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Evaluation of genetic diversity among Iranian pomegranate (*Punica granatum* L.) cultivars, using ISSR and RAPD markers

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Abstract
Considering the high level of morphological diversity in Iranian pomegranate cultivars, comparison of genetic variation among 24 pomegranate cultivars was evaluated using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. RAPD primers amplified 131 DNA fragments among which 29 were polymorphic (22.14%) and ISSR markers produced 173 amplification products, out of which 64 were polymorphic (37%). Mean PIC (polymorphic information content) was 0.128 for RAPD and 0.163 for ISSR. The results suggested that the ISSR markers produced much better reproducible bands and were more efficient in grouping cultivars. Pairwise similarity index values ranged from 0.353 to 1.0 (RAPD), 0.291 to 0.930 (ISSR) and mean similarity index values of 0.604 and 0.674 for RAPD and ISSR, respectively. The analysis of molecular variance (AMOVA) for RAPD and ISSR data showed no significant differences among the geographical regions and juice acidity of the used cultivars ($P>0.05$) indicated that genetic and geographic distances were not correlated.
Key words: *Punica granatum*, genetic diversity, pomegranate, RAPD, ISSR

Introduction

Pomegranate (*Punica granatum* L.) belongs to Punicaceae family and is an important fruit tree of tropical and subtropical regions of the world which is valued highly for its delicious edible fruits. In addition, the tree is also cultivated for its pharmaceutical and ornamental usages (Levin, 1994). The pomegranate tree has a wide geographical distribution that spreads from Iran to the Himalayas in northern India, and has been cultivated since ancient times throughout the Mediterranean regions of Asia, Africa and Europe (Levin, 1994). Pomegranate may be classified according to the acidity of its fruit into sour, sour-sweet or sweet.

Development of highly reliable and discriminatory methods have become increasingly important to plant breeders for identifying cultivars and to those in the nursery industry who need sensitive tools to differentiate and identify cultivars for plant patent protection

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(Wunsch and Hormaza, 2002). In the past, cultivars were identified primarily based on horticultural, morphological and physiological descriptions. In most cases, the descriptions and measurements varied considerably due to environmental fluctuation and differences human judgment. Differences in DNA sequence among individuals could be detected by different methods. Almost all kind of DNA markers can be used for fingerprinting fruit tree species (Wunsch and Hormaza, 2002). The Random Amplified Polymorphic DNA (RAPD) technique (Williams *et al*., 1990) based on the Polymerase Chain Reaction (PCR) has been used to detect polymorphism in some species (Williams *et al.,* 1990). Thus, the RAPD technique can generate polymorphisms between very closely related genotypes. Since 1990, RAPD markers have been successfully used to identify cultivars and/or clones of various plant species (Belaj *et al.,* 2001; Besnard *et al.,* 2001; Claros *et al.,* 2001; Ozden-Tokatli *et al.,* 2010; Takeda *et al.,* 1998).

Also, Inter-simple sequence repeat (ISSR) amplification is a technique which can rapidly differentiate closely related individuals (Zietkiewicz *et al.,* 1994). ISSR markers involve PCR amplification of DNA using a single primer composed of a microsatellite sequence such as $(CA)_8$ anchored at the 3' or 5' end by 2-4 arbitrary, often degenerate nucleotides. The sequences of repeats and anchored nucleotides are randomly selected. Coupled with the separation of amplifications products on a polyacrylamide gel, ISSR amplification can reveal a higher number of fragments per primer than RAPD. ISSR markers have been used for cultivar identification and for genetic relationship studies in various plant species (Awasthi *et al.,* 2004; Martin and Sanchez-Yelamo, 2000; Weiguo *et al.,* 2007).

