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Full Length Research Paper

Genetic diversity analysis of Iranian citrus varieties using micro satellite (SSR) based markers

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Fifteen SSR Primer Pairs were used to estimate the level of polymorphism among 23 Citrus genotypes and four natural hybrids or bud mutation was selected from Kotra Germplasm Bank (IRAN) . All fifteen loci assayed in citrus plant possessed a high level of polymorphism, with the number of alleles per locus ranging from 4 in TAA41 to 12 at CAT01, ATC09, AG14 (an average, 8.27 alleles were detected per locus). Cluster analysis with SSR markers resulted in 2 cluster groups: Group A: Yuzo and *Poncirus*. Group B: There are three separate subgroups within Group B; (i) genus *Fortunella sp* (ii) Mandarin subgroup: *Citrus reticulate (Citrus clemantin), Citrus sinensis* (Pineapple, Washington Navel), Natural types (Siahvaraz, Shalmahaleh, Moallemkoh and Kotra 4 hybrids) and (iii) *Citrus Limon* (Amol lemon - pear, Eureka, Rough Lemon), *Citrus aurantifolia, Citrus aurantium, Citrus medica and Citrus grandis*. Microsatellite analysis clustered citron and sour orange cv cluster but these taxa were quiet distant from *Fortunella SP*.

Key words: *Citrus*, microsatellite, phylogeny, polymorphism, germplasm bank, genetic diversity.

INTRODUCTION

Citrus plants are cultivated in the North and South of IRAN. Little is known about the genetic variability of Iranian cultivated citrus germplasm collection. Microsatellite or SSR (Simple Sequence Repeat) markers are co-dominant, multiallelic, highly polymorphic genetic markers and appropriate for genetic diversity studies.

Citrus Cultivated since ancient times in its centre of origin in south eastern Asia, citrus production has spread over the centuries into most areas that have a suitable climate (Webber et al., 1967) . Today citrus is one of the most widely cultivated fruit in the world, and most major production areas are far removed from the original areas. Different *Citrus* species widely grown in more than 50 countries in the world. World production is increasing and has reached 70 million tones, according to FAO (Orford et al., 1995).

Citrus taxonomy and phylogeny, however, are very complicated, controversial and confusing, mainly due to sexual compatibility between *Citrus* and related genera, the high frequency of bud mutations and the long history of cultivation and wide dispersion. Citrus varieties show diversity in their morphological traits such as size and shape of canopy, color, size, type and ripening season of the fruits and the number of seeds per fruit (Orford et al., 1995).

In the past, studies on relationships between genera and species were carried out based mainly on morphological and characteristics. Numerous classification systems have been formulated, among which those of Swingle (1943) and Tanaka (1977) have been the most widely accepted. Even these two researchers, however, have quite different concepts with respect to species classification, as Swingle included only 16 species in Citrus while Tanaka described 162. Later phylogenetic analysis by Scora (1975) and Barrett and Rhodes (1976) suggested that there were only 3 true species within the cultivated *Citrus*, that is, citron (*Citrus medica* L.), mandarin (*C. reticulata* Blanco) and pummelo [*C. grandis* (L.) Osb.] (in 1988 Scora added another true species: *C. halimii* Stone). In addition, other genotypes were derived from hybridization between these true species (Scora, 1988). More recently, biochemical data (Potvin et al.,

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Table 1. Cultivars, species, natural hybrids and bud mutation used in this study.

