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Assessment of Genetic Variation within Commercial Iranian Pomegranate (*Punica granatum* L.) Cultivars, Using ISSR and SSR Markers

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ABSTRACT

Pomegranate is one of the most important horticultural crops in Iran, and has been cultivated for thousands of years in this country. At this period due to selection of superior cultivars from nature or mutation emerged in these cultivars, and their vegetative propagation, substantial genetic variation has occurred within and among the cultivars. Thus, each cultivar may consist of different clones. According to this issue, diversity within four commercial cultivars of pomegranate was analyzed. Two molecular marker systems including ISSR and SSR were used to evaluate variability between 36 samples of four commercial cultivars. ISSR markers produced 114 amplification products, out of which 97 were polymorphic (83.23%). Mean resolving power was 2.96 for ISSR markers. 19 SSR molecular markers were used, 15 of which amplified polymorphic products, while the remaining ones monomorphic., The number of polymorphic alleles per locus ranged from two to four (average 3.6). The observed and expected heterozygosities ranged from 0.04 to 0.92 and 0.14 to 0.62, respectively. In addition, mean polymorphic information content was 0.45 for SSR loci. Our results showed that commercial Iranian pomegranate have different clones. Therefore, ISSR and SSR markers can be a useful tools for detecting clones of each cultivar.

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Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest known edible fruits. It is among fruit species mentioned in the Holy Quran and is capable of growing in different agro-climatic conditions ranging from the tropical to sub-tropical regions due to versatile adaptability, hardy nature, low cost maintenance and high returns. Botanically, the pomegranate is included in the family Punicaceae with 2n = 16 or 18. The genus Punica is known to include two species, namely *P. protopunica* and *P. granatum* (Mars, 2000). Although, it is native of Iran, it is cultivated extensively in the Mediterranean and central Asian countries. Pomegranate is also important in human medicine and its components have a wide range of clinical applications. The anthocyanin from pomegranate fruits have been shown to have higher antioxidant activity than vitamin E, vitamin C or carotene (Shukla et al. 2008). Moreover, commercial pomegranate juice has been shown to have three times higher antioxidant activity than green tea and red wine (Gil et al. 2000). Iran as a center of pomegranate growth possesses more than 760 genotypes which have been collected and maintained in Yazd and Saveh germplasm (Behzadi-Shahrebabaki, 1998), Among them there have been multiple homonyms and synonyms, therefore a comprehensive plan to identify the genotypes is essential., This problem has been observed in the commercial Iranian pomegranate, where a pomegranate cultivar has different names in various regions and several different genotypes are known by the same name. In addition, due to the long historical cultivation and various environmental conditions in which, these cultivars are growing, natural mutations is expected to occur in some genotypes and caused genetic variability in different genotypes. Some of these mutations can be very useful, so that the mutant genotypes have desirable traits and it can be a significant improvement in performance and product quality of a particular genotype.

Molecular markers have overcome the limitations of morphological and biochemical markers due to avoiding the influence of environment on the performance of genotypes. A wide range of molecular markers has been used to assess genetic diversity of pomegranate cultivars as well as wild genotypes from different parts of the world. Random amplified polymorphic DNA (RAPD) markers have provided reliable and highly polymorphic information to discriminate pomegranate cultivars (Narzary et al., 2009; Hasnaoui et al., 2010a). AFLPs (Amplified Fragments Length Polymorphism) are another marker, which has been used to evaluate genetic diversity within and among Chinese pomegranate populations (Yuan et al., 2007) and Tunisian cultivars (Jbir et al., 2008). Up to now, more than 137 microsatellite loci in pomegranate genome have been identified (Soriano et al.,

