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Genetic Diversity of Grapevine Accessions from Iran, Russia and USA Using Microsatellite Markers

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Abstract: To discover marker information content and differentiation among grapevine accessions from Iran, USA and Russia, nine microsatellite markers were used. A total of 75 alleles were detected, giving a mean of 8.3 alleles per 9 loci. The total number of alleles per locus varied between 6 to 11 and the polymorphism information content ranged from 0.65 to 0.88, indicating that these loci were highly informative. A positive correlation (r = 0.870) was observed between the number of alleles and the level of polymorphism. Two SSRs loci including SSrVrZAG47 and VVMD27 were found to be probably synonymous. Gene diversities were high in all populations with values ranging from 0.709 to 0.784. In all populations, the mean number (averaged over loci) of heterozygous individuals was higher than expected. PCO analysis could not be so clearly differentiated accessions from Iran and Russia. The pattern of clustering of the *Vitis vinifera* populations was according to their geographic distribution. It is suggested that accessions could possibly be assigned to their regions of origin according to their genotypes.

Key words: *Vitis vinifera*, genetic polymorphism, population analysis, SSRs

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is economically the most important fruit crop in the world. There are 8,000 to 10,000 grape cultivars existing worldwide today (Vouillamoz *et al*., 2004) and the origin of most of them is generally wrapped in legends. The main center of diversity of *V. vinifera* is believed to stretch from Afghanistan to the South of the Caspian Sea, through the South Caucasus to the South Coast of the Black Sea (Fatahi *et al*., 2003). Characterization and determination of grapevine cultivars are sometimes difficult, using conventional methods. Since, morphological markers are time-consuming and prone to equivocal interpretations, molecular approaches should be implemented in cultivar identification and breeding programs (Laimer *et al*., 2005). The high polymorphism of microsatellites (or SSRs-simple sequence repeats), a special class of tandem repeat loci involving a base motif of 1-6 bp of DNA repeated up to 100 times (Xu *et al*., 2004), allows cultivar identification and their co-dominant Mendelian inheritance allows the reconstruction of crosses (Piljac *et al*., 2002; Vouillamoz *et al*., 2003). Since, the development of microsatellite markers in grape cultivars (Thomas and Scott, 1993), the origin and parentage of economically important cultivars such as Cabernet Sauvignon (Bowers and Meredith, 1996), Chardonnay and Syrah (Bowers *et al*., 1999) have been uncovered. The importance of microsatellites as genetic markers and their potential in various applications such as diversity analysis or genetic mapping are well established and documented generally in plants and particularly in grapevine (Thomas and Scott, 1993; Scott *et al*., 2000; Cipriani *et al*., 1994; Bowers *et al*., 1999; Sefc *et al*., 1999, 2000; Grando *et al*., 2003; Adam-Blondon *et al*., 2004; Riaz *et al*., 2004). So far, Vitis SSRs primers have been developed by several groups (Thomas and Scott, 1993; Bowers and Meredith, 1996; Sefc *et al*., 1999; Bowers *et al*., 1999; Pellerone *et al*., 2001; Merdinoglu *et al*., 2005) and their usefulness has been assessed in samples of grapevine varieties cultivated in the regions of Australia, California, Central Europe, Calabria and Sicily, respectively. However, the information content of a given marker may be varied for cultivars from different regions due to the predominance of certain alleles or the occurrence of null alleles in some populations (Sefc *et al*., 2000).

In this research, nine microsatellite loci were employed to analyze the genetic diversity in a collection of 43 grape (*Vitis vinifera*) accessions from Iran, Russia and California (USA).

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MATERIALS AND METHODS

Plant material and DNA extraction: A total of 43 grapevine accessions was used in this study as follows:

- Thirty four accessions from Iran (grapevine collection of the Shiraz Agricultural Research Institute, Shiraz, Iran)
- Six accessions from Russia (grapevine collection of the Takestan Grapevine Research Institute, Gazvin, Iran)
- Three accessions from USA (grapevine collection of the research farm of the Horticultural Department, University of Tehran, Karaj, Iran, Table 1)

Total DNA was extracted from young leaves according to Lodhi *et al.* (1994). The purified total DNA was quantified by gel electrophoresis and its quality verified by spectrophotometry.

