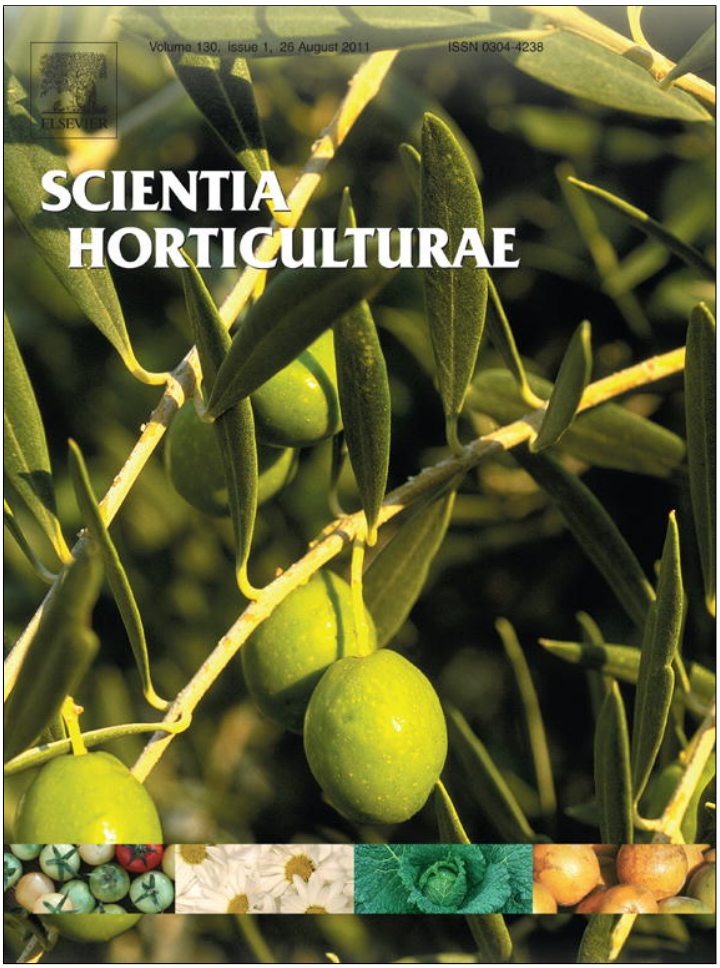

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Analysis of genetic diversity among some Persian walnut genotypes (*Juglans regia* L.) using morphological traits and SSRs markers

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ABSTRACT

There is a high diversity among cultivated walnut trees in Iran due to its long time of seed propagation and vast area of cultivation. In this study some morphological characters as well as Simple Sequence Repeat (SSRs) markers were used to analyze the genetic diversity and relationships among 31 Iranian walnut genotypes along with four foreign cultivars. The nut weight ranged from 7.52 to 17.73 g, kernel weight from 4.00 to 9.83 g, and kernel percentage ratio from 38.78 to 67.05% among studied genotypes. In SSRs analysis, nine primer pairs were tested that produced 39 alleles ranging from 2 to 8, with a mean value of 5.10 allele per primer. The Iranian genotypes showed relatively high diversity both for their SSRs loci and morphological traits. Although the foreign cultivars ('Serr', 'Vina', 'Franquette' and 'Lara') clustered with each other, they also laid close and within the Iranian genotype. The results of the study provided us with valuable diversity among our genotypes which could be used for breeding studies and also showed the power of genetic markers for analysis and evaluation of this diversity.

1. Introduction

Persian walnut (*Juglans regia* L.) is a monoecious tree with a long history of cultivation in Middle East and Europe and currently one of the major nut crops in Iran. The major producers of this species are USA, China, Turkey and Iran, respectively (www.fao.org). This species is naturally distributed from East Turkey through North of Iran, Afghanistan, Pakistan, mountains of Nepal and central Asia (McGranahan et al., 1998). Until recently walnut trees have been mostly seed propagated across the Iran plateau which has resulted in a diverse gene pool of walnut suitable for selection and breeding programs. Characterization of genotypes is a prerequisite for designing of breeding experiments. Morphological characteristics often do not result in a clear diagnosis among accessions due to changes that usually results from the effects of different environmental conditions (Kumar, 1999). Regarding the fact that walnut is highly divergent due to its open-pollination and seed propagated method, utilization of both morphological traits and molecular markers is recommended to enhance the explorations of diversity among genetic resources.