2011; Curro et al., 2010; Hasnaoui et al., 2010b) showing different ranges of genetic polymorphism in the genotypes studied. Inter-Simple Sequence Repeats (ISSR) analysis is considered as another efficient molecular marker, showing genetic variation in the wild pomegranate populations studied in Western Himalaya region (Narzary et al., 2010; and Ajal et al., 2014). Morphological, cytological and DNA markers (RAPD, AFLP, SSR and ISSR) have been used to evaluate the genetic variability of Iranian pomegranates. These studies showed the occurrence of high genetic diversity among Iranian genotypes studied at both cytogenetic (Sheidai and Noormohammadi, 2005) and molecular levels including RAPD (Sarkhosh et al., 2006; Sheidai et al., 2007; Noormohammadi et al., 2010; Zamani et al. 2013), AFLP (Moslemi et al., 2010) and SSR markers (Koohi-Dehkordi et al., 2007; Pirseyedi et al., 2010; Ebrahimi et al., 2010). The present study was performed with the aim to identify genetic diversity within four commercial pomegranate cultivars of Iran and attempt to evaluate the usefulness of the two molecular markers (ISSR and SSR) that will help breeders to recognize superior cultivars for breeding programs.

Materials and Methods

Plant Materials

This work was carried out on 36 trees of four commercial pomegranate cultivars (each cultivar was represented by 9 trees or samples), plus 3 trees of the three non-commercial pomegranate genotypes as an out group (Black, Seedless white skin, Sweet white skin) to present other samples in realistic distances in clustering, from four different regions in Iran (Table 1).The nine trees were chosen from three different gardens and each garden included three samples. The trees were between 30 and 80 years-old, vigorous, almost uniform, and free from pathogens.

DNA Isolation and ISSR Amplification

Genomic DNA was extracted from the young leaves using Vroh Bi et al. (1996) method. PCR reactions were performed in 15 µl total volumes containing 20 ng of each template DNA, 0.3 µl of primers, 8.5 µl of PCR kit master mix (CinnaGen Co., Iran), and DNA-free water. Amplifications were performed in a thermocycler (iCycler, Bio Rad, Hercules, CA, USA) programmed for a first denaturation step of 4 min at 94°C, followed by 35 cycles of 92°C for 1 min, 48-54°C (varied for each primer according to Table 2) for 50 s, 72°C for 1 min and final extension at 72°C for 10 min and then held at 4°C until the tubes were removed. Amplified products were separated by electrophoresis in 2% (w/v) agarose gels at constant voltage (80 V) in 1×TBE buffer for approximately 150–180 min, stained with ethidium bromide and photographed under UV light (Fig. 1). The size of produced fragments were determined by comparing to a size marker (GeneRuler 100bp DNA ladder, SM0313, Fermentase).

SSR Amplification

Amplification of microsatellites was performed in PCR reactions in a total volume of 20 µl, containing 20 ng genomic DNA, 1X supplied PCR buffer (Bioron, Germany), 2 mM $MgCl_2$, 200 µM of each dntp (Bioron, Germany), 1 unit of *Taq* DNA polymerase (Bioron, Germany) and 0.2 µM of forward and reverse primers (fluorescently labeled). PCRs were carried out on a thermocycler (iCycler, Bio Rad, Hercules, CA, USA) programmed with a denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min (varied for each primer according to Table 3, 72°C for 1.5 min and final extension at 72°C for 15 min, then restrain at 4°C until the tubes were removed. The PCR products were then denatured by the addition of 7.5 µl formamide loading dye (95% deionized formamide 10 mM EDTA pH 8, 0.05% xylene cyanol, 0.5% bromophenol blue), heated for 5 min at 94 ºC, cooled on ice and then 5 µl for denatured preparations were loaded on a pre-warmed (50°C) polyacrylamide sequencing gel (Bio Rad, Sequi-Gen GT). Gels were run for 2-2.5 h at 75 V and the DNA banks were visualized by silver staining as described by Bassam and Caetano-Anolles (1993). A permanent record of gels was made using a gel scanner. The size of the produced bands was estimated by comparison to size marker (Fermentase Co.).

