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Studying of Genetic Diversity in Satsuma (*Citrus unshiu*) Mandarin Utilizing Microsatellite Markers

[1]A. Ghanbari, [2]N.B. Jelodar and [2]H. Rahiman
[1]Department of Horticulture,
[2]Department of Plant Breeding, College of Agriculture, University of Mazandaran,
P.O. Box 578, Sari, Iran

Abstract: Genetic diversity of forty-four genotypes of Satsuma cultivars from Mazandaran was assessed using Simple Sequence Repeat (SSR) markers. SSR markers in different genotypes determined 2-5 alleles. The average of Polymorphic Information Content (PIC) in SSR markers were 0.88. Also, Principal Component Analysis (PCA) showed that SSR with four primary factors explained 80% of variations. Dendrograms showed that SSR markers could completely separate the Unshiu population. All the genotypes were grouped in 3 distinct clusters in cluster analysis. The control population, owari Genotypes were located in a separated cluster, while local Unshiu genotypes, unknown Unshiu, Wase and Wase derived genotypes were put together in another cluster. These results confirm the close relationship between local unshiu genotypes and Wase group or one of its similar varieties.

Key words: Unshiu mandarin, molecular marker, genetic diversity, SSR

INTRODUCTION

Citrus and its close relatives are represented by 28 genera in the tribe Citreae of the subfamily Aurantioideae in the family Rutaceae (Swingle and Reece, 1967). Before the advent of molecular data, *Citrus* was classified based on morphology or biochemical techniques such as isozymes. There are currently two-dimension (2D) commonly used classifications of *Citrus*: Swingle (Swingle and Reece, 1967) and Tanaka (Tanaka, 1977). The Swingle system recognizes 16 species in the genus *Citrus*, whereas the Tanaka system recognizes 162 species in the genus *Citrus*. Scora (1975) and Barrett and Rhodes (1976) suggested that there are only three basic true species of *Citrus* within the subgenus *Citrus* as defined by Swingle: citron (*C. medica* L.), mandarin (*C. reticulata* Blanco), and pummelo (*C. maxima* L. Osbeck). Other cultivated *Citrus* species within the subgenus *Citrus* are believed to be hybrids derived from these true species, species of the subgenus *Papeda*, or closely related genera. This idea has gained support in recent years from data derived from molecular markers (Federici *et al*., 1998; Nicolosi *et al*., 2000).

Mandarins (*Citrus* spp.) are the second most important group of citrus plants in the world, with the highest climatic adaptation among the cultivated citrus. Iran is the 6th large producer of citrus in the world. Indeed, the culture and its commercialization are restricted to few varieties, offered in a small period during the year, causes the needs to import the product. For these reasons, an Iranian mandarin breeding program is essential to investigate the available genetic resources, and to supply the market demands.

Microsatellites, or Simple Sequence Repeats (SSR), are arrays of hypervariable short (1-5 bp) repeat motifs that can be found in both coding and non-coding DNA sequence of higher organisms.

Corresponding Author: Nadali Babaeian Jelodar, Department of Plant Breeding, College of Agriculture, University of Mazandaran, P.O. Box 578, Sari, Iran
Tel: ++98-151-3822574 Fax: ++98-151-3822577

These single-locus markers are principally characterized by high frequency, Mendelian inheritance and codominance. During the last decade, microsatellites have proven to be the marker of choice in plant genetics and breeding research, because of their variability, ease of use, accessibility of detection and reproducibility (Zane, 2002). There are now many well-known examples of initiatives using microsatellites for different plant species, including *Citrus* sp. (Kijas, 1994; Kantety, 2002; Cristofani, 2003). 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.

In this research, we studied genetic variability within forty-four Satsuma mandarin (*Citrus unshiu*) genotypes utilizing SSR markers. Principal Component Analysis (PCA) showed that SSR with four primary factors explained 80 % of variations. Dendrograms showed that SSR markers could completely separate the Unshiu population. All the genotypes were grouped in 3 distinct clusters in cluster analysis. The control population, owari Genotypes were located in a separated cluster, while local Unshiu genotypes, unknown Unshiu, Wase and Wase derived genotypes were put together in another cluster.