Several types of molecular markers are employed for assessment of genetic diversity and relationships in walnut, including Isozymes (Aleta et al., 1990; Foroni et al., 2001), RFLP (Fjellstrom and Parfitt, 1994), RAPDs (Woeste et al., 1996; Nicese et al., 1998; Fatahi et al., 2010), ISSR (Potter et al., 2002), AFLP (Kafkas et al., 2005; Bayazit et al., 2007) and SSRs (Gerald et al., 2005; Ross-Davis and Woeste, 2008; Pollegioni et al., 2009), among which simple sequence repeats (SSRs) are considered as the one of the most important molecular markers since they are co-dominant, PCR-based, constantly reproducible, widely distributed in genome, and also amenable to semi or full automation (Rafalski et al., 1996). Since Iranian walnuts have been poorly characterized for their diversity, in this study and as a confirmation for our previous report (Fatahi et al., 2010), some morphological and SSR markers were applied to survey genetic relationships among walnuts sampled from different regions of Iran along with some foreign cultivars.

2. Materials and methods

2.1. Plant materials and evaluation

The samples were collected from non-grafted trees with the age of about 25–50 years from five geographical regions of four provinces. During harvest season, 10–15 nuts from each tree were randomly collected and evaluated for nut characteristics according to the walnut international descriptor (Eriksson, 1998), with some minor modifications. Finally 35 superior genotypes including 16 from Fars province (10 from Neyriz and 6 from Bavanat), 7 from Chahar-Mahal province (Shahrekord) and 11 from Tehran province (Karaj), one from Azarbayejane-sharghi province (Mianeh) as well

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Table 1
Genotype code, cultivar/genotype name of walnut and their collection sites.

No.	Genotype/cultivar* code	Collection site and origin
1	CODK4	Shahrekord, Iran
2	CCGM1	Shahrekord, Iran
3	COKH5	Shahrekord, Iran
4	CCEM1	Shahrekord, Iran
5	CCHA2	Shahrekord, Iran
6	COYYH5	Shahrekord, Iran
7	CCGM2	Shahrekord, Iran
8	NFE1	Neyriz, Iran
9	NFE2	Neyriz, Iran
10	NFE3	Neyriz, Iran
11	NFE4	Neyriz, Iran
12	NFE5	Neyriz, Iran
13	NFE6	Neyriz, Iran
14	NFE7	Neyriz, Iran
15	NFE8	Neyriz, Iran
16	NFE9	Neyriz, Iran
17	NFE10	Neyriz, Iran
18	BFE1	Bavanat, Iran
19	BFE2	Bavanat, Iran
20	BFE3	Bavanat, Iran
21	BFE4	Bavanat, Iran
22	BFE5	Bavanat, Iran
23	BFE6	Bavanat, Iran
24	Jahan1	Karaj, Iran
25	Jahan2	Karaj, Iran
26	Serr*	Karaj, USA
27	Vina*	Karaj, USA
28	Z30	Karaj, Iran
29	K72	Karaj, Iran
30	Franquette*	Karaj, France
31	Lara*	Karaj, France
32	Jahad	Karaj, Iran
33	Ziaabad	Karaj, Iran
34	Daneshkade	Karaj, Iran
35	Mianeh	Mianeh, Iran

as four foreign cultivars ('Vina', 'Serr', 'Franquette' and 'Lara') were used as our material in this study (Table 1).