ISSR and SSR Primers and Data Analysis

Thirteen synthesized ISSR and fifteen SSR loci primers were used for polymorphism detection on the samples. A list of primers and their information are presented in Tables 2 and 3. Only reproducible and well-defined bands in the replications were considered as potential polymorphic markers. For ISSR primers the polymorphic bands were scored as present (1) or absent (0) and for SSR primers scoring alleles was performed as letters (A, B, C, D). In each sample for instance: the presence of AA as a homozygote and AC as a heterozygote on that location. A similarity matrix using the similarity coefficient of Jaccard (Sneath and Sokal, 1973) was constructed from the whole ISSR and SSR data. The similarity matrix was used for the cluster analysis and construction of dendrogram using unweighted pair-group method (UPGMA) (Sneath and Sokal, 1973) using the NTsys-pc version 2.02 (Rohlf, 2000). The total number of generated fragments, the number of polymorphic bands and polymorphism percentage were calculated by GenAlex 6.4 (Marshall et al., 1998) for each primer. The number of alleles, percentage of polymorphic loci, observed heterozygosity (Ho), Expected heterozygosity (He), and Polymorphic Information Content (PIC) were calculated based on frequency of alleles of each locus. POPGENE version 1.31 (Yeh et al., 1997), was used for these analyses. The ability of the most informative primers to differentiate the accessions was assessed by calculating their resolving power (Rp) (Prevost and Wilkinson, 1999) using the following formula (Gilbert et al., 1999): Rp= ΣIb were Ib= 1– (2× |0.5 – p|), and p is the proportion of the genotypes containing the present (1) band.

Table 1 List of Pomegranate cultivars and their samples, codes and origin areas

Cultivar Names	Origin	Garden numbers with chosen codes		
		Garden Number 3	Garden Number 2	Garden Number 1
Malas Saveh	Arak(Saveh)	Mls1, Mls2, Mls3	Mls4, Mls5, Mls6	Mls7, Mls8, Mls9
Alek Yosef Khani	Arak(Saveh)	Alk1, Alk2, Alk3	Alk4, Alk5, Alk6	Alk7, Alk8, Alk9
Ghojagh Qom	Qom	Gho1, Gho2, Gho3	Gho4, Gho5, Gho6	Gho7, Gho8, Gho9
Chandab Varamin	Tehran(Varamin)	Chn1, Chn2, Chn3	Chn4, Chn5, Chn6	Chn7, Chn8, Chn9
Black Pomegranate	Karaj	Black	-	-
Seedless white skin	Karaj	Seedless	-	-
Sweet white skin	Karaj	Sweet	-	-

Table 2 ISSR primers successfully used in this study and characteristic of primers

R	Primer	Sequence primer	AT	TBN	NPB	PB	RP
1	UBC810	GAGAGAGAGAGAGAGAT	52	6	4	66	2.05
2	UBC831	CTCTCTCTCTCTCTCTT	49	6	6	100	1.58
3	UBC868	GAAGAAGAAGAAGAAGAA	49	12	12	100	4.61
4	UBC873	GACAGACAGACAGACA	52	8	6	75	2.97
5	UBC880	GGAGAGGAGAGGAGA	52	8	7	87.5	1.38
6	ISO	ACACACACACACACACACC	53	9	7	77.7	2.05
7	IS7	ACGACGACGACGACGG	50	11	11	100	5.48
8	IS8	ACGACGACGACGACGC	49	9	9	100	3.69
9	IS10	TCGTCGTCGTCGTCGC	48	8	7	87.5	4.92
10	IS13	AGAGAGAGAGAGAGAGYT	50	9	5	55.5	0.46
11	IS15	ACACACACACACACACT	52	4	3	75	0.40
12	IS23	CTCCTCCTCCTCRC	51	13	11	84.6	4.76
13	IS25	GGATGGATGGATGGAT	53	11	9	81.8	4.15
	Total			8.7	7.4	83.23	
	Mean			114	97		2.96

R: Row, AT: Annealing temperature (ºC); TBN: Total Band Number, NPB: Number of Polymorphic Band, PB: Polymorphic Band (%), RP: Resolving Power(Rp)