SSRs amplification: Nine SSRs primer pairs including VVS2 (Thomas *et al*., 1994), VVMD14, VVMD27, VVMD28, VVMD32, VVMD36 (Bowers *et al*., 1999), VrZAG21, VrZAG47 and VrZAG79 (Sefc *et al*., 1999) were selected for genotyping assays through preliminary screening of 23 primer pairs. These were generated consistently reproducible clear fragments with high polymorphism (Fig. 1). Clear fragments were generated only on three (VVS2, VVMD27 and VrZAG79) reference SSR markers out of six (This *et al*., 2004). PCR reactions were performed in a 20 μL volume, with 30 ng of genomic DNA, 1.25 mM $MgCl_2$, 0.1 mM dNTP mix, 100 ng of each primers, 1 unit *Taq* polymerase and finally 2 μL 10xPCR Buffer (all from CINNAGEN Company). The amplification reactions were performed using a Primus thermocycler (MWG), under the following conditions: 4 min at 94°C; 10 cycles of 30 sec at 94°C, 30 sec at T_m+10°C (decreasing 1°C per cycle to T_m) and 1 min at 72°C; 25 cycles of 30 sec at 94°C, 30 sec at T_m and 1 min at 72°C and a final extension step at 72°C for 5 min. All amplification reactions were repeated two times under identical conditions included a negative control. Amplification reaction products were separated on 6% denaturing polyacrylamide gels. The amplified fragments were detected using the silver staining method as described by Bassam and Gresshoff (1991).

Data analysis: Polymorphic fragments were coded by 1 and 0 for presence or absence, respectively. The No. of Alleles (NA), probability of identity (PI; Pollefeys and Bousquet, 2003) and polymorphism information content (PIC; Oliviera *et al*., 2006) were estimated as follows:

Table 1: Grapevine accessions characterized using microsatellite markers

Name	Origin
Rajabi Aghighi Zarghan	Iran
Sorkhak Nishabor	Iran
Rotabi Zarghan	Iran
Askari Lirak Zarghan	Iran
Rishbaba Oroomie	Iran
Kaleii Zarghan	Iran
Domrobahi Zarghan	Iran
Shahpasand Ghoochan	Iran
Rishbaba Siah Shiraz	Iran
Rishbaba Siah Doodaj	Iran
Askari Abade	Iran
Sahebi Oroomie	Iran
Dooshabe Ghoochan	Iran
Rajabi Siah Zarghan	Iran
Askari Mashhad	Iran
Askari Nishabor	Iran
Sahebi Nishabor	Iran
Gieii Ghalat	Iran
Sefide Abade	Iran
Keshmeshi Ghoochan	Iran
Askari Kashmar	Iran
Siah Ghoochan	Iran
Laele Ghoochan	Iran
Ite Siah Zarghan	Iran
Monagha Shiraz	Iran
Rishbaba Sefid Zarghan	Iran
Rishbaba Siah Bavanat	Iran
Ite Riz Zarghan	Iran
Rishbaba Siah Zarghan	Iran
Yaghoti Zarghan	Iran
Divane Kashmar	Iran
Rotabi Sefid Zarghan	Iran
Alhaghi Siah Abade	Iran
Rishbaba Arsenjan	Iran
Goudovng Pendji Kentsky	Russia
Moukhtchaloni	Russia
Druzhba	Russia
Noulizok	Russia
Volgo Don	Russia
Tuy Tish Gngchinskii	Russia
Flame seedless	USA
Perlet	USA
Beauty seedless	USA

$$PI = \sum_{i} P_i^4 + \sum_{i=1}^{n} \sum_{j>i}^{n} (2P_iP_j)^2$$

$$PIC = 2\sum_{i=1}^{k-1} \sum_{j=i+1}^{k} P_iP_j(1-P_iP_j)$$

where, P_i and P_j are the frequencies of the ith and jth alleles in a given population.

The gene diversity (discriminatory power) of each microsatellite locus was expressed as the expected heterozygosity that was calculated from the sum of squares of allele frequencies, as:

$$He = 1 - \Sigma p_i^2$$

where, P_i is the frequency of allele i in the set of the 43 accessions (Nei, 1973).