2.2. SSRs analysis

Genomic DNA was extracted from leaves using large-scale CTAB based method of Murray and Thompson (1980). Nine primer pairs introduced by Woeste et al. (2002) were used to amplify genomic DNA (Table 2). Primers were synthesized by MWG, Germany. Amplifications were performed in 25 μl volumes containing 1 unit of *Taq* DNA polymerase, 2.5 μl 10× PCR buffer, 2 mM $MgCl_2$, 100 μM dNTPs (CinnaGene, Iran), 0.4 μM each primer pair and 1 μl (50 ng) template DNA. PCR reactions were carried out using touchdown method programmed as follow: 4 min at 94 °C, afterward 10 cycles of 1 min at 94 °C, 1 min started at 10 °C higher than the recommended annealing temperature for each primer pair and decreased by 1 °C per cycle, and 72 °C for 1 min; followed by 25 cycles at 94 °C for 1 min, 1 min at specific annealing temperature of each primer pair and 1 min at 72 °C; followed by a final extension at 72 °C for 7 min. PCR products were denatured by adding 7.5 μl formamide loading dye (95% deionized formamide, 10 mM EDTA pH: 8, 0.05% xylene cyanol, 0.05% bromophenol blue), heated for 5 min at 94 °C, then 5 μl of denatured preparations were loaded on a pre-warmed (50 °C) 6% polyacrylamide sequencing gel (Bio Rad, Sequi-Gen GT). Gels were run for 1.5–2 h at 90 W and the DNA bands were visualized by silver staining as described by Bassam and Caetano-Anolles (1993). Permanent record of gels were made using gel scanner (Bio Rad, GS800 calibrated densitometer).

2.3. Data analysis

The mean values for each parameters of a given genotype were used to perform statistical analysis of morphological traits. Morphological pair-wise distances of the genotypes were measured using Ward's method (Anderberg, 1973). Data processing was performed by XLSTAT 2010 software (http://www.xlstat.com). The polymorphic SSRs alleles were also scored as presence (1) or absence (0). Cluster analysis was performed using the UPGMA method, based on Dice similarity coefficients (Nei and Li, 1979) using bootstrap analysis with 1000 replications and FREETREE program (ver. 0.9.1.50; Pavlicek et al., 1999). The co-phenetic correlation coefficient was calculated, and Mantel test (Mantel, 1967) was performed to check the goodness of fit for the cluster analysis with the similarity matrix on which it was based. The number of alleles (na) and the number of effective alleles (ne) were determined. Loci information content was estimated by expected heterozygosity using formula $H_e = 1 - \sum p_i^2$ (where p_i is the frequency of the *i*th allele). Discrimination power (DP) was evaluated according to Kloosterman et al. (1993).

3. Results and discussion

3.1. Morphological analysis

Based on the 22 qualitative and quantitative traits, the 35 walnut genotypes were classified into three main clusters (Fig. 1a), reflecting a high morphological variability in the collected genotypes. In the first group, 22 genotypes were clearly distinguished mainly by traits such as high shell diameter, small size of shell and closed shell seals. In the second group, the eight genotypes denominated by their high seed and kernel weight, high shell length, fleshy and filled kernels. Nine genotypes in third group showed low shell thickness with high kernel percentage (Fig. 1a). The mean of nut weight varying 7.52 gr (Ziaabad) to 17.7 gr (NFE9) were over 13 gr in 17 selections, over 15 gr and 17 gr in two selections. On the other hand in terms of kernel weight, all the selection had kernel weights more than 4 gr. Z30 and NFE10 were in first rank (9.8 and 9.3 gr), followed by CCGM1, NFE3 (8.7 gr) and CCGM2, NFE9 (8.3 gr). These kernel weights were higher than 5–6 gr in 8 selections, over 6–8 gr in 16 selections and over 8 gr in 11 selections. Kernel percentage varied 38.8 (COYYH5) to 67 (Z30) respectively. Daneshkade (66.3), COKH5 (60.8) and NFE3 with Jahan1 (60.7) genotypes had the highest kernel percent. In addition, kernel percent were over 50–60% in 22 selections and over 40–50% in 12 selections (Table 3).

