Molecular genetic diversity assessment of Citrus species grown in Iran revealed by SSR, ISSR and CAPS molecular markers

Evaluación de diversidad genética molecular de especies de cítricos cultivadas en Irán reveladas por marcadores moleculares SSR, ISSR y CAPS

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***Abstract*—In this study, genetic diversity in 19 citrus cultivars was analyzed using Simple Sequence Repeat (SSR), Inter-simple Sequence Repeat (ISSR) and cleaved amplified polymorphic sequence (CAPS) markers. Nine primers for SSR, nine ISSR primers and two primers for CAPS were used for allele scoring. One chloroplast DNA region (rbcL-ORF106) and one mitochondrial DNA region (18S-5S) were analyzed using cleaved amplified polymorphic sequence (CAPS) marker in 19 citrus accessions grown in Iran. In total, 45 SSR and 131 ISSR polymorphic alleles and tree organelle genome types were detected. Cluster analysis of SSR and ISSR data was performed using UPGMA method and based on Jaccard's coefficient. The result of this investigation showed that the SSR and ISSR primers were highly informative and efficient in detecting genetic variability and relationships of the citrus accessions. CAPS marker analysis results showed that Bakraee and one of off type Mexican lime had banding pattern similar to Clementine Mandarin, while Pummelo regarded as maternal parent of other studied genotypes Citron regarded as father parent showed definite banding pattern among 19 studied genotypes which it confirmed Cytoplasmic inheritance from mother cellular organelles.**

***Keywords:*—Markers CAPS, Citrus, genetic diversity, ISSR markers, SSR markers**

***Resumen*—En este estudio, se analizó la diversidad genética en 19 cultivares de cítricos utilizando Simple Sequence Repeat (SSR), Inter-simple Sequence Repeat (ISSR) y marcadores de secuencias polimórficas amplificadas (CAPS). Se usaron nueve cebadores para SSR, nueve cebadores ISSR y dos cebadores para CAPS para la puntuación de alelos. Se analizaron una región de ADN de cloroplastos (rbcL-ORF106) y una región de ADN mitocondrial (18S-5S) usando un marcador de secuencia polimórfica amplificada escindida (CAPS) en 19 accesiones de cítricos cultivadas en Irán. En total, se detectaron 45 alelos polimórficos SSR y 131 ISSR y genoma de los árboles. El análisis de conglomerados de los datos de SSR e ISSR se realizó utilizando el método UPGMA y se basó en el coeficiente de Jaccard. El resultado de esta investigación mostró que los cebadores SSR e ISSR fueron altamente informativos y eficientes para detectar la variabilidad genética y las relaciones de las accesiones de cítricos. Los resultados del análisis del marcador CAPS mostraron que Bakraee y uno de Lima tipo off tenían un patrón de bandas similar al Clementine Mandarin, mientras que Pummelo considerado como el padre materno de otros genotipos estudiados Citron considerado padre padre mostró un patrón de bandas definido entre 19 genotipos estudiados que confirmaron herencia citoplásmica de organelos celulares de la madre.**

***Palabras claves*—Marcadores CAPS, Citrus, Diversidad Genética, Marcadores ISSR, Marcadores SSR.**

INTRODUCTION

Simple Sequence Repeat (SSR) and Inter-Simple Sequence Repeat (ISSR) markers have been used in citrus in a wide range of applications including cultivar identification (Fang and Roose, 1997; Novelli et al., 1998; Shahsavar et al., 2007) (Biswas et al., 2010; Uzun et al., 2009), phylogenetics (Xiao-Ming et al., 2003; Marak and Laskar, 2010), zygotic and nucellar seedlings differentiation (Ruiz et al., 2000; De Oliveira et al., 2002; Krueger and Roose, 2003) and the construction of linkage maps for marker assisted breeding and map–based cloning of genes (Kijas et al., 1997; De Riek et al., 2001).