SSR markers are particularly useful for characterization of germplasm collections because they are highly polymorphic and usually codominant (Brown *et al*., 1996; Hokanson *et al*., 1998; Liu *et al*., 2003), but they have not been widely used in citrus, whereas we assessed genotype variety intro mandarin accessions.

MATERIALS AND METHODS

Plant Materials

A total of forty-one genotypes belonging to mandarin varieties including 6 different groups: (1) 7 unknown unshiu, (2) 12 native unshiu, (3) 10 unshiu Wase, (4) 7 derivations of Wase, (5) 5 Owari, (6) 3 controls (*Citrus clementina*, *Citrus reticulate*, Citrus Page) were collected from Mazandaran Province. This study was carried out in College of Agriculture, University of Mazandaran, Sari, Iran at 2007.

The complete list of accessions used in this study along with cultivar names, genus and species classification are presented in Table 1.

Table 1: Mandarin varieties and number of plants analyzed

Taxonomic classification	Varieties	Taxonomic classification	Varieties
Citrus spp. (Unknown Unshiu)	Ramsar (UR1)	*C. unshiu* Wase	Ramsar (VR)
	Ramsar (UR2)		Tonekabon (VT1)
	Ramsar (UR3)		Tonekabon (VT2)
	Tonekabon (UT)		Tonekabon (VT3)
	Sari (US1)		Tonekabon (VT4)
	Sari (US2)		Tonekabon (VT5)
	Sari (US3)		Sari (VS1)
Citrus spp. (Local Unshiu)	Amol (UA1)		Sari (VS2)
	Amol (UA2)		Ghaemshahr (VG1)
	Amol (UA3)		Ghaemshahr (VG2)
	Amol (UA4)	*C. unshiu* (derivations of Wase)	Miagava Sari (MS1)
	Babol (UB1)		Miagava Sari (MS2)
	Babol (UB2)		Hashimoto Sari (HS)
	Babol (UB3)		Okitsu Sari (OS)
	Babol (UB4)		Okitsu Ramsar (OR)
	Babol (UB5)		Closiana Ramsar (CR)
	Babol (UB6)		Sugiama Ramsar (SR)
Control	Behshahr (UBH1)	Citrus Page	Ramsar (PR)
	Behshahr (UBH2)	*C. clementina*	Clementina Ramsar (KR)
		C. reticulata	Ponkan Ramsar (UPR)

DNA Extraction

Total DNA was extracted using a modified CTAB method (Clark, 1997). About 5 g of tissue-sample from young unfurled leaves were collected, immediately submerged in liquid nitrogen, and subsequently ground in liquid nitrogen with a mortar and pestle. The ground tissue was added to 15 mL of pre-heated extraction buffer (4% CTAB (hexadecyltrimethylammonium bromide), 100 mM of Tris-HCl (pH 8.0), 1.4 M of NaCl, 20 mM of EDTA and 4 ml mL^{-1} of mercaptoethanol) in an oak ridge tube and incubated at 65°C for 30 min. Samples were extracted with 15 mL of chloroform: isoamyl alcohol (24:1, v/v) and centrifuged at 6,000 rpm for 5 min. The supernatant was transferred to a new tube and extracted as before with chloroform: isoamyl alcohol. The DNA was precipitated by adding a 2D-thirds volume of ice-cold isopropanol and followed by centrifugation at 6,000 rpm for 5 min. The recovered DNA pellet was dissolved in 600 mL of TE buffer (10 mM of Tris, 1.0 mM of EDTA, pH 8.0) and RNA contamination removed by digestion with 10 mg mL^{-1} of RNAse for 30 min at room temperature. The purified DNA was precipitated by adding one-tenth volume of 3 M sodium acetate (NaOAc, pH 5.2), followed by 2 volume of cold ethanol and collected by centrifugation at 6,000 rpm for 5 min. The DNA pellet was washed with 70% ethanol, air-dried briefly and suspended in 200 mL of TE buffer. DNA concentrations were quantified by measuring absorbance at 260 nm wavelengths using a Pharmacia Gene Quant II spectrophotometer (Pharmacia Biotech, Cambridge, UK). An aliquot of the isolated DNA for each sample was diluted to a final concentration of 40 ng mL^{-1} in TE buffer for use in the subsequent assays.

