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Genetics Diversity of some Grape Genotypes by ISSR and RAPD Markers

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Summary

Grape is an important fruit crop with various consumptions. Classical methods of identification, based on ampelographic characterization, are not always the most accurate way due to instability of morphological characteristics under the influence of the internal and external environments. The superiority of molecular markers over ampelometry for the characterization of grape genotypes is their capability to identify the diversity at genomic level free of environmental factors. Genetic variation of 15 grape genotypes (including 7 Khorasan native grape genotypes and 8 imported genotypes from Turkmenistan) were studied based on RAPD and ISSR markers. PCR amplification of DNA by using 7 primers for RAPD analysis produced 59 bands while the same number of primers used for ISSR analysis resulted in 58 bands. These bands could be scored in all genotypes. The average number of polymorphic bands per RAPD and ISSR primers was 8.4 and 8.2, respectively. The RAPD and ISSR data were combined for UPGMA cluster analysis. Dendrogram based on genetic distance (UPGMA) indicated segregation of the 15 grape genotypes into two main clusters. Turkmen genotypes formed cluster I and native Khorasan genotypes grouped in cluster II. Based on Jaccard's similarity coefficient, the highest similarity observed between the Turkmen 6 and Turkmen 8 and lowest similarity observed between Turkmen 3 and Sahebi e Rezaieh.

Key words. genetic variation – grapevine – ISSR – PCR – RAPD

Introduction

Grape (*Vitis* spp.) is a fruit crop of worldwide importance. There are about 60 known *Vitis* species, primarily distributed in the Northern Hemisphere. *Vitis vinifera* L. is one of the oldest and most important perennial crops in the world. *Vitis vinifera* L., which originated from West Asia, dominates most of the grape production in the world. Other *Vitis* species, however, are also important in grape improvement. Grapes have great nutritional value, and they can be used in many ways. Apart from being consumed as a fruit, they can be preserved for long periods of time as wine, grape juice, and pekmez (grape juice boiled to sugary solid or heavy syrup). One of the important grape-growing regions in Iran is Mashhad in Khorasan Razavi province. Cultivation of grapevines in Mashhad has an ancient history. Turkmen genotypes, were rapidly established and were well suited to Khorasan soil and environmental conditions. It is interesting to study the degree of diversity between the material originally introduced into Khorasan and that more recently re-introduced from Turkmenistan, particularly as phenotypic divergence has been observed in the vineyard. Classical methods of identification, based on botanical and ampelographic characterization not always were the most accurate way due to instability of morphological characteristics under the influence of the internal and external environment. The superiority of molecular markers over ampelometry for the characterization of grape cultivars is well established. In grape, molecular markers like RFLP (Bourquin et al. 1993) RAPD (Jean-Jaques et al. 1993; Vidal et al. 1999; Luo and He 2001) microsatellite or SSR (Bowers et al. 1993, 1996; Thomas and Scott 1993; Pellerone et al. 2001) and AFLP (Cervera et al. 2000) are widely used for characterization of cultivars, parentage analysis, identification of clones, establishing the genetic relationship and molecular mapping. Techniques based on DNA-DNA hybridization, such as restriction fragment length polymorphism (RFLP), and those based on polymerase chain reaction (PCR) are all insensitive to environmental change and so have largely replaced isozymes as the methods of choice. Moreover, the requirement for large quantities of high quality DNA, its slow speed and widespread use of radio-labelling for detection has also led to the abandonment of RFLP for such studies (Bourquin et al. 1993; Bowers et al. 1993). The *in vitro* amplification of short sections of DNA by the PCR technique is dependent on the annealing of inversely oriented oligonucleotide primers homologous to those bordering the target region, and the subsequent repeated synthesis of this DNA. There are two widely used PCR based techniques that are both cheap and generate polymorphisms of value for genetic distance analyses. In the first, Randomly Amplified Polymorphic DNA (RAPD), a ten base long decamer of ran-