Although a wide range of morphological and physiological characters show variabilities in the pomegranate, molecular studies of the pomegranate have been restricted to examinations of RAPD (Dorgac *et al.,* 2008; Zamani *et al.,* 2007; Sarkhosh *et al.,* 2009), ISSR (Talebi Bedaf *et al.,* 2005), AFLP (Jbir *et al.,* 2008; Rahimi *et al.,* 2006) and SSR (Koohi-Dehkordi *et al.,* 2007; Ebrahimi *et al.,* 2010; Pirseyedi *et al.,* 2010) to investigate the population dynamics of economically important cultivars. To meet various breeding programs and to conserve the existing genetic resources of pomegranate, the objectives of this study were to assess the levels of polymorphisms detected by RAPD and ISSR markers, comparison of information content the marker systems and using them to identify 24 Iranian pomegranate cultivars.

Materials and Methods

Plant materials and DNA extraction

Twenty four cultivars of *P. granatum* were collected from Agricultural Research Center of Yazd province, Yazd, Iran. The selection of cultivars was based on the acidity of fruits and the morphological characteristics, such as color and shape of fruit (Table 1).

Total DNA was extracted from young leaves following the CTAB (Hexadecyltrimethylammonium bromide) method described by Murray and Thompson (1980) with modifications. The purified total DNA was quantified by agarose gel electrophoresis and its quality was verified by spectrophotometry. DNA samples were diluted to 25ng/µl and stored at -20 ºC.

Table 1: Iranian pomegranate genotypes included in the study

No.	Cultivar codes	Cultivar names	Acidity	Origin
1	PGpsy	Poust syah yazdi	Sweet	Yazd
2	PGgsh	Goroch shahvar	Sweet	Yazd
3	PGtl	Tab va larz	Sweet	Yazd
4	PGbl	Bihasteh ladiz	Sweet	Systan va Balouchestan
5	PGas	Asali sarvestan	Sweet	Fars
6	PGghr	Golabi hasteh riz	Sour-sweet	Systan va Balouchestan
7	PGna	Nabati ardakan	Sour-sweet	Yazd
8	PGdhv	Dokhtar hamoumi varamin	Sour-sweet	Tehran
9	PGggn	Galu gandeh neiriz	Sour-sweet	Fars
10	PGakh	Amaneh khatouni	Sour-sweet	Yazd
11	PGtg	Togh gardan	Sour-sweet	Yazd
12	PGda	Dom anbarouti	Sour	Khorasan
13	PGpa	Panjeh arous khafr	Sour	Fars
14	PGapgh	Ardestani poust ghermez	Sour	Esfahan
15	PGvns	Vahshi narak sarvestan	Sour	Fars
16	PGds	Dabbei sarjangal	Sour	Kerman
17	PGgf	Golnar fars	Ornamental	Fars
18	PGkgs	Kaleh gavi sangan	Sour-sweet	Systan va Balouchestan
19	PGsh	Shahvar shirin	Sweet	Yazd
20	PGhm	Hasibi mehriz	Sour-sweet	Yazd
21	PGbs	Bihasteh sangan	Sweet	Systan va Balouchestan
22	PGaps	Ardestani poust sefid	Sweet	Esfahan
23	PGtmz	Torsh mamuli zabol	Sour	Systan va Balouchestan
24	PGsb	Sabi bam	Sour	Kerman

RAPD assay

One hundred-four 10mer oligonucleotide primers among sets A, B, C, AC, AD, AE, P and S (Operon technologies, Inc, USA), AJ, MG and UBC (Roche Molecular Biochemicals, Germany) were used as single primers for DNA amplification and 13 of them were selected based on clear and reproducible banding patterns. The PCR was performed in a Genius (FGENO5TD) Thermal Cycler, in a 25µl volume containing of 2.5µl of 1X reaction buffer [100mM Tris-HCl, 15mM $MgCl_2$, 500mM KCl, pH 8.3 (20ºC)], 0.5mM $MgCl_2$, 200µM each of dNTPs (Roche, Germany), 0.4µM of 10mer primer, 0.75 units of *Taq* DNA polymerase (Roche, Germany) and 100ng of template DNA, overlaid with 25µl of sterile mineral oil. The amplification condition was: initial step of denaturation at 94ºC for 2 min followed by 40 cycles of denaturation at 92ºC for 1 min, primer annealing at 35ºC for 1 min and extension at 72ºC for 2 min, followed by an extended elongation step at 72ºC for 5 min. The amplification products were analysed on 1.2% MP agarose gel (Roche, Germany) in 1X TBE buffer running at 60 volts for three hours and stained in ethidium bromide (0.5mg/ml) and visualized under UV light and photographed. The DNA size marker used was 1Kb ladder (Life technologies).