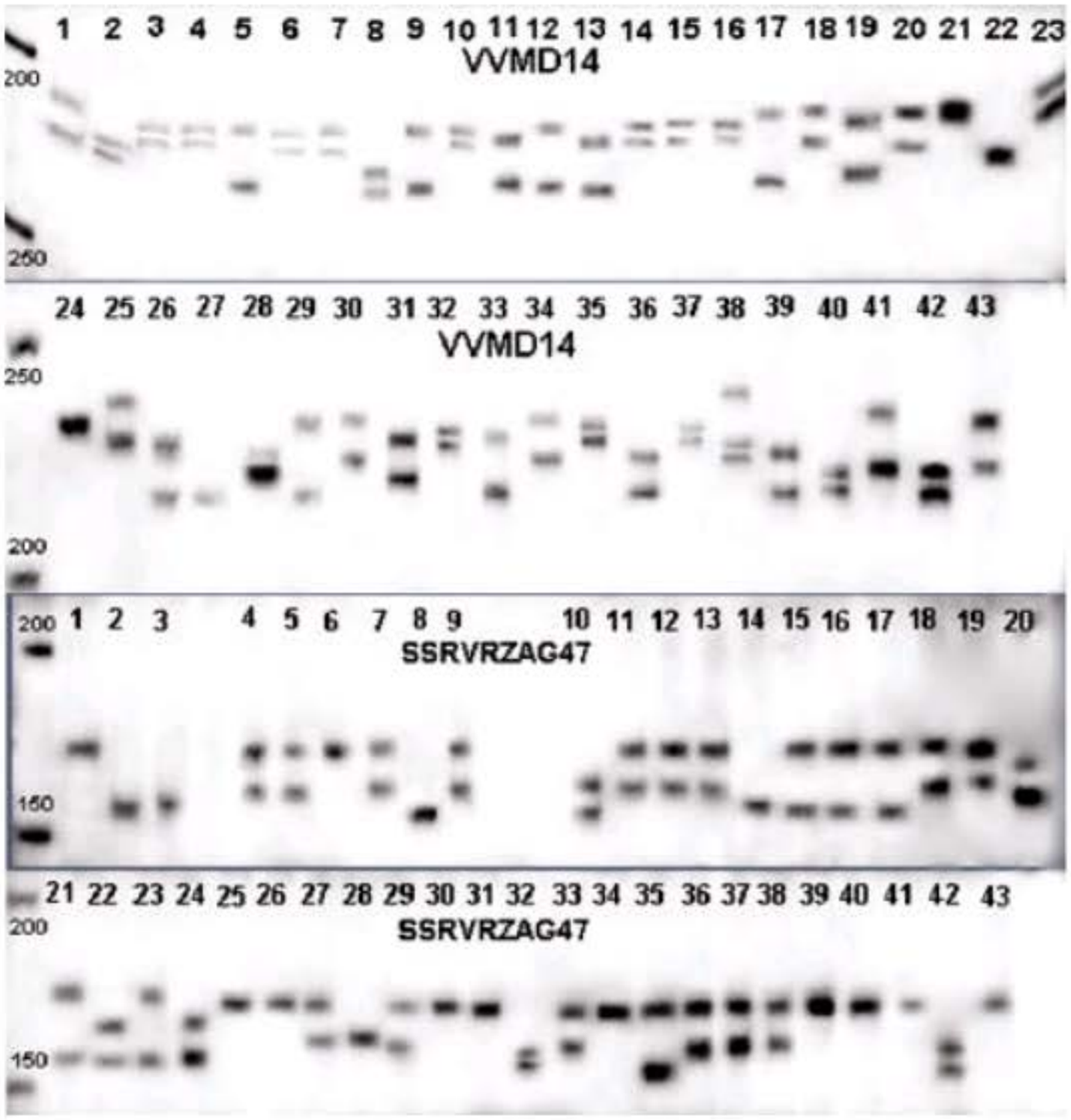

Fig. 1: SSR analysis (VVMD14 and SSRVRZAG47) in 43 grapevine accessions

To evaluate the degree of genetic differentiation among populations, we calculated Nei's (1973) coefficient of gene differentiation (G_{ST}). G_{ST} utilizes information from several loci simultaneously and calculated from allele frequencies rather than genotype frequencies (assuming Hardy-Weinberg equilibrium in all subpopulations). G_{ST} measures the proportional amount of variation within subpopulations as compared with the total population and does not specify the identity of alleles involved (Mohammadi and Prasanna, 2003). From these values, we also derived estimates of gene flow (N_m = The No. of migrants per generation). Shannon's information index (Lewontin, 1972), the percentage of polymorphic loci (PI) and population relationships were also calculated using POPGENE version 1.21 (Yeh *et al*., 1997). Principal Co-Ordinates (PCO) analysis were carried out using the similarity matrices generated with SSR data using NTSYS-PC software (Rohlf, 1998). Population relationships were inferred using the UPGMA clustering method on the basis of Nei's (1978) unbiased genetic distance.

RESULTS AND DISCUSSION

Nine primer pairs including VrZAG21, VrZAG47, VrZAG79, VVMD14, VVMD27, VVMD28, VVMD32, VVMD36 and VVS2 were produced clear bands and were selected among 23 preliminary primer pairs (Fig. 1). All of the nine loci displayed polymorphism among three populations with a total of 75 alleles identified. The number of alleles per locus was ranged from 6 to 11, with an average of 8.3. Polymorphism Information Content (PIC) ranged from 0.65 to 0.88, indicating that these loci were highly informative (Table 2). The expected heterozygosity over all loci was high and ranged from 0.697 (SSRVrZAG47 and VVMD27) to 0.884 (SSrVrZAG21). Locus SSrVrZAG21 that has the most number of alleles, exhibited the greatest level of polymorphism (He = 0.884, I = 0.493 and PIC = 0.876), whereas, loci SSrVrZAG47 and VVMD27 that have only 6 alleles in 43 accessions, exhibited the lowest level of polymorphism (He = 0.701, I = 0.366 and PIC = 0.658). In fact, a positive correlation