Common name	Type of cultivar Genus and species	Natural hybrid or bud mutation
Yuzo	*Citrus junos* Sieb	
Trifoliate Orange	Poncirus trifoliata	
Kumquat	*Fortunella Sp*.	
Clemantin	*Citrus reticulata* Blanco	
Satsuma Mandarin	*Citrus unshiu* Marcovich	
King or Sweet Orange	*Citrus nobilis*	
Pineapple Orange	*Citrus sinensis* (L.) Osbeck	
Washington Navel Orange	*Citrus sinensis*	
Siahvaraz	*Citrus sinensis*	Bud mutation
Moallemkoh	*Citrus sinensis*	Bud mutation
Kotra 2 - 4	-	Natural hybrid
Kotra 1 - 4	-	Natural hybrid
Shalmahaleh	-	Natural hybrid
Mexican Lime	*Citrus aurantifolia*	
Sweet Lime	*Citrus aurantifolia* (L.)	
Sour Orange	*Citrus aurantium* (L.)	
Amol Lemon-Pear	Near to *Citrus limon*	Bud mutation or natural hybrid
Citrus King (Pumelo)	*Citrus grandis* (L.) Osb	
Cluster Lemon	*Citrus limon* Burn.f	
Rough Lemon	*Citrus limon* Burn.f	
Eureka Lemon	Citrus limon	
Etrag Citron	*Citrus medica* (L.)	
Nova	*Near to Citrus reticulata*	Complex hybrid of Mandarin

1983), protein electrophoresis (Handa et al., 1986), isozymes (Torres et al., 1978; Fang et al., 1993; Herrero et al., 1996), microsatellites (Kijas et al., 1995), organeller genome analysis (Green et al., 1986; Yamamoto et al., 1993) and (Fang et al., 1997; Fang et al., 1998) have been used to examine relationships among *Citrus* taxa. Microsatellites, or simple sequence repeats (SSRs), are short sequence elements composed of tandem repeat units one to seven base pairs (bp) in length (Tautz, 1989). These repeats sequences have been shown to be highly polymorphic within and between species, a property that has permitted their application as molecular markers in population genetics (Goldstein et al., 1999), systematics (Goldstein and Pollock, 1997), and genome mapping (Weissenbach et al., 1992).

Microsatellites are present in high numbers in mammals and in plant genomes too, but appear to be less abundant than in mammalian or insect systems (Van Treuren et al., 1997). Thomas et al., 1994 distinguished 20 grapevines varieties by using four microsatellite loci. They proposed the use of microsatellites for establishing an international database for description of grapevine varieties, based on its high level of polymorphism, co-dominance, simplicity of analysis and repeatability (Thomas et al., 1994).

Little is known about the genetic variability of the Iranian Citrus Germplasm Collections. We investigated the phylogenetic relationships among 23 citrus plants of the Kotra Germplasm Bank (IRAN) and studied the origin of some important Citrus species of this Collection, using microsatellites markers.

MATERIALS AND METHODS

Plant materials and DNA isolation

In this study, 23 cultivars, species, natural hybrids or bud mutations were used (Table 1), which held at Germplasm Collection of Kotra (Iran) . From each accession, 50 mg of young expanding leaves were collected and stored at -80°C before DNA isolation. Genomic DNA was isolated from leaf samples in accordance with the CTAB (Hexadecyltrimethyl ammonium bromide) method described by Doyle and Doyle (1987). DNA was quantified by comparing it with lambda DNA (Promega Corporation, Madison, Wis) on ethidium bromide stained agarose gels.

PCRs and electrophoresis

Fifteen primer pairs (TAA15, TAA27, TAA41 CAC23, CAC15, CAC33, CAC39, CCT01, CAT01, ATC09, AG14, CTT01, CT21, TC26 and CT19) (MWG Biothec, Germany) were used in this

Table 2. SSR loci characterization, size of amplified fragments; primer pairs repeat motifs and total number of alleles.