ISSR assay

A total of 15 primers were tested to amplify DNA from which six primers with considerable polymorphism and reproducibility were selected for further analysis (Table 2). PCR were performed in 15 µl volume consisted of 1X PCR buffer, 2mM $MgCl_2$, 200 µM each of dNTPs, 1 µM primer, 1 U of *Taq* DNA polymerase (Roche, Germany), 2% formamide and 25 ng of template DNA. Each reaction mixture was overlaid with 25 µl of sterile mineral oil. Amplification was performed in a Genius (FGENO5TD) Thermal Cycler under the following conditions: 4 min at 94ºC for 1 cycle, followed by 30 s at 94ºC, 45 s at

52ºC, and 2 min at 72ºC for 30 cycle, and 5 min at 72ºC for a final extension. Amplification products were separated on 6% denaturing polyacrylamide gels containing 7 M Urea and 1X TBE buffer in Biometra sequencing gel (S2 model). Amplified DNA segments were detected using silver staining (Bassam *et al.,* 1991).

Data analysis

A marker index was calculated for the RAPD and ISSR markers to characterize the capacity of each primer to detect polymorphic loci among the cultivars. As such, the marker index was the sum of the polymorphism information content (PIC) values for all the selected markers produced by a particular primer. The PIC value was calculated using the formula $PIC=1-\Sigma pi^2$, where *Pi* is the frequency of the *i* allele (Smith *et al.,* 1997).

Only reproducible and clear bands in the replications were considered as potential polymorphic markers. The data obtained by scoring the presence (1) or absence (0) of amplified fragments from the RAPD, ISSR and also collective of marker profiles were subjected to the construction of a similarity matrix using Jaccard's (Jaccard, 1908) coefficients of similarity. The matrices were then used for a cluster analysis. Sequential agglomerative hierarchical nonoverlapping (SAHN) clustering was performed using the unweighted pair group method with arithmetic averages (UPGMA), and then, the results were summarized as dendrograms using NTSYSpc software 2.02 (Raholf, 1998).

The pertinency of the dendrograms to the original similarity matrix was calculated by computing the cophenetic values (rcoph) using the cophenetic (COPH) and matrix comparison (MXCOMP) modules of NTSYSpc.

Finally, the frequency of occurrence of each marker in each cultivar was computed, to render a matrix of 24 cultivars by RAPD and ISSR markers. These matrices were afterward subjected to principal component analysis (PCA).

Analysis of molecular variance (AMOVA) was performed to estimate variance components for RAPD and ISSR data and partitioning the variation into within and among local regions and acidity of cultivars, using Arlequin 3.1 software (Excoffier *et al.,* 2005).

Results

One hundred-four 10 mer RAPD primers were screened and among which 13 were chosen for their clear and reproducible band patterns (Table 2). The thirteen selected primers generated 131 RAPD fragments, an average of 10.08 bands per primer. The size of the amplified products ranged from 400 to 3,000 bp. The total number of polymorphic markers and percentage of polymorphism were 29 and 22.14%, respectively (Table 2). Primers OPAD02 and MG16 amplified maximum number of polymorphic bands. Primers OPAD02 and OPAE10 put out the highest level of distinguishable polymorphism. The PIC values, a reflection of the allele diversity and frequency among the cultivars, were not uniform with respect to for all the RAPD loci tested. The PIC values ranged from 0.012 (MG01) to 0.373 (OPAD02) with a mean of 0.128. The result showed that the minimum similarity (0.353) existed in the two local cultivars "Dom anbarouti" and "Poust syah yazdi" and the maximum similarity (1.00) occurred in the cultivars "Tab va larz" and "Bihasteh ladiz". The mean similarity index was 0.604. The data obtained from RAPD analysis of 24 pomegranate cultivars was subjected to UPGMA analysis. The cophenetic correlation coefficient (0.91) indicated little distortion between the original similarity values from the similarity matrix and the values used to construct the dendrogram. A cluster analysis was performed based on Jaccard's similarity coefficient matrices, calculated from the RAPD

markers. At the similarity of 59%, twenty cultivars were categorized in one group and the four remaining cultivars were placed in the separate groups (Figure 1a).