dom sequence is used as a primer for PCR. Several studies using RAPDs have reported the analysis of genetic relations between grape cultivars (Aras et al. 2005; Ercisli et al. 2008; Jaladet et al. 2009; Karatas et al. 2010). The second technique, Inter Simple Sequence Repeat Polymerase Chain Reaction (ISSR-PCR) has been used to assess genetic relationship among 16 table grape varieties (*Vitis* ssp.) grown in Turkey (Sabir et al. 2008). Also several authors have reported the use of ISSR marker to analyse the diversity of the *Vitis vinifera* species (Dhanorkar et al. 2005; Sabir et al. 2009) ISSR primers are generally complementary to dinucleotide simple sequence repeat motifs that are abundant in genomic DNA, but also contain a one to three base „anchor" to ensure the primer anneals to either the 5'or 3' end of the SSR (Charters et al. 1996). However, several authors have reported the use of both ISSR and RAPD marker to analyse the diversity of the *Vitis vinifera* species (Herrera et al. 2002). The aim of the present work was to survey the genetics diversity of *Vitis vinifera* L. genotypes grown in Khorasan by ISSR and RAPD markers.

Materials and Methods

Fifteen rootstock genotypes (Khorasan and Turkmen genotypes) were used for the study. The genotypes were collected from Mashhad Agricultural Research Institute, Iran. Some important characteristics of the genotypes are presented in Table 1. The young leaf was used for extracting DNA from these genotypes.

DNA extraction

Total genomic DNA was extracted using modified cetyltrimethylammonium bromide (CTAB) procedure as described by Dellaporta et al. (1983). Finally, the DNA samples were stored at −20 °C. DNA concentrations were determined at 260 nm spectrophotometrically and the quality verified by electrophoresis on a 0.8 % agarose gel.

RAPD-PCR

A set of 25 random primers were screened initially for polymorphism with the template DNA of mother plants of rootstocks. From which, 7 primers were selected and used for amplifcation (Table 2). PCR was carried out in a standard reaction of 25 µl containing 25 ng template DNA, 2 mM primer,1.5 mM $MgCl_2$, 0.2 mM each of the dNTP's, 1 unit Taq polymerase (Cinnagene, Tehran, Iran), 10x PCR buffer and sterile MQ water. Amplification by PCR was performed using Primus thermocycler under the following condition: 4 min at 95 °C then 40 cycles of 1 min at 95 °C (denaturation), 1 min at 37 °C (annealing) and 2 min at 72 °C (extension) and final elongation step at 72 °C for 8 min. RAPD analysis using each primer was repeated at least twice to establish the reproducibility of banding pattern of the DNA sample studied.

ISSR-PCR

Out of 20 ISSR primers used for initial screening, only 7 primers produced reproducible and scorable bands (Table 2). They were used for PCR amplification using the following protocol. PCR reaction mixture (20 µl) contained the following components/concentrations: 1 unit of Taq DNA polymerase (Cinnagene, Tehran, Iran), 1.5 mM $MgCl_2$, 10x PCR buffer, 0.2 mM of each dNTP, with 0.5 mM of primer and 10 ng template DNA. Optimum annealing temperature for these ISSR primers obtained using gradient PCR and varied from 44.5 to 54.5 °C. PCR amplifications were performed using Primus thermocycler under the following condition: 3 min at 94 °C then 35 cycles of 1 min at 94 °C (denaturation), 1 min annealing temperature (variable for each primer), 2 min at 72 °C (extension) and final elongation step at 72 °C for 8 min.

Table 1. Morpholical characteristics of grape genotypes.