PCR Amplification

Ten SSR primers (Table 2) were used to amplify DNA in a Polymerase Chain Reaction (PCR). The PCR reaction for SSR consisted of 0.5 ng μL^{-1} of template DNA, 0.25 μL^{-1} dNTPs, 0.2 μL^{-1} of each of forward and reverse primers, 2.5 μL^{-1} PCR buffer, 1.5 μL^{-1} magnesium chloride and 0.2 μL^{-1} (1 unit) Taq DNA polymerase. Cycling conditions consisted of 95°C for 5 min; 30 cycles of: 95°C 1 min, 58-64°C for 30 sec (annealing temperature was specific for each primer) and 72°C for 1 min; and one cycle of 72°C for 5 min. Electrophoresis was also carried out on polyacrylamide-gel (10% PAGE, (50 × 21)), at 18 mA for 2 h, as described in Ruiz *et al*. (2000), stained with $AgNO_3$.

Data Analysis

The molecular results were analyzed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) version 3.2 (Rohlf, 1998). Dendrograms were generated by the Unweighted Pair Group Method with Arithmetic Average (UPGMA) algorithm (Sneath and Sokal, 1973) using the Manhattan distance and following the procedures detailed in the NTSYS program.

Table 2: SSR primers sequence (Scarano *et al*., 2003) used for unshiu genotypes

Locus	Forward (5'->3')	Reverse (5'->3')	Allele size	Tm (°C)
CaC23	ATCACAATTACTAGCAGCGCC	TTGCCATTGTAGCATGTTGG	237-270	58.0
CAC39	AGAAGCCATCTCTCTGCTGC	AATTCAGTCCCATTCCATTCC	147-180	58.5
AG14	AAAGGGAAAGCCCTAATCTCA	CTTCCTCTTGCGGAGTGTTC	119-163	58.5
CAT01	GCTTTCGATCCCTCCACATA	GATCCCTACAATCCTTGGTCC	122-164	59.5
TC26	CTTCCTCTTGCGGAGTGTTC	GAGGGAAAGCCCTAATCTCA	122-154	59.0
TAA1	GACAACATCAACAACAGCAAGAGC	AAGAAGAGCCCCCATTAGC	147-190	63.0
TAA3	AGAGAAGAAACATTTGCGGAGC	GAGATGGGACTTGGTTCATCACG	133-172	62.5
TAA15	GAAAGGGTTACTTGACCAGGC	ATTCCCAGCTGCACAAGC	141-240	59.5
TAA41	AGGTCTACATTGGCATTGTC	ACATGCAGTGCTAATGAATG	122-185	56.5
CAGG9	AATGCTGAAGATAATCCGCG	TGCCTTGCTCTCCACTCC	103-121	57.0

RESULTS AND DISCUSSION

Microsatellite markers produced polymorphic and monomorphic bands ranged from 103 to 270 base pairs (Table 2). A maximum of 5 alleles were amplified by CAT01 and TAA15 primers, while minimum alleles (2 alleles) were observed when amplified by CAC23, TC26 and CAGG9 primers (Table 3). Figure 1 shows locus segregation.

For Microsatellite markers, Maximum PIC value was observed in TAA41 that was 0.98 and minimum one was observed in CAC23 (0.69). The mean value of PIC was 0.87 for Microsatellite markers (Table 4).

Genetic Distance and Similarity

Table 5 shows genetic distance between different groups calculated using with Nei value (Nei, 1972). In this study, we illustrate that genetic distance in control and owari groups was most

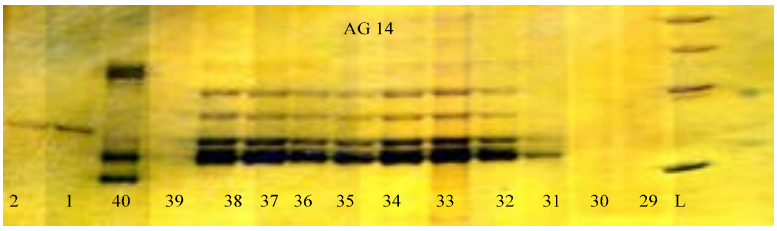

Fig. 1: SSR AG14 alleles banding pattern on polyacrylamide-gel (10% PAGE). 1, 2 and 40: control and others are Unshiu samples; L: DNA size marker (Fermentas).