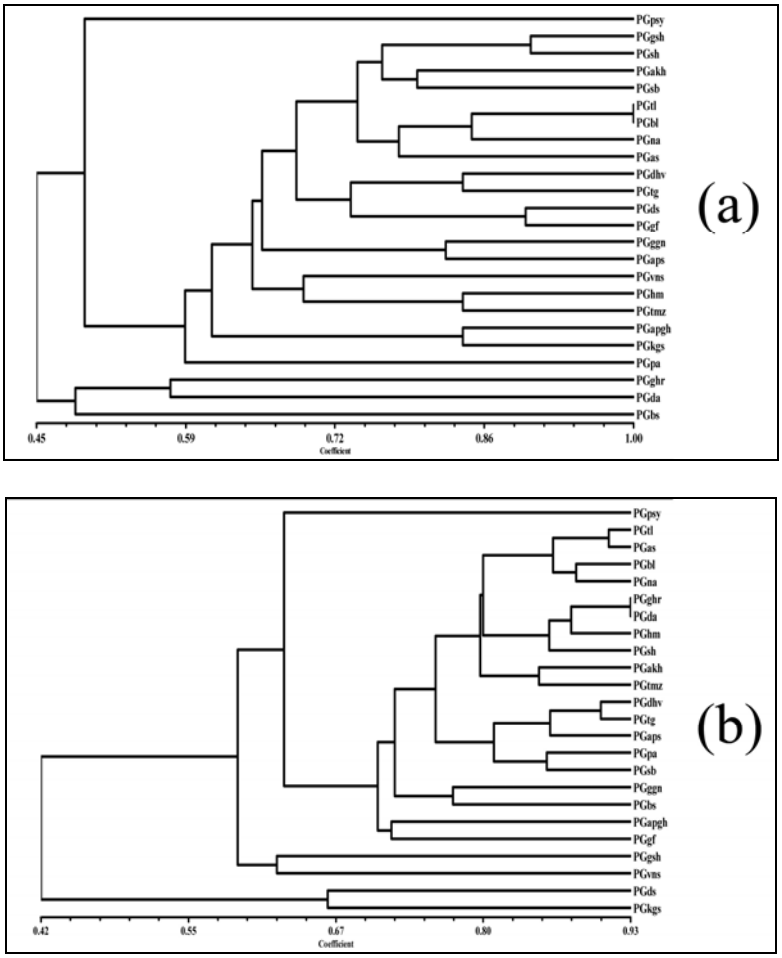

Figure 1: UPGMA dendrogram showing relations among Iranian pomegranate cultivars using RAPD (a) and ISSR (b) data and Jaccard's similarity coefficient.

Eighteen ISSR primers were initially tested using pomegranate DNA as single or combined. Three primers as single and three as combined were based on $(AG)_n$, $(GT)_n$, $(GA)_n$, $(AC)_n$, $(CT)_n$ or $(CA)_n$ repeats, each anchored by various nucleotides used in this study as polymorphic primers (Table 2). The six selected primers generated 173 fragments, an average of 28.83 bands per primer. The size of the amplified products ranged from 80 to 3,000 bp with the scoreable region being from 100 to 2,000 bp accordingly, and the total number of polymorphic markers and percentage of polymorphism were 64 and 36.99%, respectively (Table 2). In the case of the ISSR analysis, the mean PIC value was 0.163, and the lowest and highest PIC values were 0.099 (ISSR5 and ISSR6) and 0.257 (ISSR11), respectively. The similarity coefficients for 24 pomegranate cultivars based on the ISSR fragments ranged from 0.291 ("Dabbei sarjangal" and "Poust syah yazdi") to 0.930 ("Dom anbarouti" and "Golabi hasteh riz"). The mean similarity index was 0.674. Twenty four cultivars were clustered in five distinct groups at the similarity level of 65%, 19 of which were placed in one group (Figure 1b).