Code	Genotypes name	Present seed in berry	Marketing	Berry colour	Kind of use
T1	Turkmenistan No. 1	1–2	Good	Black	Industrial
T2	Turkmenistan No. 2	1–2	Good	Pink	Table grape
T3	Turkmenistan No. 3	None	Good	Black	Industrial
T4	Turkmenistan No. 4	None	Good	Black	Industrial
T5	Turkmenistan No. 5	None	Good	Black	Table grape
T6	Turkmenistan No. 6	1–2	Very good	Pink	Table grape
T7	Turkmenistan No. 7	1–2	Very good	Pink	Table grape
T8	Turkmenistan No. 8	1–2	Very good	Pink	Table grape
YR	Yazid ozum Rezaeieh	1–2	Good	Green	Industrial
KO	Kolahdari e Bojnourd	2–3	Very good	Cream	Table grape
KQ	Keshmeshi e Qouchan	None	Good	Cream	Raisin
SN	Sahebi e Neyshabur	None	Very good	Purple red	Table grape
SR	Sahebi e Rezaeieh	2–3	Good	Purple	Table grape
SD	Siyah Saadi e Dargaz	2–3	Good	Dark red	Industrial
KGH	Keshmeshi ghermez	2–3	Good	Red	Raisin/Table grape

Table 2. Primers survey for determination of different grape genotypes.

Polymorphism (%)	No. of polymorphic bands	No. of total bands	Sequence	Kind of marker	Primers
100.0	11	11	AGGTGACCGT	RAPD	OPM11
100.0	11	11	GGGTAACGCC	RAPD	OPA9
100.0	11	11	GTGACGTAGG	RAPD	OPA8
100.0	7	7	GTCCACACGG	RAPD	OPB6
100.0	5	5	GTCCCGTGGT	RAPD	OPJ12
100.0	6	6	TGTAGCAGGG	RAPD	OPJ15
100.0	8	8	GTCCACACGG	RAPD	A9
100.0	8	8	CGCTGTGTGTGTGTGTG	ISSR	S1
100.0	8	8	GAGCACACACACACACA	ISSR	S2
88.8	8	9	AGAGAGAGAGAGAGAGG	ISSR	S3
100.0	9	9	GAGAGAGAGAGAGAGAA	ISSR	S4
100.0	10	10	ACACACACACACACACCG	ISSR	S5
40.0	2	5	GAGAGAGAGAGAGAGATG	ISSR	S6
77.7	7	9	GAGAGAGAGAGAGAGAT	ISSR	S7

Separation of PCR products by electrophoresis

RAPD and ISSR products were loaded into a 1.2 % and 2 % agarose gel in 0.5 × TBE buffer during 2 h at 65 V. Then gel stained with ethidium bromide (0.05 %) and visualized on a UV transilluminator.

Data Analysis

Amplified DNA fragments were scored as 1/0 (presence/absence). The genetic associations were evaluated by calculating the Jaccard's similarity coeficient for pairwise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to the cluster analysis of unweighted pair group method with arithmetic averages (UPGMA) and a dendrogram was generated by using NTSYS-PC Ver. 2.1 software.

Results

RAPD Band Pattern

PCR amplification of DNA, using 7 primers for RAPD analysis produced a total of 59 bands that could be scored in 15 grape genotypes. Average number of bands created for each primer 8.4 bands with size ranging from 400 to 2000 bp. Band pattern obtained from primer OPJ12 shown in Fig. 1B. The dendrogram obtained from RAPD data which resulted in two main clusters (Fig. 2). The first cluster (Group I) was divided into two subgroups so that Turkmen genotypes 1–5 were clustered in sub-group A and Turkmen 6, Turkmen 7, Turkmen 8, with Yazid ozum Rezaeieh, Kolahdari e Bojnourd, Keshmeshi Qouchan and Sahebi Rezaeieh were clustered into sub-group B. The second cluster (Group II) included Siyah Saadi e Dargaz, Keshmeshi ghermez and Sahebi e Neyshabur.