Organellar restriction fragment length polymorphisms (RFLPs), either conventional or PCR-based, have been used to study phylogenetic relationships of Citrus and its related genera (Green et al., 1986; Nicolosi et al., 2000; Abkenar et al., 2004a). Analysis of chloroplast DNA (cpDNA) variant is especially valuable in phylogenetic and maternal similarity studies of suspected hybrids due to its evolutionary conservatism and predominant uniparental inheritance, maternal in citrus. (Palmer et al., 1988; Bayer et al., 2009; Lu et al., 2011). More recently, different molecular markers such as cleaved amplified polymorphic sequence (CAPS) of cpDNA (Yamamoto et al., 2013; Ninomiya et al., 2015; Fujii et al., 2016) (Nonaka et al., 2017; Dorji and Yapwattanaphun, 2015; Froelicher et al., 2011), have been used for identification, parentage analysis and phylogeny of citrus species or cultivars.

In this study, the potential of cellular organelles DNA

polymorphism, using CAPS marker which is RFLPs based on polymerase chain reaction (PCR), a simple but reliable method of DNA analysis, was investigated to provide new information onrelatedness of citrus species.

MATERIALS AND METHODS

Plant material and DNA isolation In the present study, 19 accessions of citrus species or cultivars were used (Table 1). Total genomic DNA was extracted from fresh leaves according to the modified method of Murray and Thompson (1980); Sharafi et al. (2014). After quantity determination of DNA using a spectrophotometer Nano Drop 2000, Thermo Scientific), the DNA templates were diluted to 25 ng/ul for using in PCR reactions.

SSR

SSR assays were performed as described by Ahmad et al. (2003) using 7 primers from the 26 sets described by these authors. They were: CMS-4, CMS-7, CMS-16, CMS-19, CMS-24, CMS-45 and CMS-46. Two other primers described by Kijas et al. (1997) (TAA15 and TAA 41) were also used (Table 4). Polymerase chain reactions (PCR) were performed in 15 ul volumes comprising 1x PCR buffer, 2 mM MgCl, 0.2 mM each dNTP, 0.1 mM of each forward and reverse primer, 1 unit of Taq DNA polymerase and 50 ng of genomic DNA. The amplifications were performed with a first denatureation at 94ºC for 5 min followed by 35 cycles of 30 sec at 94ºC, 30 sec at 55C, and 30 sec at 72ºC, with a final extension at 72ºC for 5 min. The PCR products were separated on 6% denaturing polyacrylamide gel in TBE buffer (1x) (45 mMTris- Boric, 1 mM EDTA pH 8.0) followed by staining with silver nitrate.

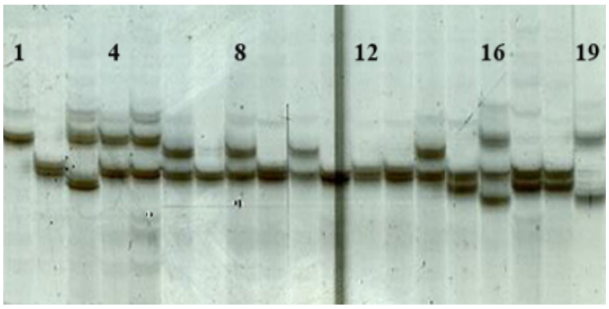

Figure 1. Polymorphism of SSR (locus CMS-7) in the citrus accessions of this study. Three alleles in No. 16 are related to 'Persian' lime, a triploid citrus species. The numbers are related to the accessions of Table 1.

Source: Prepared by the authors.

ISSR

A total of nine ISSR primers were used for PCR amplifications of genomic DNAs (Table 5) (Awasthi et al., 2008). The concentration of PCR reagents was almost same as that of SSR excepting 0.3 mM of ISSR primer and 2.5 mM of MgCl2 were used. The amplifications were performed with a first denaturation at 94°C for 2 min followed by 35 cycles of 30 sec at 94°C, 45 sec at 50°C, and 2 min at 72°C, with a final extension at 72 °C for 10 min. The PCR products were separated on 1.5%agarose gels (Top Vision) in TBE buffer (1) followed by staining with ethidium bromide.

Table 1. List of plant materials used in this study.