Table 2: RAPD and ISSR primers successfully used in this study and the number of total and polymorphic bands amplified in pomegranate cultivars.

Marker name	Primer name	Sequence (3'-5')	Total band number	Number of polymorphic bands	Polymorphic bands (%)	PIC value
RAPD	OPAC11	CCTGGGTCAG	12	3	25	0.188
	OPAD02	CTGAACCGCT	8	5	62.5	0.373
	OPAD04	GTAGGCCTCA	8	2	25	0.246
	OPAD13	GGTTCCTCTG	5	2	40	0.172
	OPAD15	TTTGCCCCGT	6	1	16.67	0.014
	OPAD16	AACGGGCGTC	10	2	20	0.110
	OPAE10	CTGAAGCGCA	11	2	18.18	0.081
	OPB10	CTGCTGGGAC	12	1	8.33	0.013
	OPP02	TCGGCACGCA	10	2	20	0.135
	OPP14	CCAGCCGAAC	12	2	16.67	0.096
	MG01	AGCGCCGACG	14	2	14.29	0.012
	MG11	AGGAGCTGCC	16	2	12.5	0.024
	MG16	GAAGAACCGC	7	3	42.86	0.200
	Total		131	29		
	Mean		10.08	2.23	22.14	0.128
ISSR	LK7	5'–CCA$(CT)_8$-3'	32	10	31.25	0.256
	ISSR5	5'–CCA$(AG)_8$T-3'	25	8	32	0.099
	ISSR6	5'–$(GA)_8$C-3'				
	ISSR10	5'–$(GT)_8$A-3'	38	11	28.94	0.106
	ISSR12	5'–$(AG)_8$YT-3'				
	ISSR11	5'–$(AG)_8$YT-3'	24	6	25	0.156
	ISSR12	5'–$(GA)_8$YT-3'				
	ISSR11	5'–$(AG)_8$YT-3'	26	15	57.69	0.257
	ISSR12	5'–$(GA)_8$YT-3'	28	14	50	0.103
	Total		173	64		
	Mean		28.83	10.67	36.99	0.163

The matrices for RAPD and ISSR markers were also compared using Mantel's test (Mantel, 1967) for matrix correspondence. The correlation between the matrices of cophenetic values relating to the dendrograms based on RAPD and ISSR data was very low (r=0.02).

The relationships among cultivars were initially defined by the first three principal vectors of the PCA, which together accounted for 65% (RAPD), 73% (ISSR) and 66% (RAPD+ISSR) of the total variation at the molecular level (data not shown).

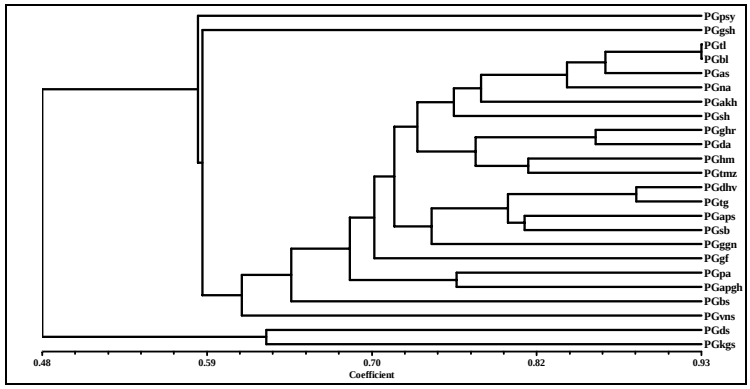

Figure 2: UPGMA dendrogram of 24 pomegranate cultivars based on RAPD and ISSR pulled data and Jaccard's similarity coefficient.