Table 2: Characterizations of microsatellite makers used in present study

Locus	NA total	MNA (±SD)	He (1-ΣPi^2)	Ho	I	PIC	PI	G_{st}	NM
SSrVrZAG21	11.0	9.0±1.73	0.884	0.953	0.493	0.876	0.025	0.133	3.259
SSrVrZAG47	6.0	4.0±1.73	0.701	0.721	0.366	0.658	0.136	0.135	3.204
SSrVrZAG79	10.0	7.0±2.64	0.826	0.953	0.401	0.804	0.053	0.273	1.332
VVMD14	8.0	6.0±2.64	0.844	0.884	0.398	0.825	0.430	0.185	2.203
VVMD27	6.0	4.0±1.73	0.701	0.721	0.366	0.658	0.136	0.135	3.204
VVMD28	8.0	6.7±1.53	0.794	0.767	0.359	0.778	0.070	0.056	8.429
VVMD32	8.0	5.3±2.31	0.791	0.860	0.377	0.768	0.071	0.107	4.173
VVMD36	10.0	6.3±2.51	0.807	0.977	0.352	0.783	0.610	0.174	2.374
VVS2	8.0	5.7±2.51	0.807	0.744	0.334	0.783	0.063	0.137	3.150
Mean	8.3	6.0	0.795	0.842	0.383	0.770	0.177	0.217	1.808
SD	1.732	1.548	0.060	0.105	0.046	0.072	0.203	0.059	2.029

NA: No. of Alleles, MNA: Mean No. of Alleles, He: Expected Heterozygosity, Ho: Observed Heterozygosity, I: Shannon's Information Index, PIC: Polymorphism Information Content, PI: Probability of Identity, NM: Gene flow and Nei's (1973) coefficient of gene differentiation G_{ST}

Table 3: Genetic characteristics of grapevine populations based on nine microsatellite loci

Population	N	MNA	He	Ho	I	P (%)	SE
Iran	34	8.11	0.784±0.05	0.834±0.10	0.3650±0.21	97.33	0.0251±0.00060
Russia	6	5.778	0.756±0.11	0.900±0.12	0.3281±0.27	62.67	0.0087±0.00013
USA	3	4.222	0.709±0.08	0.814±0.29	0.2270±0.28	41.33	0
Total	43	6.037±1.957	0.795±0.06	0.842±0.10	0.3871±0.19	100.00	

N: Sample size, MNA: Mean No. of Alleles, He: Expected Heterozygosity, Ho: Observed Heterozygosity Ho, I: Shannon's Information Index, P: Percentage of polymorphic loci SE: Wright's fixation index

was observed between the number of alleles and the level of polymorphism (r = 0.870 between NA and He, r = 0.867 between NA and PIC and r = 0.579 between NA and I). When calculated across all accessions, the PI was low for all loci (PI values from 0.025 to 0.136) except for loci VVMD36 (PI = 0.61) and VVMD14 (PI = 0.43).