Nº	Common or Local name	Scientific name	Location of sampling	Accession's code (or name in figures)
1	Balang	Citrus medicaL	Kotra collection*	Citron
2	Clementine	C. reticulata Blanco	Kotra collection	Clementine
3	Darabi	A local Citrus cultivar	Kotra collection	Darabi
4	Sweet lime	C. limettioides Tan.	Kotra collection	Sweet lime
5	Bakraii	A local lime-like cultivar	Kotra collection	Bakraii
6	Off-type of Mexican lime	C. aurantifolia Swingle	Kotra collection	N6
7	Mineola tangelo	C. paradisix C. reticulata	Kotra collection	Mineola Tangelo
8	Eureka lemon	C. limon[L.] Burm. F	Kotra collection	Eureka lemon
9	Siavaraz	A local orange cultivar	Kotra collection	Siavaraz
10	Lisbon lemon	C. limon[L.] Burm. F	Kotra collection	Lisbon lemon
11	Orlando tangelo	C. paradisix C. reticulata	Kotra collection	Orlando tangelo
12	Frost Valencia orange	C. sinensis(L.) Osb	Kotra collection	Valencia Orange
13	Washington Navel Orange	C. sinensis(L.) Osb	Kotra collection	Washington
14	A local lemon-like cultivar	Citrus spp.	Kotra collection	N14
15	A local citrus cultivar	Citrus spp.	Kotra collection	N15
16	Persian lime	C. latifolia Tan	Ramsar collection*	Persian Lime
17	Duncan grapefruit	C. paradisi Macf.	Ramsar collection	Duncan
18	Sour orange	C. aurantiumL.	Ramsar collection	Sour Orange
19	Mexican lime	C. aurantifolia Swingle	Kotra collection	Mexican Lime

*Both Kotra and Ramsar collections are located in north of Iran, Mazandaran province.

Source: Prepared by the authors.

CAPS

One chloroplast DNA region (rbcL-ORF106) and one mitochondrial DNA region (18S-5S) were amplified using universal primers as described by Abkenar et al. (2004b) (Table 2). The PCR amplifications were performed in 25 ul volumes comprising 1x PCR buffer, 2.5 mM MgCl, 0.2 mM each dNTP, 0.1 mM of each forward and reverse primer, 1 unit of Taq DNA polymerase and 50 ng of genomic DNA. The amplifications were performed with a first denaturation at 92ºC for 2 min followed by 35 cycles of 1 min at 92ºC, 1 min

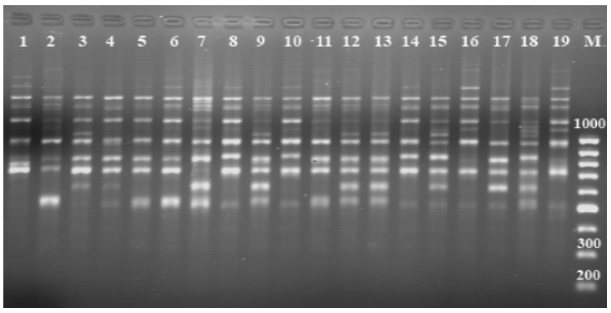

Figure 2. ISSR polymorphism resulted from (AC)8G primer. The numbers are related to the accessions of Table 1.

Source: Prepared by the authors.

at 55ºC, and 4 min at 72ºC, with a final extension at 72ºC for 7 min. The primer pairs evaluated in this investigation produced clear single bands within the specified size range. The approximate size of PCR fragments (bps) amplified by the primer pairs were as following; rbcL-ORF106 (3100), 18S-5S (1177) (Table 2). The amplified products were digested for more than 3 h with different restriction enzymes according to manufacturer's instructions. Amplification products were electrophoresed on 1.5% agarose gels while their restricted fragments were electrophoresed on 3% agarose gels. The gels were stained by ethidium bromide.

