polymorphic information content ($PIC = 1- \Sigma^n Pi^2$) according to Liu (1998).

RESULTS AND DISCUSSION

A total of 52 putative alleles were detected by the 7 citrus microsatellite markers with an average of 7.42 putative alleles per locus. The number of putative alleles per locus ranged from a minimum of three for *CAC23* to a maximum of 10 for the most polymorphic marker *TAA45*. At nearly all putative loci, primers successfully amplified the target sequences across sweet oranges and mandarins. The alleles of marker *TAA15* with 14 different accessions are shown in Fig. 1 and the size of the 14 alleles ranged from 141 to 174 bp. The length of alleles in the 14 accessions ranged from 120 to 300 bp. Except for *CAC23* and *TAA33*, all other microsatellite markers showed a high value of *PIC* ranging from 0.876 to 0.950 with a mean of 0.818 (Table III). Table II showed that majority of accessions were heterozygous, with all of two different sizes, such as in *TAA3*, *TAA15*, *CAC23*, *TAA33*, *TAA41* and *TAA45*. The mandarins ' Bami I ' and ' Mohali I ' were homozygous for loci *TAA45* and *TAA33* respectively. There were nine homozygous and five heterozygous alleles for locus *TAA52*. Nearly all microsatellites amplified fragments in all accessions, which showed that a high level of sequence conservation exists within the primer sites flanking the microsatellites in citrus genome. A large amount of structural and functional homology between citrus genome has been suggested in the past (Jarrel *et al.*, 1992). According to Kijas *et al.* (1997), SSR primer conservation was found to exist across a broad range of citrus and related species. For use in linkage mapping, microsatellites must be informative across many species and cultivars within the genus or family, especially where a wide range of genetic variability exists, as is the case within citrus.

Analysis was performed in this study using highly polymorphic microsatellites according to Kijas *et al.* (1997) and low variation was detected within eight *C. sinensis* (sweet orange) accessions at any of seven markers tested. Variation was revealed between a number of *C. reticulata* (mandarin) accessions.

Fig. 1. SSR profile of 14 Citrus accessions using primer pair *TAA15*

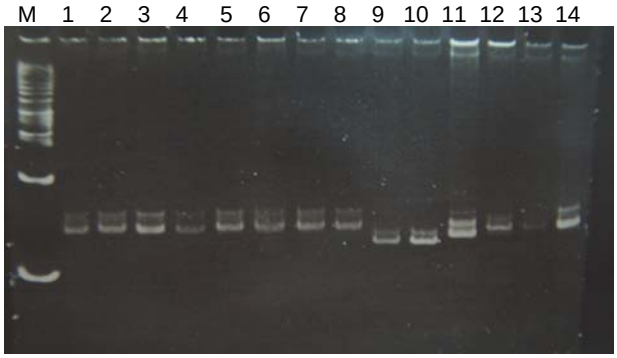

Fig. 2. Cluster analysis of sweet orange and mandarin accessions

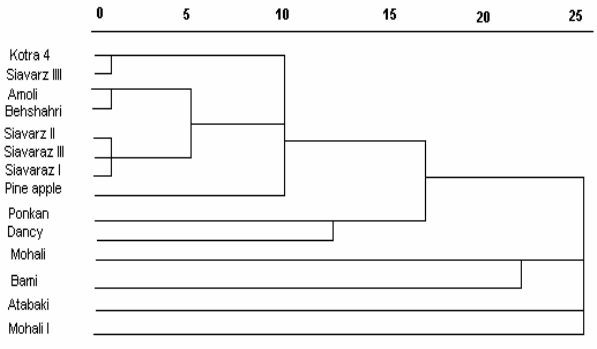

Table I. Plant material used in the study with some morphological characterization

Common name	Swingle system	Shape and Size of fruit	Fruit color	Season of maturation
Pine apple	*Citrus sinensis*	round - medium	orange	midseason
Amoli	*Citrus sinensis*	round - medium	yellow - orange	midseason
Siavaraz I	*Citrus sinensis*	round - medium	yellow	midseason
Siavaraz II	*Citrus sinensis*	round - medium	yellow - orange	midseason
Behshahri	*Citrus sinensis*	round - medium	yellow - orange	midseason
Siavaraz III	*Citrus sinensis*	round - medium	yellow - orange	midseason
Kotra 4	*Citrus sinensis*	round - medium	orange	midseason
Siavaraz IIII	*Citrus sinensis*	round - medium	orange	midseason
Ponkan	*Citrus reticulata*	moderately oblate - large	yellow - orange	early-midseason
Duncy	*Citrus reticulata*	oblate – medium to small	deep orange-red	mid-season
Bami I	*Citrus reticulata*	round - medium to small	yellow - orange	mid-season
Atabaki	*Citrus reticulata*	round - medium to small	yellow - orange	mid-season
Mohali	*Citrus reticulata*	round - medium to small	yellow - orange	mid-season
Mohali I	*Citrus reticulata*	round - medium to small	yellow - orange	mid-season