Two SSRs loci including SSrVrZAG47 and VVMD27 were found to be probably synonymous, since the polymorphisms observed for both were identically expressed. The fragments at locus VVMD27 were always 21 bp longer than those at locus SSrVrZAG47. Similar results in the assessment of genetic diversity of French grapevine hybrids have been reported (Pollefeys and Bousquet, 2003).

A comparison of allelic data among the grapevine populations revealed relatively similar levels of genetic variation for all populations (Table 3). The Mean No. of Alleles (MNA) per population ranged from 4.22 (USA) to 8.11 (Iran). The lowest number of alleles was detected in the population with the smallest sample size and a positive correlation was observed between the MNA and the sample size of each population (r = 0.949).

Expected heterozygosity (He) and Shannon's information index (I) were used as two useful intrapopulational gene diversity indices. Gene diversities were high in all populations with values ranging from 0.709 to 0.784. The distribution of gene diversities among the populations was similar to that of the allele numbers: there was no significant difference between the populations, the highest values (both He and I) were obtained for the Iranian accessions and the lowest variation was observed in the USA population. USA population exhibited the lowest level of polymorphism (P = 41.33%, He = 0.709 and I = 0.227), whereas Iran population exhibited the greatest level of polymorphism (P = 97.33%, He = 0.784 and I = 0.365). In all populations, mainly Russian grapevines, the mean number (averaged over loci) of heterozygous individuals was higher than expected. In the Russian group, a heterozygosity of 100% was observed at loci SSrVrZAG21, SSrVrZAG79 and VVMD32. There were only heterozygous cultivars in the USA population at loci SSrVrZAG21, SSrVrZAG79 and VVS2. In contrast, USA cultivars display a low level of heterozygosity at SSrVrZAG47 and VVMD27, with an observed heterozygosity of 33%, while the expected percentages were 75 and 63%, respectively. As pointed out by Roa *et al.* (2000), heterozygote deficiency can be explained as a result of different factors: unrecognized genetic structure within populations, inbreeding due to consanguineous mating and presence of null alleles, etc. Non-amplifying or null alleles have been highlighted in the literature from human studies. The occurrence of null (non-amplifying) alleles can be a result of divergence in the sequences flanking the microsatellite and undetectable or unqualified DNA template. These cause heterozygotes to be scored incorrectly as homozygous. In this study, the null alleles were confirmed after several repetitions with different amplification conditions (to ensure that no reaction failure existed).

Coefficient of gene differentiation (G_{ST}), the proportion of total genetic variation due to differences among populations, ranged from 0.056 (VVMD28) to 0.273 (SSrVrZAG79), with an average of 0.217 across all loci, indicating that about 22% of the total microsatellite variation was among populations and most of genetic diversity (78%) occurred within populations. The above