Table 3: Observed alleles in Unshiu mandarin populations evaluated with SSR markers

Locus	Forward sequences	Reverse sequences	Observed alleles
CAC23	ATCACAATTACTAGCAGCGCC	TTGCCATTGTAGCATGTTGG	2
CAC39	AGAAGCCATCTCTCTGCTGC	AATTCAGTCCCATTCCATTCC	3
AG14	AAAGGGAAAGCCCTAATCTCA	CTTCCTCTTGCGGAGTGTTC	3
CAT01	GCTTTCGATCCCTCCACATA	GATCCCTACAATCCTTGGTCC	5
TC26	CTTCCTCTTGCGGAGTGTTC	GAGGGAAAGCCCTAATCTCA	2
TAA1	GACAACATCAACAACAGCAAGAGC	AAGAAGAAGAGCCCCCATTAGC	3
TAA3	AGAGAAGAAACATTTGCGGAGC	GAGATGGGACTTGGTTCATCACG	3
TAA15	GAAAGGGTTACTTGACCAGGC	CTTCCCAGCTGCACAAGC	5
TAA41	AGGTCTACATTGGCATTGTC	ACATGCAGTGCTATAATGAATG	3
CAGG9	AATGCTGAAGATAATCCGCG	TGCCTTGCTCTCCACTCC	2

Table 4: PIC value for SSR and ITS markers in evaluated varieties and genotypes

SSR primers	PIC value	Primers	PIC value
CAC23	0.686	TAA1	0.707
CAC39	0.922	TAA3	0.801
AG14	0.911	TAA15	0.934
CAT01	0.904	TAA41	0.975
TC26	0.922	CAGG9	0.981
Mean	0.874		

Table 5: Genetic and similarity distance for Unshiu Mandarin population with SSR markers

Population of study	Unknown unshiu	Local unshiu	Wase	Wase derived	Owari	Control
Unknown unshiu	*******	0.9262	0.9123	0.9681	0.9068	0.8399
Local unshiu	0.0767	*******	0.9709	0.9490	0.9158	0.9185
Wase	0.0917	0.0295	*******	0.9263	0.8997	0.8898
Wase derived	0.0325	0.0524	0.0766	*******	0.9390	0.8524
Owari	0.0979	0.0879	0.1057	0.0630	*******	0.7808
Control	0.1745	0.0851	0.1167	0.1597	0.2474	********

Genetic identity (above diagonal) and genetic distance (below diagonal)

(0.25) and in local unshiu and Wase was least (0.97). These data shows that genetic similarity of local unshiu and Wase is high. Most of genetic distance observed for SSR analyzed data between control and owari population

Cluster Analyzed Based Molecular Data

The POPGEN software program version 1.31(Yeh *et al*., 1999) was utilized to cluster based on six populations. The data showed that populations derived to three groups. The primary group consists of unknown unshiu, local unshiu, Wase and derived Wase; second group consists of owari and third group containing control populations.

Genotypes analyze with NTSYS software based on UPGAMA method showed high diversity between genotypes. The genetic similarity based on Dies similarity correlation ranged from 0.35 to 1. Also, slight analysis is done alone and without primary grouping by NTSYS software according to UPGAMA method and similarity index with due regard to all genotypes. Figure 2 and 3 show the grouping of genotypes. The range of cultivars and studied kinds similarity, according to similarity index is changeable from 0.35-1. This range is showing high diverse among genotypes.

With due attention to top clusters, the suegiyama cultivar settled close to local and also Wase cultivars. While this cultivar has extra distance with the cultivars of owari's group.

Principal Component Analysis (PCA)

Principal Component Analysis was done according to achieved similarity matrix according to the Dis coefficient. As observed, first component alone accounts for 45.39% of all changes. Which is considerable, because usually in the data of molecular markers, possibility of accounting for more amounts of primary indignation's variance by several principal component exists rarely (Table 6).