Locus code	Repeat	Forward Primer	Reverse Primer	Alleles	Size range (bp)
TAA15	TAA	GAAAGGGTTACTTGACCAGGC	CTTCCCAGCTGCACAAGC	5	123 - 130
TAA27	TAA	GGATGAAAAATGCTCAAAATG	TAGTACCCACAGGGAAGAGAGC	10	158 - 230
TAA41	TAA	AATGCTGAAGATAATCCGCG	TGCCTTGCTCTCCACTCC	4	242 - 265
CAC23	CAC	ATCACAATTACTAGCAGCGCC	TTGCCATTGTAGCATGTTGG	6	105 - 135
CAC15	CAC	TAAATCTCCACTCTGCAAAAGC	GATAGGAAGCGTCGTAGACCC	10	135 - 190
CAC33	CAC	GGTGATGCTGCTACTGATGC	CAATTGTGAATTTGTGATTCCG	8	77 - 109
CAC39	CAC	AGAAGCCATCTCTTCTGCTGC	AATTCAGTCCCATTCCATTCC	6	120 - 165
CCT01	CCT	TCAACACCTCGAACAGAAGG	CCCACATGCTAGCACAAAGA	8	93 - 119
CAT01	CAT	GCTTTCGATCCCTCCACATA	GATCCCTACAATCCTTGGTCC	12	138 - 172
ATC09	ATC	TTCCTTATGTAATTGCTCTTTG	TGTGAGTGTTTGTGCGTGTG	12	130 - 210
AG14	AG	AAAGGGAAAGCCCTAATCTCA	CTTCCTCTTGCGGAGTGTTC	12	110 - 172
CTT01	CTT	TCAGACATTGAGTTGCTCG	TAACCACTTAGGCTTCGGCA	7	226 -252
CT21	CT	CGAACTCATTAAAAGCCGAAAC	CAACAACCACCACTCTCACG	8	130 - 170
TC26	TC	CTTCCTCTTGCGGAGTGTTC	GAGGGAAAGCCCTAATCTCA	7	93 - 119
CT19	CT	CGCCAAGCTTACCACTCACTAC	GCCACGATTTGTAGGGGATAG	9	175 - 205

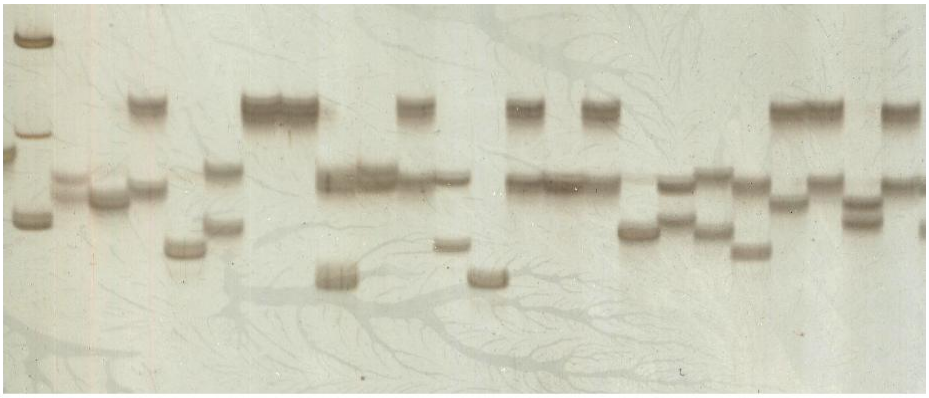

Figure 1. Microsatellite polymorphism (locus CAC15) A is size marker. Lane 1 = Yuzu; Lane 2 = Rough Lemon; Lane 3 = Clemantin; 4 = Etrag Citron; 5 = Sour Orange; 6 = Satsuma Mandarin; 7 = Kumquat; 8 = Amol Lemon-Pear; 9 = Siahvaraz; 10 = Cluster lemon; 11 = Trifoliate Orange; 12 = Pinapple Orange; 13 = Citrus king (Pumelo); 14 = Washington Navel Orange; 15 = Eureka Lemon; 16 = Sweet Lime; 17 =cluster sour orange; 18 = Moallemkoh; 19 = Shalmahaleh; 20 = Kotra 2-4; 21 = Nova; 22 = Kotra 1-4; 23 = Mexican Lime.