Results and Discussion

ISSR Assay

Thirteen ISSR primers produced 114 bands across the 39 samples, of which 97 were polymorphic. The number of amplified fragments varied from 4 (UBC-810) to 12 (UBC-868) across the samples. The average number of polymorphic bands per primer was 7.4. The percentage of polymorphism for primers ranged from 55.5 to 100, with an average polymorphism percent of 83.23 (Table 2). Over the 13 primers, fragment sizes ranged from 200 to 1900 bp. The ability of the most informative primer to differentiate between samples was assessed using the Rp (Prevost and Wilkinson, 1999), whereby Rp values of primers varied from 0.04 (IS15) to 5.48 (IS7). The average Rp per primer was 2.96. The similarity values varied from 0.27 for Gho4 versus Sweet to 0.89 for Mls6 and Mls7, respectively. A Jacard's similarity coefficient dendrogram based on matrix similarity analysis with ISSR data is presented in Figure 2 According to dendrogram, samples of Malas Saveh were separated into three (I) groups, when a similarity of 0.74 was considered Ghojagh's and Alak's samples in the resemblance of 67.5 were divided into 5 (II) and 6 (II) groups respectively. Chandab's samples in the similarity of 63 were detached into 4 (III)groups. In accordance with UPGMA clustering three of the out-group genotypes separated into two main clusters (Fig. 2).

SSR Assay

From 15 SSR primers, 35 alleles were identified (Table 3) The number of alleles obtained per locus varied from 2 (EPS06, EPS09, EPS19 and PGCT111) to 4 (PGCT109, PGCT080, PGCT088, PGCT093A and ABRII-MO26) with an average of 3.06 alleles per locus. The Ho and He ranged from 0.04 to 0.92 (mean value=0.50) and 0.14 to 0.62 (mean value= 0.56), respectively. The PIC values ranged from 0.14 to 0.9 with an average of 0.45. The size of the alleles products ranged from 147 to 237 bp. The Rp, used to determine the ability of primers to differentiate pomegranates, ranged from 0.3 (PGCT015) to 1.94 (PGCT080) (Table 4). Out of 15 polymorphic loci, 10 deviated significantly from the Hardy-Weinberg equilibrium (HWE) ($P<0.05$).

In the SSR similarity matrix, the highest and lowest similarity values were 0.96 (Between Chandab5 and 6) and 0.33 (Between Alak7 and Ghojagh6) respectively. The Jacard's similarity coefficient dendrogram obtained from SSR data is shown in Figure 3. With threshold similarity of 0.74 samples of Malas Saveh, Alak Yousef Khani and Chandab Varamin divided into 4, 8 and 5 (I) groups, respectively. When a similarity of 0.70 was considered, Ghojagh's samples separated into 6 (II) assemblies. The three out-group samples like the ISSR dendrogram divided into two groups. The seedless genotype was placed alone in a group in both clusters.

Table 3 Characteristic of SSR loci.

Locus Name	Repeat Motif	Primer sequence (5'-3')	Ta(°C)	Expected Product Size (bp)
PGCT015	$(CT)_{20}$	F: GACGCCTTTAGTTTGCTCCA R: CTCGGGACAGGACTTGGAAT	60	161
PGCT028	$(CT)_{15}$	F: AAAAGCTGGCACTCAAACTC R: GGCATTACTTCCAGGACAAC	57	215
PGCT080	$(GA)_{17}$	F: TGAGTGGAAGGGAAATAGGA R:TCACCCTCTCCAAAATCAAA	58	230
PGCT088	$(CT)_{20}$	F: TCTCTCTCTACCCCGACACC R: TAGCGTCAAGATTGTGAAAAGG	56	150
PGCT109	(CT)18	F:GTAGCCACTTTAGGGCGAGA R:CGTCTAAAAGCGACAGCAAG	58	230
PGCT087	(CT)24	F:GCCTTTTCCTGCTTTCCTTT R:CATACAGCGGACCACAACAC	60	181
PGCT093A	(AG)19	F:TATCTGTCGCAGGAAGGATG R:GAAGCCAATTCCTCAAAGATG	58	235
PGCT093B	$(GA)_{16}$	F: CCACTTCCCTCCTACCTTCC R: ACGTCTGCTTGCACCTCTTT	60	188
PGCT111	(CT)22	F:TCCTCCGACCCTTTCTTATC R:CCCTATCATCCTTCCCATTC	58	237
EPS06	$(CT)_{9}$	F: TGGGGATTATCGTTGTCTTCA R: TCCAAGCTGAACTCGTTCCT	58	236
EPS08	$(AG)_{15}$	F: TAATCCCATTCCAAACAAGTCC R: ATATTGACGGAGGCTTCACTGT	57	199
EPS09	(AG)18	F:TTCCCGAGAAAGTTGCATATCT R:TAGTCCGTGAGGATTTTGTCCT	58	205
EPS19	$TC)_{13}($	F: AAATCGCATCCCTCCGTCT R: CTGTTCGCCAGGGTAAAGA	63	147
ABRII-MP26	$(AG)_{25}$	F: TTTCTCGAAGAATTGGGTAA R: CTGAGTAAGCTGAGGCTGAT	57	160
ABRIIMP42	$(GA)_{9}$	F: GAGCAGAGCAATTCAATCTC R: AACAATTTCCCATGTTTGAC	57	220