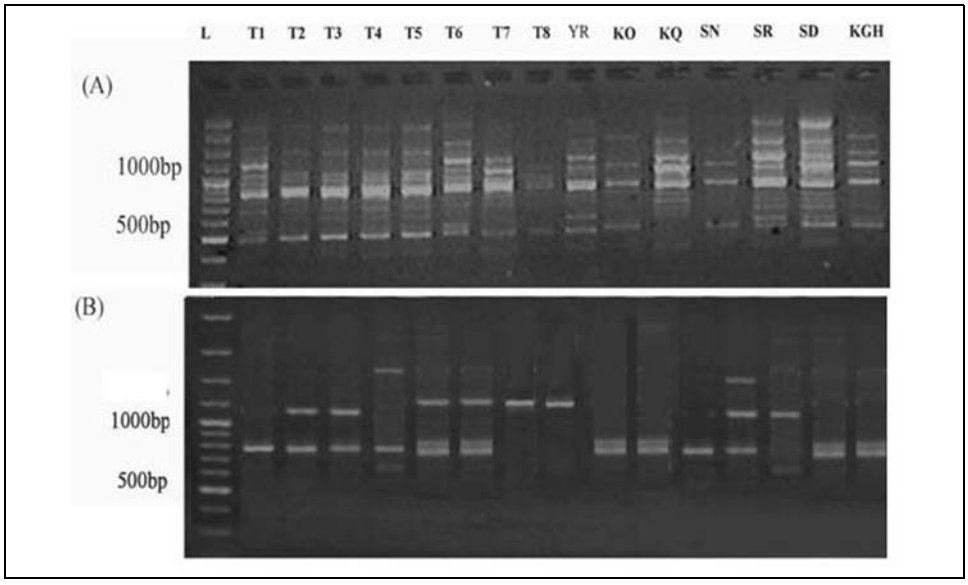


Fig. 1. A: DNA banding pattern by S7(ISSR) primer distinguished grape genotypes. B: DNA banding pattern by OPJ12(RAPD) primer distinguished grape genotypes, L: 100 bp DNA ladder

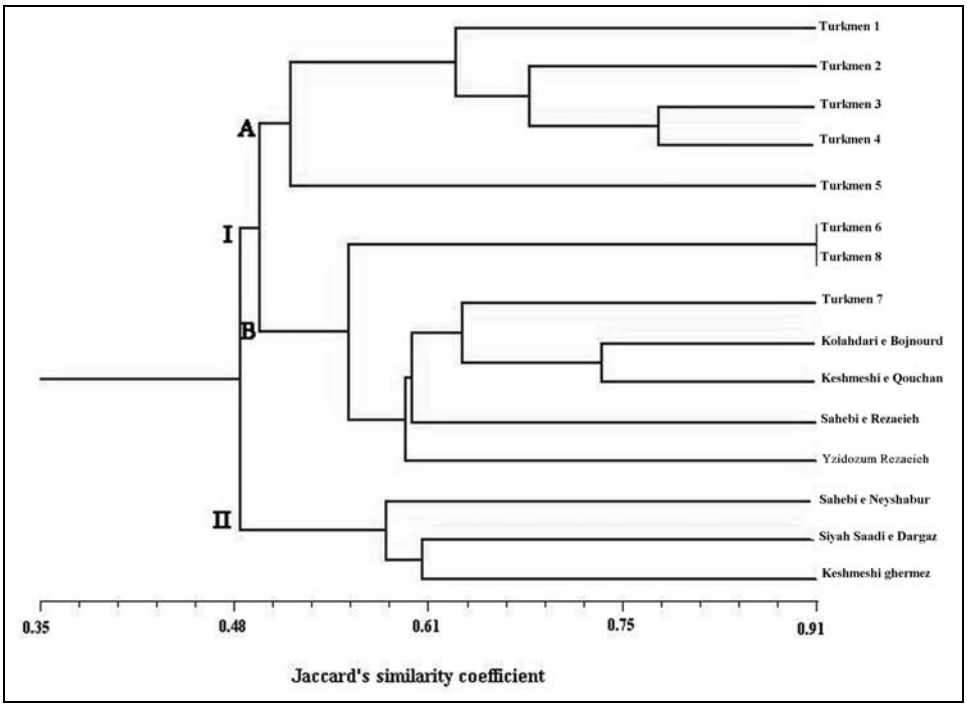


Fig. 2. Dendrogram showing relationship between 15 different grape genotypes based on RAPD analysis using UPGMA