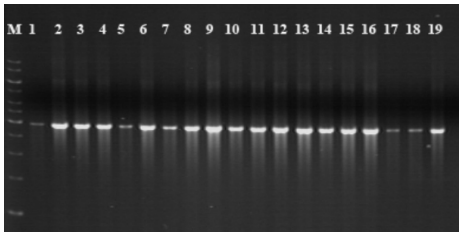

Figure 3. Amplification of genomic DNAs using rbcL-ORF106/Hinf I primers. M: 1kb size marker. The numbers are related to the accessions of Table 1.

Source: Prepared by the authors.

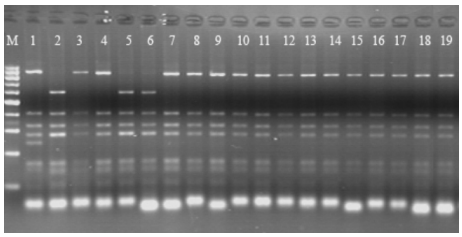

Figure 4. CAPS polymorphism resulted from rbcL-ORF106/Hinf I. The numbers are related to the accessions of Table 1.

Source: Prepared by the authors.

Data analysis

The presence of an amplified product (fragment) was identified as "1" and the absence was designated as "0" and a similarity matrix was constructed based on Jaccard's coefficient. The accessions were grouped by cluster analysis using the unweighted-pair group method with arithmetic averages (UPGMA). The computer program used was NTSYS-PC, version 2.011 (Rohlf, 2000). The cophenetic correlation coefficient, r, was calculated based on Mantel's test Mantel (1967) to check the fit goodness of the cluster analysis to the matrix on which it was based. Total number of effective alleles (Ne), expected heterozygosity (He), observed heterozygosity (Ho), and PIC (polymorphic information content) was calculated using Gen AlEx (Ver. 6.5) (Peakall and Smouse 2012) and Mstools computer programs. PIC provides an estimate of the discriminatory power of a locus. PIC values for codominant markers like SSR range from 0 (monomorphic) to 1 (very highly discriminative). It refers to the value of a marker for detecting polymorphism within a population and is equivalent to gene diversity. PIC for dominant markers like ISSR is a maximum of 0.5 De Riek et al. (2001).

For each primer pair/enzyme combination, the accessions were grouped along with the similarity of their restriction patterns firm by a letter followed by a number. For instance, by rbcL-ORF106/Hinf I combination all of the accessions were placed into two groups, a1 and a2. Finally, those accessions with all identical cpDNA restriction patterns were grouped in a common organelle genome type (OGT; OGT1, OGT2and etc.) (Table 3 and Table 6).

Table 2. Pairs of cpDNA and mtDNA primers used for PCR amplification and approximate PCR product sizes and restriction enzymes

Primers	Sequence (5'-3')	Primer pair	PCR product size (bp)	Restriction enzyme
rbcL	ATGTCACCAC AAACAGAAAC TAAAGCAAGT	rbcL-ORF106	3100	HinfI
ORF106	ACTACAGATCT CATACTACCCC	-	-	MspI
-	-	-	-	TaqI
18S	ATATGGCGCA AGACGATTCC	18S-5S	1177	HinfI
5S	GTGTTGCTGA GACATGCGCC	-	-	MspI
-	-	-	-	TaqI

Source: Prepared by the authors.

Results and discussion

SSR Polymorphism and genetic relationships

Seven primers from Ahmad et al. (2003) and two primers from Kijas et al. (1997) were selected for PCR amplification and data scoring. Using these nine selected primers, in total 45 polymorphic alleles were obtained. PIC values ranged from 0.305 for CMS-16 to 0.766 for CMS-19 (Table 4). The mean value of PIC (0.612) was relatively high which confirmed the high polymorphism among the genotypes. This high level of polymorphism again could be detected from the values of He (also called diversity index) (Table 4). Cophenetic correlation was found to be high (r = 0.928) suggesting that 93% of the similarity matrix was represented by the clustering analysis.