Shown for each primer pair are the forward (F) and reverse (R) primer sequences, repeat motif, annealing temperature (*Ta)* and expected product size respectively.

Table 4 The results of primer screening for 39 pomegranate samples.

Locus Name	N	Ho	He	Rp	PIC
PGCT015	3	0.04	0.14	0.30	0.20
PGCT028	3	0.30	0.34	0.82	0.36
PGCT080	4	0.05	0.53	1.94	0.40
PGCT088	4	0.92	0.62	1.07	0.60
PGCT109	4	0.84	0.57	1.07	0.76
PGCT087	3	0.35	0.32	0.82	0.30
PGCT093A	4	0.76	0.56	0.97	0.68
PGCT093B	3	0.61	0.56	1.43	0.64
PGCT111	2	0.47	0.39	1.02	0.38
EPS06	2	0.79	0.50	0.46	0.28
EPS08	3	0.69	0.61	1.28	0.66
EPS09	2	0.51	0.45	0. 97	0.14
EPS19	2	0.17	0.35	0.71	0.90
ABRII-MP26	4	0.17	0.20	0.46	0.24
ABRIIMP42	3	0.87	0.50	0.41	0.48
Mean	3.06	0.50	0.56	-	0.45

N, number of alleles; Ho, observed heterozygosity; He, Expected heterozygosity; Rp, resolving power; PIC, polymorphic information content values are given for each locus. The locus names are same as given in Table 3.

Table 5 Analysis of Molecular Variance (AMOVA) of 39 selected pomegranate accessions

Source of variation	df	SS	MS	Est. Var.	%
Among cultivars	4	164.80	41.20	3.31	17
Within cultivars	34	543.55	15.98	15.98	83
Total	38	708.35		19.29	100