3.2. SSRs analysis

Using SSRs primers we investigated the polymorphism among our genotypes. 39 polymorphic alleles were successfully amplified among studied genotypes. Previous studies have shown that they were useful for discriminating the variations within *regia* species (Gerald et al., 2005; Foroni et al., 2005). The number of presumed alleles ranged from 2 to 8, with a mean value of 5.10 alleles per locus. Gerald et al. (2005) applied some of these SSRs markers on different cultivars of *regia* and other walnut species and suggested a high level of diversity. The locus WGA376 had the highest number of effective alleles (6.63) while the locus WGA27 possessed the least (0.8). The observed heterozygosity ranged from 0.48 for WGA27 to 0.86 for WGA202 loci, with an average value of 0.72 for all the studied loci. The expected heterozygosity for individual loci ranged from 0.57 in locus WGA27 to 0.93 in locus WGA202, with an average of 0.80. For all loci, the observed heterozygosity was clearly lower than the expected heterozygosity in all loci. The most informative locus was WGA376, with a DP of 0.77, whereas the least informative

Table 2
Properties of the microsatellite loci used to characterize 35 walnut genotypes.

SSR locus	Primer pair sequences	Allele size (bp)	Tm (°C)
WGA1	ATT GGA AGG GAA GGG AAATG CGC GCA CAT ACG TAA ATC AC	181–195	48
WGA9	CAT CAA AGC AAG CAA TGG G CCA TTG CTC TGT GAT TGG G	236–248	48
WGA27	AAC CCT ACA ACG CCT TGA TG TGC TCA GGC TCC ACT TCC	192–210	51
WGA32	CTC GGT AAG CCA CAC CAA TT ACG GGC AGT GTA TGC ATG TA	120–196	47
WGA71	ACC CGA GAG ATT TCT GGG AT GGA CCC AGC TCC TCT TCT CT	136–210	45
WGA69	TTA GTT AGC AAA CCC ACC CG AGA TGC ACA GAC CAA CCC TC	158–180	45
WGA202	CCC ATC TAC CGT TGC ACT TT GCT GGT GGT TCT ATC ATG GG	238–275	46
WGA276	CTC ACT TTC TCG GCT CTT CC GGT CTT ATG TGG GCA GTC GT	165–190	52
WAG376	GCC CTC AAA GTG ATG AAC GT TCA TCC ATA TTT ACC CCT TTC G	218–254	46

loci were WGA276 and WGA27 which had a DP of 0.65. The average of this parameter for all loci was 0.70 (Table 4). These results are in agreement with those reported by Gerald et al. (2005), Ross-Davis and Woeste (2008) and Pollegioni et al. (2009); suggesting the successful utilization of these markers in different *Juglans* species.

Table 3
Mean values of some important morphological traits of 35 evaluated walnuts.

Genotype	Nut weight (gr)	Kernel weight (gr)	Kernel percentage
CODK4	15.6	8.0	51.2
CCGM1	17.2	8.7	50.4
COKH5	13.5	8.2	60.8
CCEM1	15.6	8.8	56.6
CCHA2	12.4	5.1	41.0
COYYH5	14.9	5.8	38.8
CCGM2	14.0	8.3	59.1
NFE1	13.3	6.5	48.9
NFE2	12.0	6.8	56.5
NFE3	14.4	8.7	60.7
NFE4	14.5	7.5	52.0
NFE5	12.8	6.1	48.0
NFE6	12.8	6.2	48.1
NFE7	11.3	6.8	60.5
NFE8	14.5	7.2	49.7
NFE9	17.7	8.3	46.8
NFE10	14.3	9.3	65.1
BFE1	17.7	8.8	49.6
BFE2	15.5	7.7	50.1
BFE3	15.9	6.9	43.7
BFE4	16.8	8.0	47.7
BFE5	15.3	7.2	47.3
BFE6	15.2	8.4	54.9
Jahan1	13.0	7.9	60.7
Jahan2	10.3	5.7	55.7
Serr	10.2	6.8	66.9
Vina	9.9	4.6	47.0
Z30	14.7	9.8	67.0
K72	15.0	8.3	55.3
Franquette	11.2	5.3	47.3
Lara	12.5	6.6	52.8
Jahad	13.1	7.8	59.5
Ziaabad	7.5	4.0	53.3
Daneshkade	12.8	8.5	66.3
Mianeh	13.1	7.0	53.4

The observed heterozygosities represented by used SSRs markers (the average value of 0.72), were partly similar to those reported previously by Foroni et al. (2005) (0.68, on average), who showed a high level of heterozygosity among different genotypes.