Calculation shows that three first principal component totally accounts for 78.69% of changes. whereas in quantitative traits because of high correlation of primary indignations, it is suggested if 2 to 3 first principal component account for changes upper than 80%, the PCA will have high efficiency. In studying genetic variety by molecular method, designing monotonous distribution of markers in genome, selection of markers from different ports of genome and low correlation among them and few number of used markers with regard to whole genome can be the reasons of much number of principal component in accounting for primary indignations (Rohlf, 1998). In this research 6 first principal components account for 85.98% of primary changes that is considerable in molecular method and is the reason of good efficiency of markers. With due attention to Table 6, first component accounts for 45.39% of all changes and second component accounts for 29.19% of principally changes. In this manner, totally by 7 principal components which their Eigenvalue are more than 1, 87.58% of changes can be accounted for.

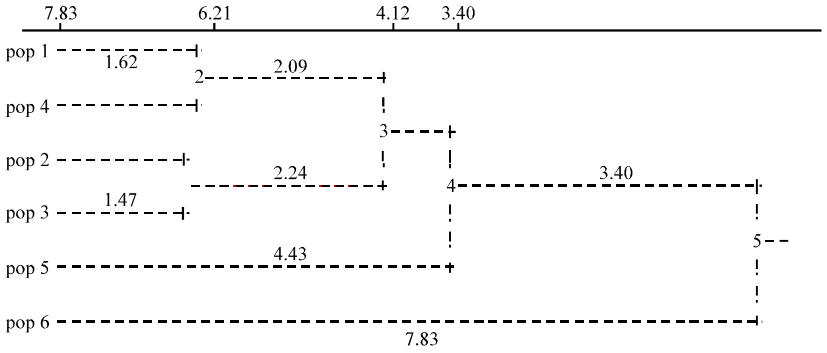

Fig. 2: Dendrogram for 6 genotypes of unshiu mandarin by UPGMA method with SSR markers in POPGEN software. POP means population

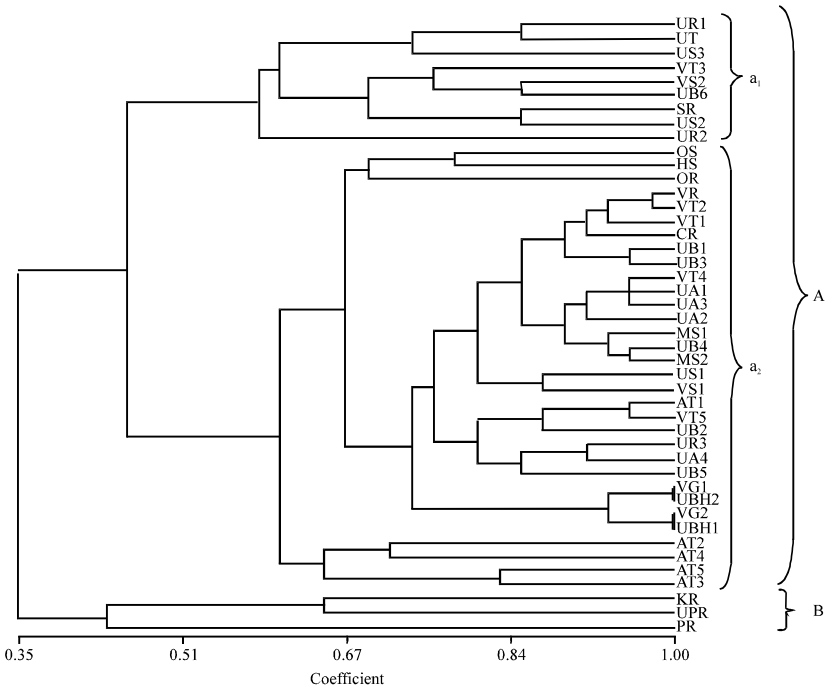

Fig. 3: Dendrogram for 44 Samples by UPGMA method with SSR markers. Coefficient COFH Matrix correlation (r = 0.85)