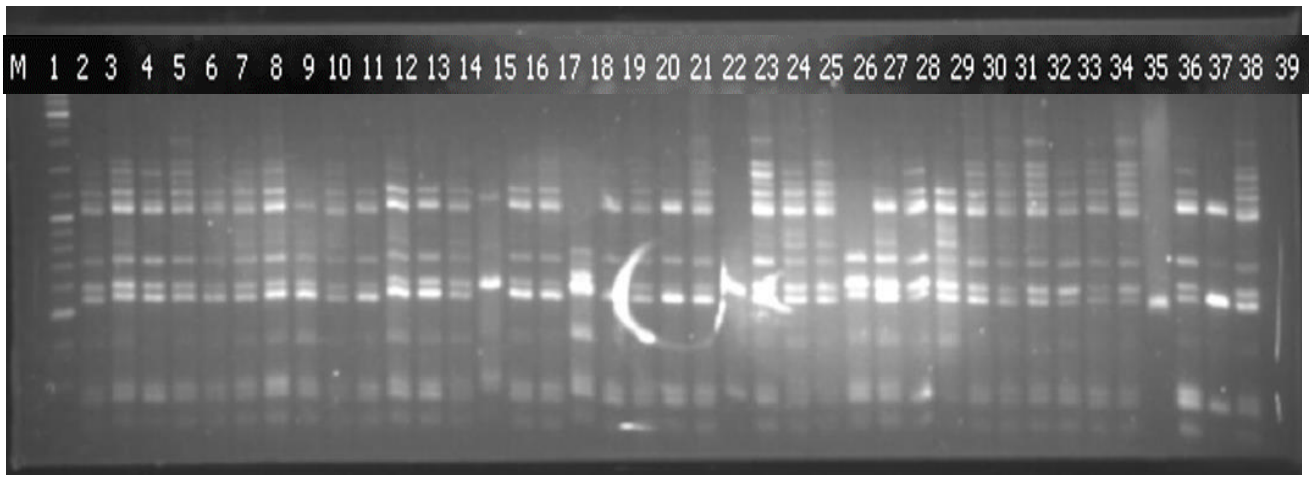

Fig. 1 ISSR amplification profile for primer UBC868 on 39 samples of pomegranate contains 7 pomegranate genotypes: M: 100bp DNA ladder, (1- 9) Malas Saveh, (10- 18) Alak Yousef Khani, (19-27) Ghojagh Qom, (27- 36) Chandab Varamin, 37 black pomegranate, 38 Seedless white skin, 39 Sweet white skin.