research (Table 2). PCRs were performed in a final volume of 10 L, containing the following: 20 mmol Tris–HCl/L (pH 8.4); 50 mmol KCl/L; 1.5, 2.5, or 5 mmol MgCl2/L, depending on the primers; 0.1 mmol/L of each dNTP (deoxynucleoside triphosphate); 0.8 mol/L of each primer; 20 ng of genomic DNA; and 1 U *Taq* polymerase (Invitrogen, Carlsbad, Calif). The following temperature profile was used: 95°C for 1 min, then 35 cycles of 94°C for 45 s, 45 – 63°C for 45 s and 72°C for 75 s), ending with 72°C for 7 min (Progene; Techne, Cambridge, UK). PCR products were separated by electrophoresis in 6% acrylamide gels, stained with ethidium bromide (0.8 g/mL), using 1× TBE (89 mmol Tris/L, 89 mmol boric acid/L and 2 mmol EDTA/L (pH 8.0) buffer and visualized under ultra-violet light. Molecular sizes of the amplified fragments were estimated using a 100-bp ladder (Invitrogen) (Soriano et al., 2005) (Figure 1).

PIC (polymorphism information content) value

In order to determine the informativeness of the microsatellites, the PIC values were calculated. PIC value was calculated according to the formula:

$$PIC = 1-P^2_{ij}$$

Polymorphism analysis

For a single locus, the presence of amplified fragments was scored as 0.5 if the Individual was heterozygous, 1 if it was homozygous, and 0 if the allele was not present. According to these observations,

Table 3. PIC value of different Iranian *Citrus* germplasm.

	PIC*					
SSR	**Natural hybrids**	**Grape fruit**	**Lemons**	**Mandarins**	**Citrus**	**Mean PIC**
TAA15	0.69	0.50	0.50	0.50	0.50	0.65
TAA27	0.62	0.75	0.76	0.72	0.72	0.85
TAA41	0.0	0.81	0.48	0.55	0.50	0.52
CAC23	0.0	0.38	0.58	0.56	0.0	0.71
CAC15	0.72	0.63	0.85	0.83	0.72	0.86
CAC33	0.65	0.61	0.68	0.54	0.52	0.78
CAC39	0.67	0.50	0.50	0.69	0.50	0.72
CCT01	0.69	0.62	0.61	0.52	0.41	0.75
CAT01	0.85	0.81	0.80	0.87	0.27	0.89
ATC09	0.67	0.63	0.69	0.53	0.50	0.76
AG14	0.59	0.38	0.85	0.67	0.0	0.87
CTT01	0.58	0.53	0.56	0.68	0.53	0.74
CT21	0.81	0.75	0.78	0.67	0.50	0.73
TC26	0.89	0.50	0.82	0.67	0.0	0.83
CT19	0.71	0.76	0.78	0.59	0.48	0.79
Mean PIC	0.64	0.61	0.68	0.63	0.41	

*Polymorphic information content.

a similarity matrix was generated using the Nei's genetic distance (Nei, 1972). Similarity data were processed through the unweighted pair-group method (UPGMA) cluster analysis conducted using NTSYS program (Exeter Software, Setauket, N.Y.) (Rohlf, 1993), program, applying the Jaccard (1908) and Dice (Sneath and Sokal, 1973) coefficients. The goodness of fit measured by the cophenetic correlation unweighted pair-group method, arithmetic average (UPGMA) cluster analysis and finally depicted in a dendrogram (Figure 1).

RESULTS

Microsatellite polymorphism and accession variability

Microsatellites analysis clustered Citron and sour orange cv cluster but these taxa were quiet distant from *Fortunella SP*.

The present study showed the utility of microsatellite markers for the detection of polymorphisms among the Iranian citrus germplasm. The identification of similarity group could be useful for the selection of parental plants to be used in the breeding programs.

All fifteen loci assayed in citrus plant possessed a high level of polymorphism, with the number of alleles per locus ranging from 4 in TAA41 to 12 at CAT01, ATC09, AG14 (an average, 8.27 alleles were detected per locus).

Where Pij is the frequency of the jth microsatellite allele for loci. This value is referred to as heterozygosity and gene diversity (Weir 1990, Anderson et al, 1993).

The most highly polymorphic loci were: CAT01 (12 alleles, PIC=0.89), AG14 (12 alleles, PIC = 0.87), TAA27 (10 alleles, PIC = 0.85) (Table 3).