The similarity coefficients of 24 pomegranate cultivars based on 29 RAPD and 64 ISSR markers ranged from 0.338 ("Dabbei sarjangal" and "Poust syah yazdi") to 0.932 ("Bihasteh ladiz" and "Tab va larz"), and accordingly the mean similarity index value of the combined RAPD and ISSR was 0.655. A cluster analysis performed based on combination of the data for both markers, separated the cultivars into two distinct clusters. The first cluster included only two cultivars, whereas the second cluster was further divided into three subclusters. Among these subclusters, two local cultivars ("Poust syah yazdi" and "Goroch shahvar") were separated from other cultivars (Figure 2).

AMOVA for RAPD and ISSR data indicated that there were no significant differences among the geographical regions and juice acidity of the used cultivars ($P > 0.05$).

Discussion

Among markers, RAPD and ISSR are simple, which provide a quick screen for DNA polymorphism and very small amounts of DNA are required. In addition, information on template DNA sequence is not necessary. However, with respect to RAPD markers problems of reproducibility are reported (Muthusamy *et al.,* 2008). In order to assure reproducibility, optimization of PCR reaction and also its repetition is essential. In this study, each RAPD analysis was repeated in separate experiments at least twice, and only reproducible markers were considered. ISSR primers consist of 17-19 nucleotides and optimization of annealing temperature is important. The choice of annealing temperature for further ISSR analysis is based on the complexity and reproducibility of banding patterns. The primers that were based on $(AT)_n$ or $(TA)_n$ repeats amplified no products at all. Possibly, this indicates that the pomegranate genome lacks, or else has very few of these two microsatellites, although Wang *et al.,* 1994, reported that $(AT)_n$ was the most abundant microsatellite in plant nuclear genomes. Alternatively, lack of amplification products may be due to the self-complementary nature of $(AT)_n$ or $(TA)_n$ primers. In this study, when optimal conditions for PCR had been determined, reproducible patterns were obtained for both RAPD and ISSR assays.

In general, among the set of accessions investigated, the efficiency of a molecular marker technique depends on the amount of polymorphism it can detect. In our study, ISSR fingerprinting was more efficient than the RAPD assay; it detected 37% polymorphic DNA markers among the 24 cultivars analyzed, compared with 22.14% for RAPD fingerprinting. Similar results were obtained for several other plant species (Galvan *et al.,* 2003; Nkongolo *et al.,* 2005; Qian *et al.,* 2001; Raina *et al.,* 2001). However, Fang and Roose (1997) showed that RAPD had a higher level of variation in *Citrus* spp. than ISSR, and Metais *et al.,* (2000) demonstrated that the two techniques produced similar levels of polymorphism in *Phaseolus vulgaris*. The correlation between the matrices of cophenetic values for the dendrograms based on RAPD and ISSR data was also very low ($r=0.02$). It is probably due to the nature of different marker systems. RAPD markers cover the entire genome, revealing length polymorphisms in coding or noncoding and repeated or single-copy sequences (Williams *et al.,* 1990), whereas, the origin of the amplification products in ISSR is known to be from the sequences between the two microsatellite sites (Zietkiewicz *et al.,* 1994).

Three first principle eigen vectors of the PCA, which together accounted for RAPD, ISSR and combined data showed high total variation at the molecular level, indicating the suitability of the RAPD and ISSR approaches for genetic clustering.

Based on the pairwise analysis of the amplification products which were obtained with

the 13 tested RAPD primers, all the tested pomegranate cultivars showed a very high similarity values. Different relationships were observed between various cultivars. "Bihasteh sangan", "Dom anbarouti" and "Golabi hasteh riz" were quite distinct from the rest of cultivars. "Tab va larz" and "Bihasteh ladiz" were clustered together and in the studied cultivars showed the highest average similarity value (similarity coefficient of 1.0) which indicates that although there are some morphological differences in fruit characteristics, these cultivars may probably be mutants of each other (Sarkhosh *et al.,* 2009). All the remaining cultivars showed very limited differences, but sufficient to distinguish the different cultivars.