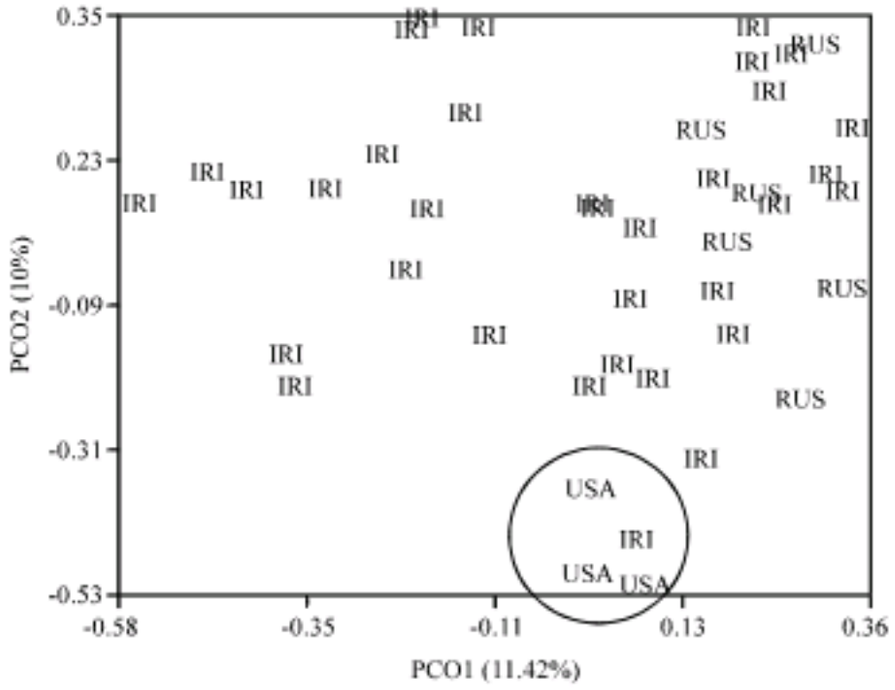

Fig. 2: Principle Co-Ordinate (PCO) analysis of allele frequencies in three populations

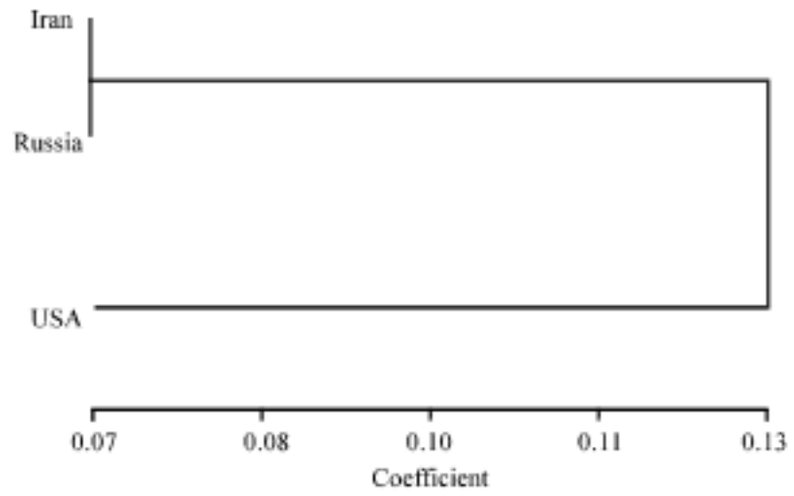

Fig. 3: A UPGMA dendrogram based on Nei's distance showing genetic relationships among the three populations of grapevine

results, together, suggest that there has been significant isolation and restriction of gene flow among *Vitis vinifera* regional populations. It is important to note that these results may be affected by the population sample size as Iran population has 34 accessions whereas Russia and USA has only 6 and 3 accessions, respectively.

Genetic relationships among the 3 populations were also investigated by means of Principal Coordinate Analysis (PCOA; Fig. 2). The first (PCO1) and the second (PCO2) principal coordinates accounted for 11.42% and 10% of the total variation, respectively. Principle coordinate analysis showed that accessions from USA are completely different than Iranian and Russian accessions. One of Iranian accessions is very similar to accessions from USA. A possible explanation is that this Iranian accession may be developed from cultivars introduced from USA or it could be explain by germplasm transferring among these two countries.

A dendrogram was generated by UPGMA based on Nei's genetic distance (Table 3) to show the genetic relationships of the populations studied (Fig. 3). Genetic identity values among pairs of populations ranged from 0.843 to 0.936 (Table 4). The similarity among populations can be seen in the UPGMA dendrogram, where, total populations cluster at a genetic distance below 0.13. The dendrogram was clearly separated into two major clusters, one included USA population and the other cluster consisted of populations from Iran and Russia which clustered together. The pattern of the *Vitis vinifera* accessions clustering is according to their geographic distribution. It suggested that accessions could possibly be assigned to their regions of origin according to their genotypes.

Table 4: Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

Population	Iran	Russia	USA
Iran	***	0.9358	0.9163
Russia	0.0663	***	0.8425
USA	0.0874	0.1713	***

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