Table II. Amplification products using *TAA3, TAA15, TAA33, TAA41, TAA45, TAA52* and *CAC23* primers

Accession	*TAA3*	*TAA15*	*TAA33*	*TAA41*	*TAA45*	*TAA52*	*CAC23*
Pine apple	150-165	150-168	120-135	162-174	153-165	132- -	258-279
Amoli	150-165	150-168	120-132	162-174	132-144	132- -	258-279
Siavaraz I	150-165	150-168	120-135	162-174	132-144	132- -	258-279
Siavaraz II	150-165	150-168	120-135	162-174	132-144	132- -	258-279
Behshahri	150-165	150-168	120-132	162-174	132-144	132- -	258-279
Siavaraz III	150-165	150-168	120-135	162-174	132-144	132- -	258-279
Kotra 4	150-165	150-168	120-132	162-174	147-156	132- -	258-279
Siavaraz IIII	150-165	150-168	120-132	162-174	147-156	132- -	258-279
Ponkan	141-150	141-150	120-135	- -	153-168	150-174	258-279
Duncy	141-150	141-150	120-135	150-162	153-168	147-168	258-279
Bami I	156-171	147-159	120-135	138-153	153- -	144-165	258-300
Atabaki	147-159	153-162	120-126	141-159	141-150	144-159	258-279
Mohali	153-165	153-162	120-135	153-171	156-171	150- -	258- -
Mohali I	156-174	156-174	120- -	153-171	156-171	150-168	- -

Table III. Microsatellite loci in sweet oranges and mandarins

Primers	Alleles size variation	Alleles number	homozygous	heterozygous	PIC
TAA3	141-174	9	0	14	0.876
F^1- AGAGAAGAAACATTTGCGGAGC					
R^2- GAGATGGGACTTGGTTCACACG					
TAA15	141-174	9	0	14	0.897
F- GAAAGGGTTACTTGACCAGGC					
R- CTTCCCAGCTGCACAAGC					
TAA33	120-135	4	1	13	0.646
F- GGTACTGATAGTACTGCGGCG					
R- GCTAATCGCTACGTCTTCGC					
TAA41	138-174	9	0	13	0.910
F- AGGTCTACATTGGCATTGTC					
R- ACATGCAGTGCTATAATGAATG					
TAA45	132-171	10	1	13	0.950
F- GCACCTTTTATACCTGACTCGG					
R- TTCAGCATTTGAGTTGGTTACG					
TAA52	132-174	8	9	5	0.945
F- GATCTTGACTGAACTTAAAG					
R- ATGTATTGTGTTGATAACG					
CAC23	258-300	3	1	12	0.505
F- GGTGATGCTGCTACTGATGC					
R- CAATTGTGAATTTGTGATTCCG					

F^1= Forward -R^2= Reverse

Studies conducted by Kijas *et al*. (1997), Filho *et al*. (1998), Novelli *et al*. (1999) and Riaz *et al*. (2003), showed that SSR markers could not distinguish between *C. sinensis* cultivars but *C. reticulata* cultivars did. These results are most likely a direct reflection on breeding history of the various accessions tested. During detailed morphological investigation of citrus, *C. reticulata* was found to be a heterogenous species which had cultivars ranging from facultative apomicts to completely sexual types (Barret & Rhodes, 1976). A different conclusion was drawn for *C. sinensis*. The members of this species are thought to have undergone only minor somatic mutational variations from the original biotype resulting in such variants as seedlessness, pigmentation and time of maturity (Barrett & Rhodes, 1976).

Genetic relationships have been presented as a dendrogram (Fig. 2.). It could be seem from Fig. 2. that all the sweet orange accessions could be grouped into one main group consisting of three sub-groups: (1) ' Kotra 4 ' and ' Siavaraz IIII ', (2) ' Amoli ', ' Behshahri ', ' Siavaraz I ', ' Siavaraz II ' and ' Siavaraz III ' and (3) ' Pine apple '. These results suggest that these local accessions i.e. sub-groups 1 and 2 represent variations of a single clone with different names which are grown in different regions. Another explanation could be concluded that they are in fact different cultivars which were derived from somatic mutations that were not detected by the molecular markers used in this study.

In mandarin group, four main groups were identified. ' Ponkan ' and ' Dancy ' were clustered in one group. The genetic distance of ' Ponkan ' with ' Dancy ' (Gd=12) suggests that these mandarins are similar cultivars, rather than a distinct species as suggested by Tanaka (1954). Luro *et al*. (1995) obtained similar result among ' Ponkan ' and ' Dancy ' accessions. ' Mohali ' with ' Bami ' were grouped in the same group. Consequently, it supposed that these local accessions represent variations of a single clone with different names. ' Atabaki ' and ' Mohali I ' were clustered in two groups separately.

Our results showed conservation of tested microsatellite loci among the citrus species. Microsatellites were able to efficiently identify cultivars at the species level, but individual cultivars within each species, believed to have evolved through mutations, were undistinguishable. Consequently, like other genetic markers, it seem that primer amplified microsatellite loci are not useful for the detection of point mutations (Breto *et al*., 2001).

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