ISSR Band Pattern

The PCR amplification using ISSR primers gave rise to reproducible amplification products. ISSR primers produced different numbers of DNA fragments, depending upon their simple sequence repeat motifs. The 7 primers produced 58 bands across 15 genotypes, of which 52 bands were polymorphic. Bands varied in size from 500 to 2500 bp. Average number of bands per primer were 8.2. Dendrogram obtained from ISSR data has been classified genotypes tested in two major groups I, II (Fig. 3). Group I included Turkmen except turkmen 4 and group II included other genotypes. Band pattern obtained from primer S7 are shown in Fig. 1A.

Combined (ISSR and RAPD) Data

To obtain reliable results, the RAPD and ISSR data were combined for UPGMA cluster analysis. The UPGMA dendrogram obtained from the cluster analysis of ISSR and RAPD data gave similar clustering pattern, with Jaccard's similarity coefficient ranged from 0.35 to 0.91. The fourteen primers generated 117 bands with size ranging from 400 to 2500 bp. Out of the 117 bands, 111 scorable bands were found to be polymorphic and 6 bands were found to be monomorphic. Dendrogram based on Jaccard's similarity coefficient genetic distance (UPGMA) indicated segregation of the 15 grape genotypes into two main clusters (Fig. 4). Turkmen genotypes formed cluster I and native Khorasan genotypes grouped together in cluster II. In cluster II Yazidozum Rezaeieh, Kolahdari e Bojnourd and Sahebi e Neyshabur formed sub cluster I and Keshmeshi Quochan, Sahebi e Rezaeieh, Siyah Saadi e Dargazand and Keshmeshi Ghermez grouped together in sub cluster II. Again, in sub cluster II Keshmeshi Quochan and Sahebi e Rezaeieh grouped together in sub-sub cluster I and Siyah Saadi e Dargazand and Keshmeshi ghermez grouped together in sub-sub cluster II. The highest similarity observed between the Turkmen 6 and Turkmen 8 with 91.5 % similarity and lowest similarity observed between Turkmen 3 and Sahebi e Rezaeieh with 27.7 % similarity. Similarity coefficient values among 15 grape genotypes based on data from 14 primers shown in Table 3.

Discussion

DNA molecular markers have been used to characterize a wide range of plant species. In this study, RAPD and ISSR-PCR were used to study variation in some Khorasan native and non – native genotypes of *V. vinifera*. The most plausible explanation lies in the careful selection of primers and markers for reproducibility and the strict standardisation of the protocol used. Indeed, the consistency and reproducibility of the amplification technique used has been reported even when two different thermocyclers were employed (Herrera 1998). It is important to bear in mind, however, that the occurrence of errors when RAPD is used, sometimes leads to an overestimation of variability and is particularly important for fingerprinting applications (Pooler and Scorza 1995; Halldén et al. 1996) Duplicate runs, optimisation experiments and replicating independent experiments all have been used to select primers and markers, and also to minimise the presence of errors during experiments. Compared with other DNA marker systems, the inter-simple sequence repeat (ISSR) marker is an intermediate method in terms of technical difficulties, reproducibility and cost (Reddy et al. 2002). ISSR markers overcome the shortcomings of the low reproducibility of RAPD, the high cost of amplified fragment length polymorphism (AFLP) and the complexity of SSR, and represent a fast and cost-efficient technique (Reddy