Among the 24 cultivars analyzed with ISSR marker, four main groups were recognized by UPGMA based on Jaccard's similarity coefficient (Figure 1b). The first group contained "Poust syah yazdi", the second group included "Goroch shahvar" and "Vahshi narak sarvestan", the third group consisted of "Dabbei sarjangal" and "Kaleh gavi sangan", while all remaining cultivars formed the fourth group. "Vahshi narak sarvestan", "Dabbei sarjangal" and "Kaleh gavi sangan" were quite distinct from the rest of cultivars and were readily separated from other cultivars. These cultivars are wild and it seems that they differ from other cultivars, morphologically e.g. fruit size, fruit color, seed color and the taste. "Dom anbarouti" and "Golabi hasteh riz" grouped and showed the highest average similarity value among the studied cultivars. "Poust syah yazdi" cultivars that have a black bark as a distinguishable marker from the other cultivars, was separate in both RAPD and ISSR analysis. In total, ISSR analysis, was more efficient than RAPD analysis.

A close genetic similarity was found in some of the cultivars analyzed as shown by high values of similarity index. Also, the similarities detected with ISSRs are greater than the similarities measured according to RAPD data. Fernandez *et al.,* (2002) and Muthusamy *et al.,* (2008) have studied barley cultivars and rice bean (*Vigna umbellata*) landraces, respectively, and they also found higher similarity index by ISSRs than by RAPDs.

Observation of no significant difference among the geographical regions based on AMOVA for RAPD and ISSR data and the clustering pattern of cultivars revealed that there are no correlation between genetic diversity and geographic distances. In the study of RAPD profiles in Iranian pomegranates by Sarkhosh *et al.,* (2009) and of AFLP profiles in Tunisian pomegranates by Jbir *et al.,* (2008), the authors could not detect any correlation between provenance of the accessions and similarity or otherwise in the PCR profiles. This can be due to the exchange of plant materials across the regions during the history of pomegranate cultivation.

In conclusion, both RAPD and ISSR are methods useful for revealing molecular relationships among pomegranate cultivars and ISSR markers exhibited higher levels of polymorphisms than RAPD. Relationships among these pomegranate cultivars revealed by ISSR markers were not generally in agreement grouping showed by RAPD markers.

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ارزیابی تنوع ژنتیکی ارقام انار ایران (*Punica granatum* L.) با استفاده از نشانگرهای RAPD و ISSR

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چکیده

با توجه به تنوع بالای مورفولوژیکی در ارقام انار ایران، تنوع ژنتیکی ۲۴ رقم انار با استفاده از نشانگرهای RAPD و ISSR مورد بررسی قرار گرفت. آغازگرهای RAPD در مجموع ۱۳۱ قطعه DNA تکثیر نمودند که ۲۹ قطعه آنها (۲۲/۱۴ درصد) چندشکل بودند. آغازگرهای ISSR نیز از مجموع ۱۷۳ قطعه تکثیر شده، ۲۹ (۳۷ درصد) چندشکلی حاصل نمودند. میانگین محتوای اطلاعات چندشکل (PIC) برای آغازگرهای RAPD و ISSR به ترتیب ۰/۱۲۸ و ۰/۱۶۳ به دست آمد. نتایج نشان داد که نشانگرهای ISSR در مقایسه با نشانگرهای RAPD الگوی نواری تکرارپذیری ایجاد می‌کنند و برای گروه‌بندی ارقام انار مؤثرترند. ضریب شباهت بین ارقام از ۰/۳۵۳ تا یک (RAPD) و ۰/۲۹۱ تا ۰/۹۳۰ (ISSR) متغیر بود و میانگین آن در نشانگرهای RAPD و ISSR به ترتیب ۰/۶۰۴ و ۰/۶۴۷ گزارش شد. تجزیه واریانس مولکولی (AMOVA) در داده‌های RAPD و ISSR اختلاف معنی‌داری بین نواحی مختلف جغرافیایی و طعم میوه ارقام مورد مطالعه نشان نداد (P>۰/۰۵) که بیانگر عدم ارتباط تنوع جغرافیایی و تنوع ژنتیکی است.

واژه‌های کلیدی: *Punica granatum*، تنوع ژنتیکی، انار، RAPD، ISSR

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