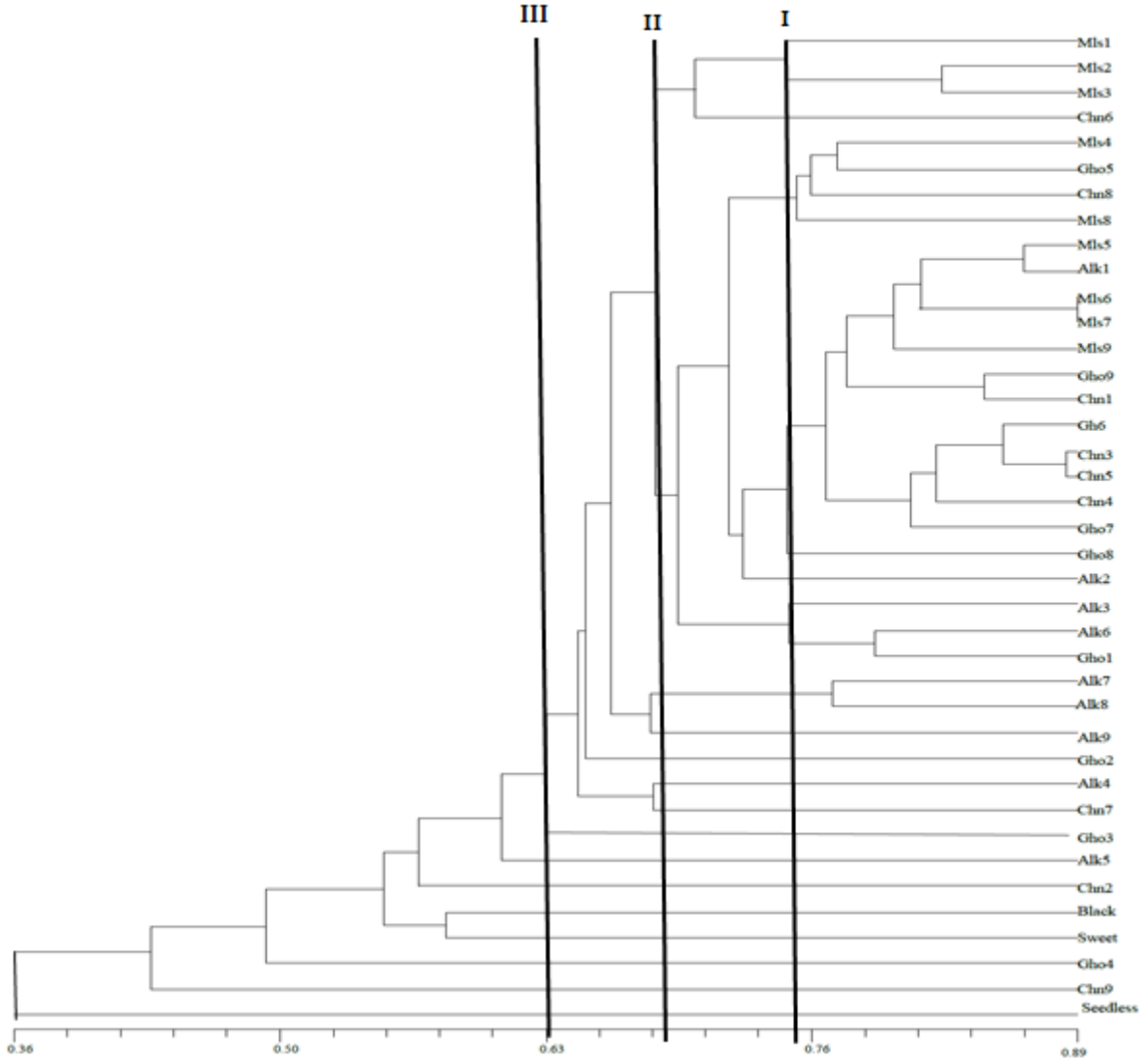

Fig. 2 Cluster of 39 pomegranate samples based on Jaccard's similarity matrix from the ISSR data.