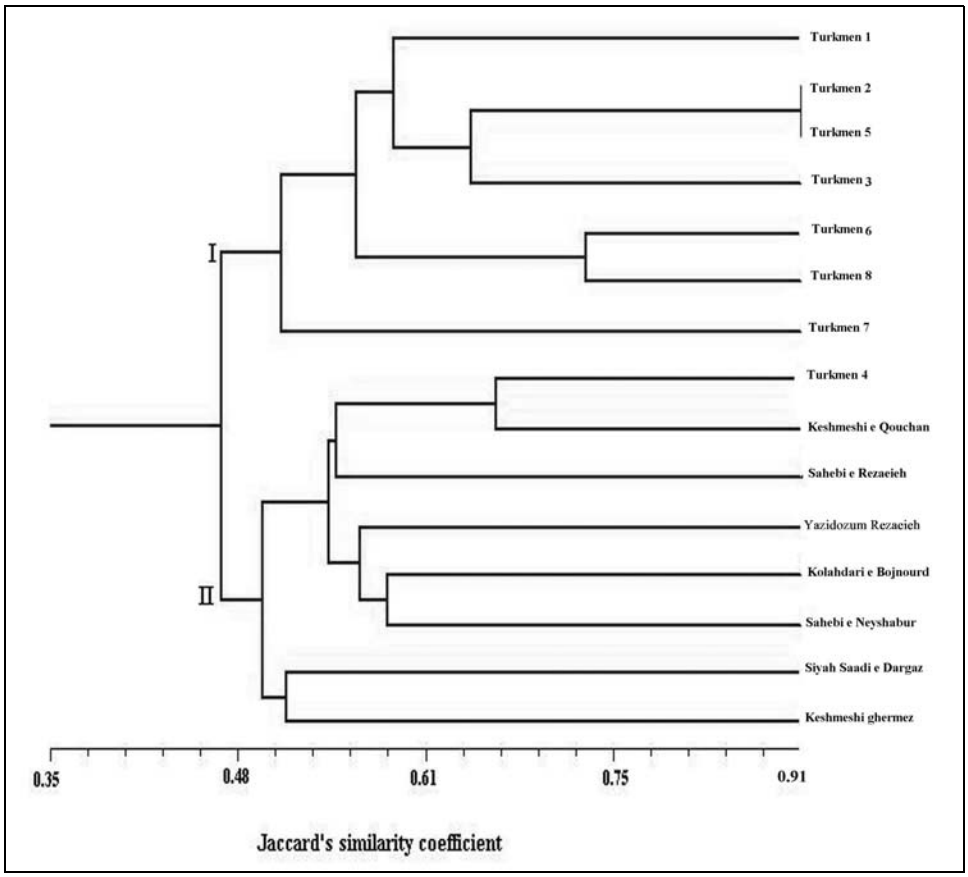


Fig. 3. Dendrogram showing relationship between 15 different grape genotypes based on ISSR analysis using UPGMA