ISSR polymorphism and genetic relationships

All of the nine ISSR primers produced easy to score frag-

Table 3. Restriction sites dividing large fragments into corresponding visible small fragments

	cpDNA (rbcl-ORF106)			mtDNA (18S-5S)		
	HinfI	MspI	TaqI	HinfI	MspI	TaqI
Citron	a1	b1	c1	d1	e1	f1
Clementine	a2	b2	c2	d1	e1	f1
Darabi	a3	b1	c1	d1	e2	f1
Sweet Lime	a3	b1	c1	d1	e2	f1
Bakraii	a2	b2	c2	d1	e1	f1
N6	a2	b2	c2	d1	e1	f1
Mineola Tangelo	a3	b1	c1	d1	e2	f1
Eureka Lemon	a3	b1	c1	d1	e2	f1
Siavaraz	a3	b1	c1	d1	e2	f1
Lisbon Lemon	a3	b1	c1	d1	e2	f1
Orlando Tangelo	a3	b1	c1	d1	e2	f1
Valencia Orange	a3	b1	c1	d1	e2	f1
Washington	a3	b1	c1	d1	e2	f1
N14	a3	b1	c1	d1	e2	f1
N15	a3	b1	c1	d1	e2	f1
Persian Lime	a3	b1	c1	d1	e2	f1
Duncan	a3	b1	c1	d1	e2	f1
Sour Orange	a3	b1	c1	d1	e2	f1
Mexican Lime	a3	b1	c1	d1	e2	f1

Source: Prepared by the authors.

Table 4. Number of total and effective alleles (Ne), He, Ho and PIC for nine SSR loci

	SSR marker						
Loci	Total alleles	Polymorphic alleles	Polymorphism range (%)	Ne	He	Ho	PIC
CMS -4	8	8	100	4,53	0,760	0,531	0,720
CMS -7	6	6	100	4,16	0,767	0,893	0,729
CMS -16	3	3	100	2,6	0,379	0,195	0,305
CMS -19	5	5	100	4,03	0,806	0,787	0,766
CMS -24	5	5	100	3,89	0,745	0,511	,0.718
CMS -45	3	3	100	2,35	0,609	0,588	0,527
CMS -46	3	3	100	2,99	0,676	0,735	0,592
TAA -15	4	4	100	1,57	0,442	0,422	0,412
TAA -41	8	8	100	5,36	0,779	0,673	0,742
Total	45	45	-	-	-	-	-
Mean	5,01	5,01	100	3,49	0,663	0,537	0,612

Source: Prepared by the authors.

Table 5. Number of total and Polymorphic alleles and PIC for nine ISSR loci

ISSR marker				
Primer	Total alleles	Polymorphic alleles	Polymorphism range (%)	PIC
(AG)8G	18	18	100	0.39
(GA)8C	17	15	88.2	0.38
(GA)8A	18	18	100	0.33
(CT)8T	12	8	66.6	0.32
(AC)8Y*T	17	16	94.1	0.3
(GT)8AGTCY	12	12	100	0.34
(GACA)4	20	15	75	0.37
(AG)8T	18	16	88.8	0.35
(AC)8G	16	3	81.2	0.34
Total	148	131	81.2	0.35

Source: Prepared by the authors.

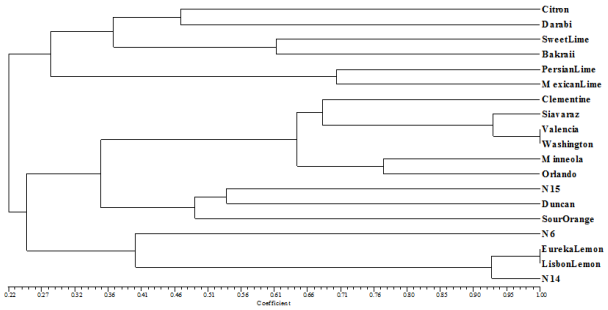

Figure 5. Dendrogram of 19 citrus accessions obtained from SSR markers using Jaccard's coefficient and UPGMA clustering method.

Source: Prepared by the authors.