3.3. Better evaluation of genetic relatedness among walnut genotypes by data integration of two molecular markers

The pair-wise comparison of accessions by RAPD marker as previously reported (Fatahi et al., 2010) showed relative genetic similarities between genotypes ranging from 0.23 (between 'NFE3' and 'Vina') to 0.90 (between 'NFE7' and 'NFE8'). In present study on the basis of SSRs marker the similarity among accessions was from 0.18 (between 'BFE3' and 'NFE5') to 0.89 (between 'BFE1' and 'BFE5'). In the study by Gerald et al. (2005) they reported genetic similarity coefficients that ranged from 0.40 to 1.00 among Californian walnut cultivars and other species. Also according to Nicese et al. (1998) 'Franquette' showed 0.55 and 0.42 genetic similarity with 'Vina' and 'Serr' respectively. In our previous report the similarity between 'Vina' and 'Serr' with 'Franquette' cultivars were 0.56 and 0.64, respectively. SSRs results showed lower genetic similarity values compared to the RAPDs marker in our studies. Represented lower genetic similarity by SSRs compared to RAPDs, may be due to the higher resolution of polymorphism detection

Table 4
Walnut SSRs loci used to study 35 walnut genotypes (na: number of alleles, ne: number of effective alleles, Ho: observed heterozygosity, H_e: expected heterozygosity, DP: Discrimination power).

Loci	na	ne	Ho	H_e	DP
WGA376	8	6.63	0.72	0.81	0.77
WGA69	7	5.42	0.79	0.86	0.74
WGA276	5	3.43	0.74	0.84	0.65
WGA71	4	2.40	0.70	0.80	0.72
WGA32	5	3.60	0.78	0.80	0.66
WGA27	2	0.80	0.48	0.57	0.65
WGA202	8	6.41	0.86	0.93	0.74
WGA9	3	1.80	0.65	0.72	0.71
WGA1	4	2.20	0.77	0.85	0.74
Mean	5.1	3.4	0.72	0.80	0.70