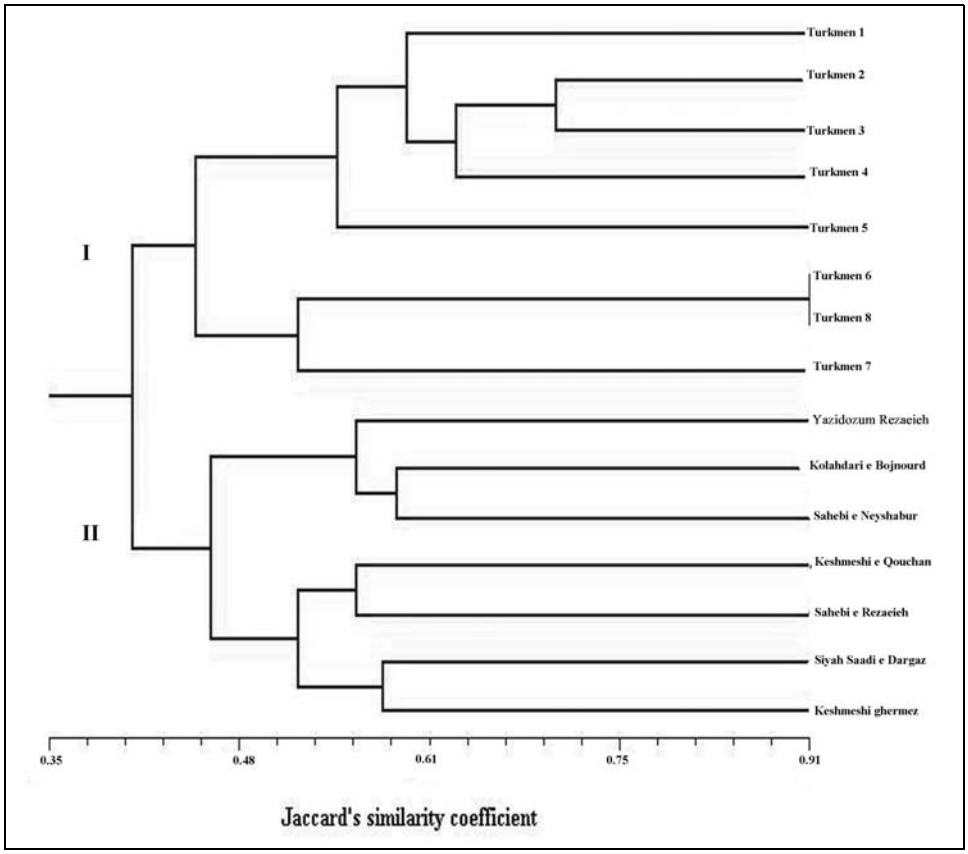


Fig. 4. Dendrogram showing relationship between 15 different grape genotypes based on combined ISSR and RAPD analysis using UPGMA

Table 3. Jaccard similarity coefficient of 15 grape genotypes based on RAPD and ISSR analysis.

T1														
T2	.667													
T3	.636	.746												
T4	.600	.676	.645											
T5	.643	.687	.600	.590										
T6	.438	.500	.438	.346	.517									
T7	.479	.466	.357	.456	.471	.500								
T8	.450	.513	.472	.392	.528	.915	.515							
YR	.420	.429	.333	.394	.356	.357	.349	.412						
KO	.378	.486	.373	.500	.386	.375	.483	.408	.491					
KQ	.476	.500	.387	.533	.516	.388	.421	.417	.384	.522				
SN	.333	.400	.303	.343	.333	.310	.317	.343	.434	.509	.456			
SR	.381	.405	.277	.410	.374	.345	.355	.341	.412	.493	.608	.469		
SD	.449	.456	.352	.447	.447	.370	.398	.366	.380	.507	.610	.372	.587	
KGH	.435	.393	.318	.349	.434	.352	.313	.348	.360	.432	.543	.389	.579	.663

et al. 2002; Chen et al. 2008). The ISSR-PCR has been described as an alternative approach for polymorphism studies. The high reproducibility with ISSR technique is attributable to the use of longer primers allowing for more stringent (higher) annealing temperatures than those of RAPDs (Kojima et al. 1998). The ISSR have been widely used to study genetic diversity of plants, such as *Elymus sibiricus* (Ma et al. 2008), *Undaria pinnatifida* (Wang et al. 2006), *Oryza meyeriana* (Wan et al. 2008) and eggplant (Albani et al. 2004). In both the ISSR- and RAPD-derived dendrograms showed high similarity with exception of the divergences among Turkmen genotypes. Therefore, either of the molecular marker techniques appeared effective for the assessment of genetic relationships within grape genotypes. Although RAPD and ISSR markers demarcated 15 genotypes into two groups, clustering of genotypes within groups was not similar. A possible explanation for the difference in resolution of RAPD and ISSR is that the two marker techniques targeted different portions of the genome. These differences may also be attributed to marker sampling errors and/or the level of polymorphism detected, reinforcing the importance of the number of loci and their coverage of the overall genome in obtaining reliable estimates of genetic relationships among cultivars (Souframanien and Gopalkrishna 2004). In all, RAPD and ISSR primers revealed polymorphism between the Turkmen genotypes and Khorasan native genotypes. It is therefore clear that the Turkmen genotypes propagate in recent years in Iran has a different genetic structure to the native genotypes from Khorasan. However, our study suggests that these two groups of grape (Turkmen and Khorasan genotypes) genotypes are not very closely related but there are close relationship between the genotypes placed in each group.

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