Cluster analysis

UPGMA cluster analysis of the similarity matrix obtained from 23 SSR alleles (Nei 1972) resulted in a dendrogram of genetic relationships that grouped cultivars in agreement with their geographic origins and pedigrees (Figure 2), producing 2 main clusters. The first cluster Included Yuzo and *Poncirus* **.** The second cluster was subdivided into 3 sub-clusters (i) genus *Fortunella sp* (ii) Mandarin subgroup: *Citrus reticulate (Citrus clemantin), Citrus sinensis* (Pineapple, Washington Navel), Natural types (Siahvaraz*,* Shalmahaleh, Moallemkoh and Kotra 4 hybrids) and (iii) *Citrus limon* (Amol lemon-pear, Eureka, Rough Lemon), *Citrus aurantifolia, Citrus aurantium, Citrus medica and Citrus grandis*.

Microsatellite analysis clustered Citron and sour orange cv cluster but these taxa were quiet distant from *Fortunella SP*.

DISCUSSION

The transportability of the microsatellites among species belonging to different genera or even families has been previously reported (Dirlewanger et al., 2002).

In our work microsatellite markers were used to study genetic diversity in 23 citrus plants of the Kotra Germplasm Collection, IRAN (Yuzo, Rough Lemon, Clemantin, Etrag Citron, Sour Orange, Satsuma Mandarin, Kumquat,