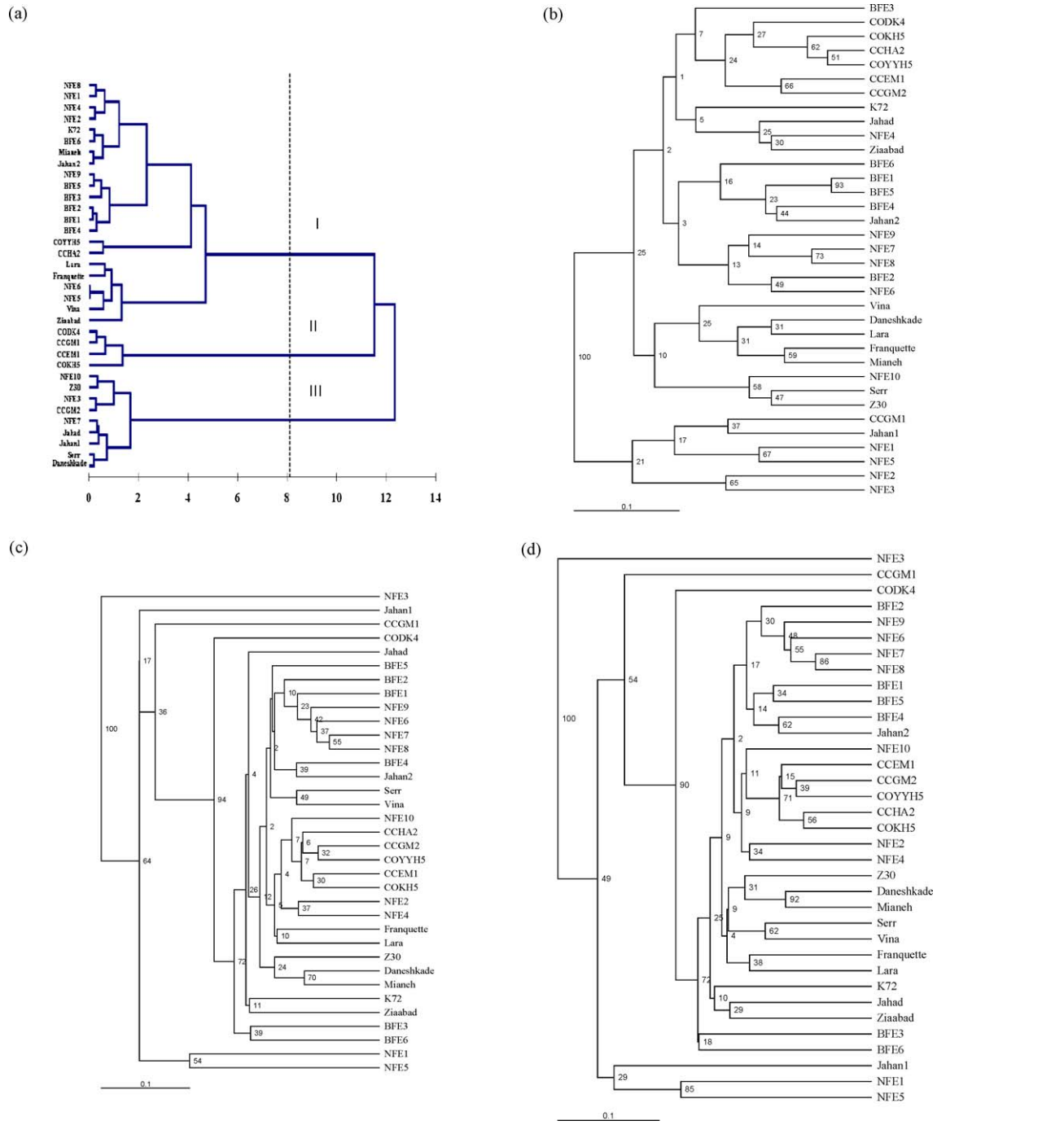

Fig. 1. Cluster analysis using morphological traits (a), SSRs (b), RAPDs (c) and RAPDs + SSRs (d) data of 35 walnut accessions (31 Iranian and 4 foreign cvs), based on Wards and UPGMA methods, bootstrap resampling replicates.

by SSRs which are co-dominant reproducible markers (Staub et al., 2000).

We observed that the genetic similarity coefficients for genotypes collected from the same regions (e.g. Shahrekord) were high (from 0.8 to 1.0) and showed the close relatedness among these genotypes. Bayazit et al. (2007) also reported a high level of similarity among studied genotypes from geographic region in Turkey, suggesting that they have been probably derived from common ancestors. In present study, most of our genotypes were collected from the superior walnut populations from the same geographic regions in Shahrekord and because of that we observed almost similar genetic background among our genotypes.

3.4. Clustering of genotypes

The SSRs data were used to produce the dendrogram of 35 walnut genotypes. In the dendrogram six sub-groups were observed (Fig. 1b). The first sub-group in SSRs markers included six genotype from Shahrekord ('CODK4', 'COKH5', 'CCHA2', 'COYYH5', 'CCEM1', 'CCGM2') and a genotype from Bavanat ('BEF3'). The second sub-group included three genotypes from Karaj ('Ziaabad', 'K72' and 'Jahad') and a genotype from Neyriz ('NFE4'). The third sub-group included four genotypes from Bavanat ('BFE1', 'BFE4', 'BFE5' and 'BFE6') and one genotype ('Jahan2') form Karaj. The forth sub-group consisted of a genotype sampled from Bavanat and four genotypes from Neyriz ('NFE6', 'NFE7', 'NFE8' and 'NFE9'). The fifth sub-group

comprised of four genotypes from different regions of Iran ('Z30', 'Mianeh', 'Daneshkade' and 'NFE10') plus four cultivars that originated from France and USA. The last sub-group included four genotypes from Neyriz ('NFE1', 'NFE5', 'NFE2' and 'NFE3') with two genotypes from Shahrekord ('CCGM1') and Karaj ('Jahan1'). The Neyriz genotypes were nested as discernible groups within the denderograms produced in each marker system. Thus, this region can be considered as a useful and special source of walnut genetic diversity for further breeding studies.