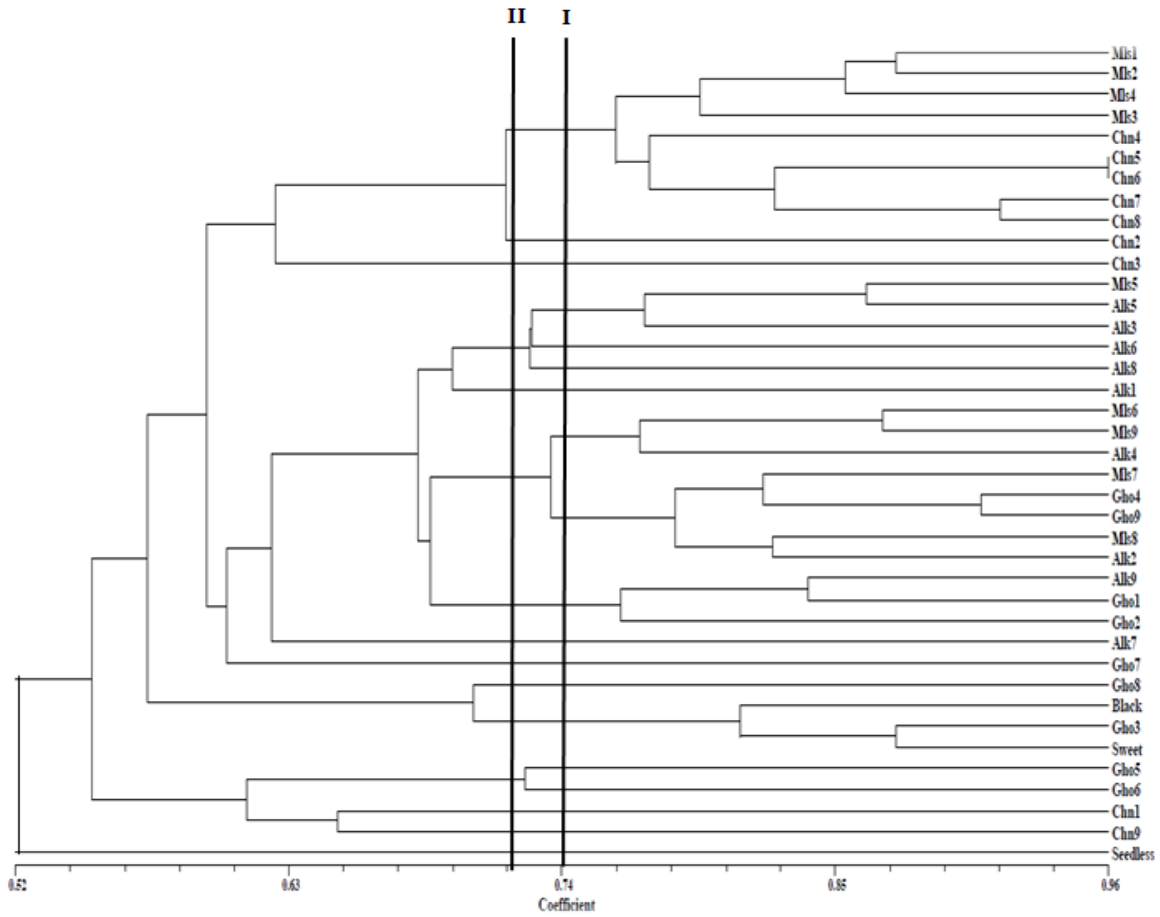

Fig. 3 UPGM dendrogram of 39 pomegranate samples. based on Jaccard's similarity matrix from the SSR data.

Analysis of Molecular Variance (AMOVA)

Analysis of molecular variance by applying molecular data of 39 pomegranate samples and selecting 13 ISSR and 15 SSR primers resulted in a genetic diversity of 17% among the cultivated accessions growing in 4 different regions with different ecological history, while 83% of molecular diversity was found within these cultivars (Table 5). These results are in accordance to the findings of Narzary et al. (2010) and may be due to the reason that these regions had a high genetic overlap as a result of relatively high gene flow. This reasoning is further strengthened by the clonal propagation of pomegranate accessions (Parvaresh et al. 2012; Ajal et al. 2014).

Conclusions

To the best of our knowledge, this is the first time that clonal diversity has been reported for *P. granatum* L. Iran hosts a great genetic diversity of *P. granatum* and more than 760 Iranian genotypes are collected at Iranian national pomegranate in Yazd, Iran. Identification of genetic variation within cultivars (clones of each cultivar) is very important, because some of them can have unique characteristics that might be economically more important.

Our results clearly demonstrate that Iranian pomegranate cultivars have many clones and that ISSR and SSR markers can be used to identify clonal diversity within cultivars. The used ISSR primers showed a higher percentage of polymorphic bands of 83.23% compared to the finding in Iran by Noormohammadi et al (2012), (Narzary et al., 2010), Talebi Bedaf et al. (2011), and Ajal et al, (2014) in Morocco. In addition, due to high PIC value obtained in the majority of ISSR and SSR markers, these markers could be used for identification and characterization of each pomegranate cultivar maybe, only due to the high number of SSR and ISSR loci studied, supporting the results obtained by Ebrahimi et al. (2010); Hasnaoui et al. (2010b); Pirseyedi et al. (2010); Soriano et al. (2011) that present low PIC values. Cophenetic correlation between Dice similarity and Jacard's similarity coefficient dendrogram showed the highest value (r = 0.86) in SSR data and in ISSR data (R= 0.92). The correlation between ISSR and SSR showed a significant retrogression (R^2=0.41) (Noormohammadi et al., 2012).

In the study herein, the result of ordination and cluster analysis (ISSR and SSR) clearly showed that there exists a high degree of genetic diversity within each cultivar, Therefore samples which were collected as a genotype can belong to other cultivars. This investigation suggested

that almost all the Iranian cultivars share similar genetic background, and are likely derived from a small number of introductions in ancient times (Hasnaoui et al., 2011b; Nafees et al., 2015). The outcomes of Jacard's dendrogram in both ISSR and SSR primers can indicate a problem of homonymy or synonymy in the cultivars appellation. Hence, despite the relative high degree of diversity, the Iranian pomegranate germplasm represents a quite homogenous population, similar results have been reported by Ajal et al., (2014); Nafees et al., (2015). In accordance with obtained results in this study there have been genetic differences within each cultivar, and these distinctions may be caused by different environmental conditions, wrong naming by pomegranate growers and mutation that cause the incorrect name.

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