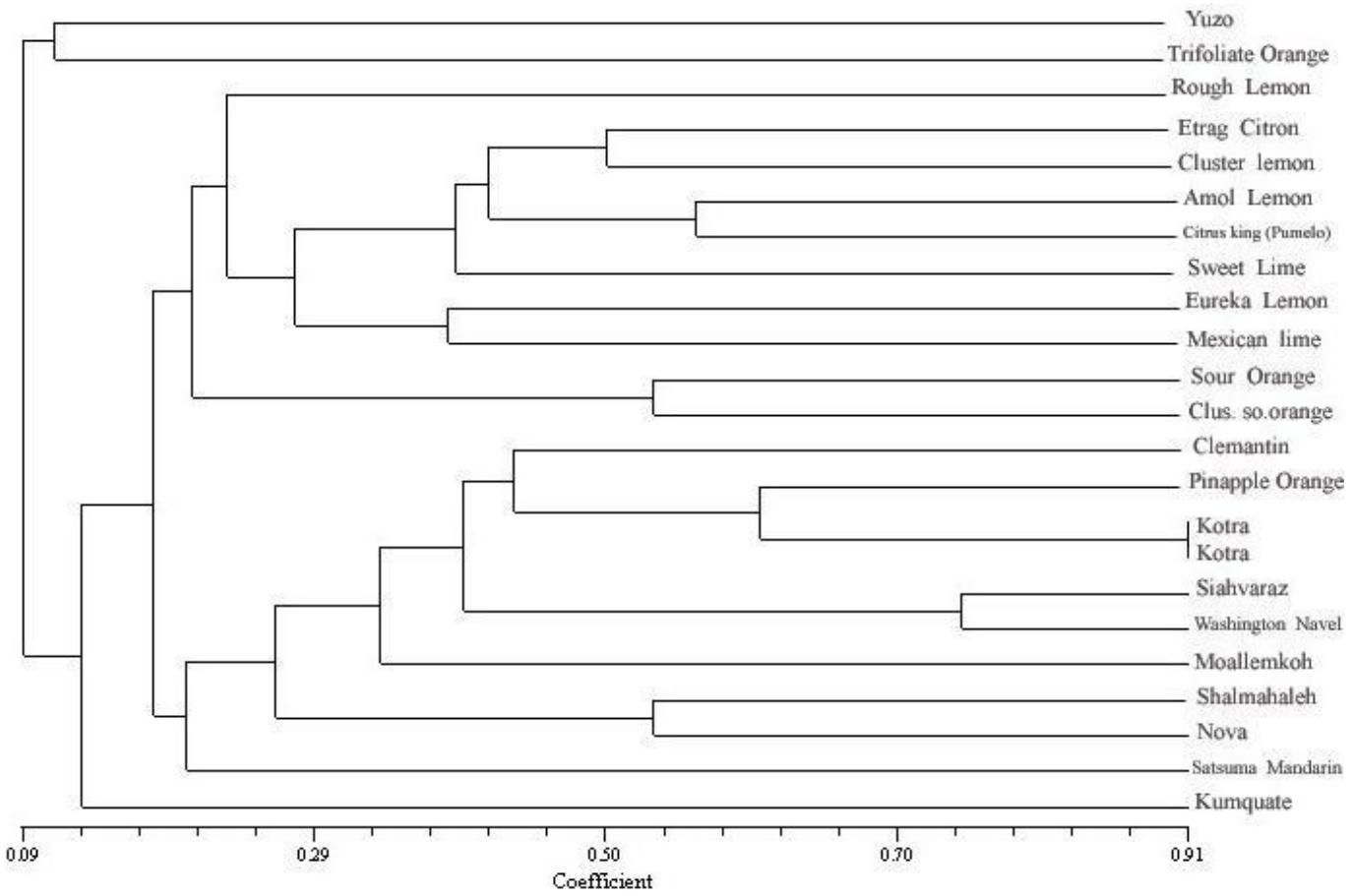

Figure 2. Dendrogram of the 23 citrus cultivars included in this study generated by unweighted pair-group method (UPGMA) cluster analysis from the similarity matrix obtained using Nei's (1972) genetic distance.

Amol Lemon-Pear, Siahvaraz; ,Cluster lemon, Trifoliate Orange, Pineapple Orange, Citrus king (Pumelo), Washington Navel Orange, Eureka Lemon, Sweet Lime, Moallemkoh, Shalmahaleh, Kotra 2 - 4, Nova, Kotra 1 - 4, Kumquate, Mexican Lime and Sour Orange var. Cluster). According to Wang et al. (1994), in plant nuclear DNA the dinucleotides sequence (AT)n is the most abundant, followed by (A)n/(T)n and (AG)n/(CT)n. In our experiments, CAT01, ATC09, AG14 gave excellent fingerprint patterns, suggesting that these repeats are abundant in citrus plants. In the study of Gulsen and Roose (2001) cpDNA indicated that *Fortunella sp* had totally different microsatellite patterns from the other taxa analysed. Although Fortunella is well differentiated from *Citrus* on the basis of detailed morphological studies, apparently there has not been the same level of divergence at the molecular level. Our experiments indicated that the genus *Citrus* is quiet distant from the related genus *Poncirus*. Kotra 1 - 4 and Kotra 2 - 4 probably originated as nucellar seedling from the same tree. Siahvaraz has a much similarity to Washington Navel orange and probably originated from bud mutation. Shalmahaleh is a natural hybrid and has a greater similarity to Nova. Moallemkoh has a similarity to Washington navel Orange and Siahvaraz and is probably hybrid between them or as a bud mutation. Shalmahaleh, Nova, Etrag Citron and Citrus King (Pumelo) are very similar and Shalmahaleh is apparently as a hybrid origin, most probably of Nova and Etrag Citron or Nova and Citrus King (Pumelo). Amol Lemon -Pear is probably derived from hybridization between Rough Lemon and Citrus King and has a similarity to them.

The percentage of PIC (polymorphic Information Content) in lemon, Mandarin, Grapefruit, Natural hybrid and sweet orange were 0.68, 0.63, 0.61, 0.64, 0.41 as observed by Novelli et al. (2000).

Heterozygosity is important to both natural and cultured populations because (1) it provides a large spectrum of genotypes for adaptive response to changing conditions and (2) heterozygous individuals usually are superior to less heterozygous individuals in many economically important characteristics like growth, fertility and disease resistance. A set of informative SSR markers detected considerable levels of genetic variability in the Iranian citrus germplasm. The identification of similarity group could be useful for the selection of parental plants to be used in the breeding programs.

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