With some minor exceptions, the clustering of genotypes by RAPDs markers in the previous study (Fatahi et al., 2010) and SSRs markers in this study showed similar dendrogram. Furthermore both marker systems were found highly effective in discriminating genotypes (Fig. 1b and c). According to the genetic similarity values from both markers (RAPDs and SSRs), five genotypes namely 'CCGM2', 'COYYH5', 'CCHA2', 'CCGM1' and 'COKH5' from Shahrekord province were closely related and thus grouped in one branch in the cluster. However, the 'CODK4' genotype by having some distinct morphological attributes separated from the other genotypes of this province. Integration of morphological and molecular data can be helpful in developing the breeding programs to more efficiently select the candidate genotypes as parents. Genotypes from different regions (such as 'NFE8', 'Franquette', 'BFE4' and 'Jahan2') grouped together indicating a low correlation between molecular grouping and their geographical origins. Dhanaraj et al. (2002) also reported a similar observation in assessment the diversity of Indian cashew collected from regions using RAPDs markers. This might be due to possible germplasm exchange between different places over the time. Dendrogram for pooled data from both SSRs and RAPDs markers sets in Fig. 1d was very similar to that from the RAPDs markers with an exception of 'BFE1' genotype separated from 'BFE2' and 'NFE9'. The two genotypes ('NFE3' and 'Jahan1') regularly clustered separately from all other genotypes using RAPDs and SSRs or pooled data from both markers. The 'NFE1', 'NFE5' and 'CODK4' genotypes clustered with the majority of genotypes in molecular dendrograms.

The association of Iranian genotypes with the foreign cultivars in both marker systems implied the possibility of gene flow or plant material exchange between countries. This also can be due to the share of some common ancestors. Foroni et al. (2005) reported a relatively high similarity between 'Sorronto' and two other cultivars ('Serr' and 'Hartly'). Therefore, they suggested that the 'Sorronto' might have originated from crosses between 'Sorrento', 'Serr' and 'Hartly' respectively. The 'Vina' and 'Serr', two walnut cultivars from USA, showed 48% and 72% genetic similarity by RAPDs and SSRs markers, respectively. The other report by Nicese et al. (1998) showed that 'PI-159568' from Iran plateau and 'Serr' from California grouped closely. McGranahan et al. (1998) and Gerald et al. (2005) confirmed that 'Serr' is a hybrid derived from the cross between 'Payne' and 'PI-159568'. These cultivars have been traditionally grown in California over the past decades. On the basis of a report by Foroni et al. (2005) 'Serr' clustered far from 'Sorronto' genotype from Italy. In the molecular study of Nicese et al. (1998) these two cultivars nested in the same group. Similarly, in present study, 'Franquette' and 'Lara' by showing a high similarity (RAPDs, 0.77 and SSRs, 0.67) were placed in the same group in both marker systems. The relatively high similarities observed between foreign cultivars and Iranian genotypes supported the hypothesis that Iran to be the central place for walnut and then it moved to other areas toward China, Russia and southern European districts (Bayazit et al., 2007).

3.5. Comparison between morphological and molecular markers

Although not very high, the correlation coefficients was statistically significant between similarity matrix of RAPDs and SSRs ($r = 0.58$). The co-phenetic correlation coefficient between the dendrogram and the original similarity matrix for RAPDs and SSRs were significant and relatively high ($r = 0.94$ and 0.87, respectively). The co-phenetic correlation between the denderogram and the original similarity matrix of pooled RAPDs and SSRs data was also significant ($r = 0.92$). The correlation between morphological similarity matrix and molecular similarity matrix (RAPDs = 0.32 and SSRs = 0.18) were not significant. A lack of correlation was also observed earlier between genetic similarities based on SSRs and morphological traits in wheat (Roy et al., 2004). Low correlation coefficient between RAPDs markers and morphological similarity matrix were also reported in pomegranate (Zamani et al., 2007, 2010). These similar results from different fruit crops support the idea that morphological traits cannot be accurate and precise for estimating genetic relationships among different cultivars. This discrepancy can be attributed to the effects of environmental conditions on morphological characteristics. This lack of fit is more pronounced when the recorded characters are restricted to a specific organ of the plant (e.g. only fruit characters).

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