Table 6: Principle component analysis for SSR markers

Principle component	Eigenvalue	Percentage	Cumulative
1	20.88	45.39	45.39
2	13.43	29.19	74.57
3	1.89	4.12	78.69
4	1.44	3.13	81.82
5	1.10	2.19	84.01
6	0.91	1.97	85.98
7	0.74	1.60	87.58
8	0.67	1.46	89.03
9	0.61	1.33	90.36
10	0.49	1.07	91.43
11	0.47	1.03	92.45
12	0.41	0.90	93.35
13	0.39	0.85	94.20
14	0.32	0.70	94.89
15	0.28	0.61	95.51
16	0.27	0.59	96.10
17	0.26	0.56	96.65
18	0.23	0.49	97.14
19	0.17	0.38	97.52
20	0.16	0.34	97.87

DISCUSSION

Microsatellite markers showed rather good diverse in different populations of unshiu Mandarins that with due attention to being imports of these cultivars and little antecedent of their cultivation and reproduction, this range of diverse is considerable. In grouping genotypes with this front hypothesis

of existing 6 primary groups (unknown unshiu, local unshiu, Wase, Wase's derivatives, Owari and non-unshiu Mandarins (control) cluster analysis with POPGEN software can divide these genotypes to 3 completely separate clusters. The population of control was separate from other populations significantly which is considerable. Also, the population of Owari's genotypes is approximately distinct from other unshiu populations, while 4 other populations as unknown unshiu, local unshiu, Wase and Wase's derivations settled in common cluster. This subject shows this matter that cultivated cultivars in Mazandaran were principally from Wase or kinds close to it and don't have much proximity to Owari's genotypes. According to achieved results and acted analysis, rather high allele Frequency and hetrozygosity observed in these populations. These results are in opposite with Herrero *et al*. (1996) observations which described the genetic basic of Mandarins limitedly by enzyme studying and microsattelite markers. Breto *et al*. (2001) also reported low diverse in studying genetic variety of Kelemantin mandarins by AFLP markers which they illustrated coefficient of all genotypes from one primary tree as its reason. In cluster analysis of entire genotypes without considering primary grouping with NTYSYS software, markers couldn't create exact clusters and applicable to primary grouping. In studying which was done by Nicoloci *et al*. (2000) by using RAPD and SCAR markers on different germplasm of citrus, all of the Mandarins settled in one cluster. Also, Colettafilho *et al*. (1998) in studying Mandarin's accessions with RAPD markers, they extremely settled them in 2 clusters. Hence, in this kind of clustering, Owari's genotypes are divided approximately in a separable part and detached from other cultivars of unshiu. Also, the cultivars of control Mandarins are completely segregated from kinds of unshiu genotypes. But cultivars belonged to 4 other groups are clustered irregularly. The considerable point is that, the suegiyama sample which in same references is settled in Owari's group, in every 3 kinds of clustering of this research is divided by side of Wase's population's clusters. So, the reason of lowering variance or observed differences in studying molecular markers, is coming out of environmental effects. In this research, SSR markers could create great and exact differentiation among populations. According to this indicator of having several Polymorphism Information Content (PIC), the markers have differences. The average of PIC for SSR was (0.87) which indicates higher efficiency of these markers in showing the existence of diverse among studied populations of unshiu. Totally we can declare that the usage of SSR technique in this research for studying genetic variety on DNA level among known cultivars and natural unknown types existing in the collection have a high efficiency and susceptibility and also by using this technique, cognition of existing cultivars in the collection, genetic proximity, their relationship with each other and recognized cultivars and running and management of existing genotypes in the collection can be promoting in order to use them correctly in the programs of citrus plant breeding. So, grouping by several principal components was done which has more portion in accounting for changes, which this grouping is brought in dendrogram. According to the opinion of Primmer *et al*. (1996), when the phylogenetic distance among close varieties increases (indeed when the relative varieties have much diverse) the efficiency of SSR markers in grouping genotypes will decrease which this subject observed in this research, so that in 3D plot, some individuals of different populations were settled close the individuals of other populations. In this research, SSR markers could greatly differentiate different genotypes. Even close individuals in a population approximately differentiated. Luro *et al*. (2000) could specify small differences of individuals in lemon populations by using Sequence Tagged Microsatellite (STMS), while these markers weren't much effective in the societies of orange and Mandarin. Moreover, Barkley *et al*. (2006) in studying variety of citrus fruits germplasm? s collection announced that SSR markers are so effective for phylogenic studying in close and attached groups and differentiation of close genotypes.

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