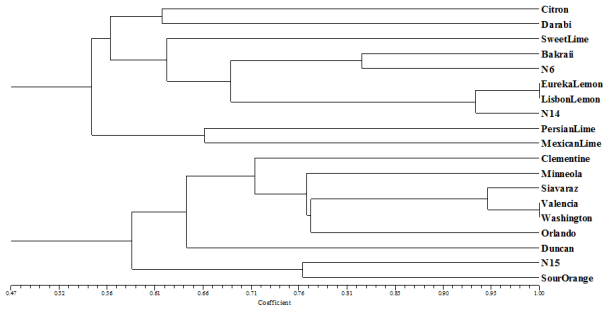

Figure 6. Dendrogram of 19 citrus accessions obtained from ISSR markers using Jaccard's coefficient and UPGMA clustering method.

Source: Prepared by the authors.

ments (Fig. 2). The PIC values of the ISSR primers were less than 0.5 as it was expected. Cophenetic correlation was found to be high (r = 0.921) suggesting that 92% of the similarity matrix was represented by the clustering analysis. The ISSR dendrogram (Fig. 6) was very similar to that obtained by SSR markers.

Both SSR and ISSR markers found no differentiation between 'Eureka' and 'Lisbon' lemons, and between 'Frost Valencia' and 'Washington Navel' sweet oranges in agreement with results of previous studies on citrus cultivar identification (Ahmad et al., 2003; Uzun et al., 2009). These markers could not differentiate those cultivars of sweet orange and grapefruit originated through mutation too (Fang and Roose, 1997; Aydin UzUn et al., 2010).

'Orlando' is a sibling of 'Minneola' tangelo, having 'Dancy' mandarin and 'Duncan' grapefruit as its parents. Remaining closely related to mandarin and grapefruit, these two tangelos were well separated from each other. The similarity value between them was 0.77.'Siavaraz' is a local cultivar of sweet orange with a long history of cultivation in northern part of Iran, Mazandaran and Guilan provinces (Sharafi et al., 2016). It is a highly seedy and juicy cultivar with unknown origin (Sharafi et al., 2016). In the present study with a slight divergence this cultivar was found very closely related to Frost Valencia' and 'Washington Navel.

The origin of 'Bakraii' is unknown. This cultivar is usually used as a rootstock for citrus cultivars especially Mexican

lime in south of Iran. Its fruits are consumed occasionally despite their bitterness (Shahsavar et al., 2007). Recently through SSR and PCR-RFLP markers, (Golein et al., 2012), found that 'Bakraii' is a hybrid between rough lemon (C. jambhiriLush.), as maternal parent, and sweet lime (C. limettioidesTan.) (Shahsavar et al., 2007) also found a close affinity between 'Bakraii' and sweet lime using ISSR markers. Our results also support a close relationship between 'Bakraii' and sweet lime. The only accession that showed close affinity to 'Bakraii' was N6 here. We suggest that it could be a hybrid of 'Bakraii'.

'Persian' lime is a unique and new cultivar to citrus industry of Iran and is not being cultivated widespread (Sharafi et al., 2016). The flowers of 'Persian' lime are devoid of viable pollen also contain exceedingly few functional ovules (Mantel, 1967). These characteristics prevent its natural hybridization with other citrus species. Probably for these reasons, no accession was closely clustered with 'Persian' lime in this study, but it was located in acid lime cluster.

Acid lime has a long history of cultivation in the south regions of Iran such as Fars, Hormozgan, Kerman, Bushehr and Sistan-Baluchestan provinces (Sharafi et al., 2016). Too many hectares of acid lime orchards have been established in these provinces. Trees of these orchards have been mostly originated by seed propagation of Mexican lime (Faghihi et al., 2011).

Table 6. Polymorphic restriction patterns of chloroplast and mitochondrial DNA and tree organelle genome types (OGTs) related to citrus accessions analyzed in this study

Restriction patterns							
OGT	rbcL-ORF106			18S-5S			No. of Accessions
	HinfI	MspI	TaqI	HinfI	MspI	TaqI	
1	a1	b1	c1	d1	e1	f1	1
2	a2	b2	c2	d2	e2	f2	3
3	a3	b3	c3	d3	e3	f3	15
							19

Source: Prepared by the authors.

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