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Characterization of Some Iranians and Foreign Walnut Genotypes Using Morphological Traits and RAPD Markers

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Abstract. Walnut, (*Juglans regia*), is one of the most important nutritive nut crops. Iran is considered as one of the centers for diversity and cultivation of walnut in middle-eastern part of the world. In this study, some morphological traits in combination with polymorphic RAPD primers were applied to evaluate genetic potential of 35 (31 Iranian and four foreign) walnut genotypes. From 14 RAPD primers, 180 DNA bands were amplified among which 174 were highly polymorphic. The lowest and the highest pair-wise similarity coefficients between examined genotypes were 0.27 and 0.89, respectively. Estimated resolving power index (Rp) were from 2.17 (opAD16) to 8.40 (opAA19) and total resolving power (Rp) 70.61 in all 14 RAPD loci. Cluster analysis based on Jaccard's similarity coefficients and UPGMA method divided the genotypes into five groups at similarity of 0.50, which the main group consisted of 31 genotypes including 4 foreign cultivars. Genetic variation among locations took 9.48% of the total genetic variation detected with Analysis of molecular variances (AMOVA) ($p<0.001$). Most Iranian genotypes grouped closely with foreign cultivars and showed high similarities indicating their close genetic relatedness. RAPD markers showed to be an efficient technique for studying genetic diversity of walnut genotypes. These genotypes with different genetic bases and suitable horticultural traits seem to be good candidates applicable for direct use as vegetatively propagated material or as parents in walnut improvement programs.

***Additional key words*:** cluster analysis, genetic diversity, *Juglans regia*

Introduction

Walnut, *Juglans regia*, is a self-fertile plant but, with dichogamous (protander or protogyne) nature in pollination system, is usually considered as an outcross species (Britton, et al., 2007). J. regia, which belongs to the genus *Juglans* including 21 species (Manning, 1978), is the most commonly cultivated species in Iran, Central Asia and Europe. Iran with a yearly production of almost 165,000 tons of walnuts, ranks as the third producer country following China and USA (FAO, 2007). Since walnut trees have been propagated using seeds for many centuries across the Iran plateau, an unambiguous opportunity is provided for growers and breeders to select promising genotypes useful for asexually propagation as well as for choosing parents in walnut breeding programs. Sexual propagation has a disadvantage of giving no uniform trees and nuts that are undesirable for orchard management as well as nut grading and marketing. Grafting of walnut seedlings dates back to 1985 for plantations new uniform walnut orchards in Iran (Atefi and Mostafavi, 1985). For this purpose, new selections such as Z30, Z60, K72, and G3 were introduced to be used in Karaj county, Tehran province (Atefi, 2001). Afterwards, Eskandari et al. (2005) also selected some genotypes according to yield and nut characteristics from natural populations in different provinces. Now, all these selections are in use to produce grafted walnut plants in some nurseries. The asexually propagated walnut trees are supported by government for production of uniform marketable nuts. The main disadvantage is the risk of facing drift of genetic resources or erosion problems over time. Evaluation of walnut genotypes and their pomological characteristics will be very useful direct for denomination, collection, preservation and utilization or cross-breeding programs of walnut. However, morpho-pomological characteristics often do not result in a clear diagnostic test between genotypes and accessions due to ambiguous measurements or phenotypic changes caused by the different environmental conditions (Kumar, 1999). Morphological traits and RAPD markers in genotyping have their advantages and drawbacks but their combined utilization is recommended to enhance the resolving power of examined diversity among genetic resources. Many different marker systems including morphological, biochemical and DNA markers have been applied on walnut. Isozymes were used to characterize walnut species and cultivars to detect inter and intera-species relationships (Aleta et al., 1990; Froni et al., 2001). RFLP markers were also used to determine the presence of apomicticness, phylogenic relationships, and assessment of genetic diversity among walnuts cultivars (Fjellstrom and Parfitt, 1994; Fjellstrom et al., 1994). With development of PCR-RAPD technique, it has been employed on walnut to find markers closely linked to hypersensitivity to cherry leaf-roll virus disease (Woeste et al., 1996b), to identify interspecific hybrids (Malvolti et al., 1997), to assess genetic relationships among the genotypes (Nicese et al., 1998), and to construct genetic linkage map (Woeste et

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al., 1996a). The RAPD markers have been used in other horticultural crops such as pomegranate (Zarei et al., 2009) olive (Hagidimtriou et al., 2005). Inter simple sequence repeats (ISSRs) were also used to characterize Californian walnut germplasm (Potter et al., 2002). Amplified fragment length polymorphism (AFLP) technique was applied to analyze diversity in walnut genotype (Bayazit, et al., 2007; Kafkas et al., 2005; Vos et al., 1995). Fourteen microsattelite loci were successfully used on walnut collection at the University of California (Dangel et al., 2005). The present study was carried out to precisely assess the genetic diversity of some potentially important walnut genotypes located in different regions of Iran by using morphological characters and also RAPD markers.

Materials and Methods

Plant materials and characters evaluation

Thirty one walnut genotypes from different production areas of Iran as well as four introduced cultivars ('Serr', 'Vina', 'Franquette', and 'Lara') were used as plant materials in this study (Table 1). Assessments of nut characteristics were carried out on three samples as replications, each 10-15 nuts and totally 30-45 nuts per tree. Twenty-two quantitative and qualitative characteristics were recorded among genotypes according to walnut international descriptor (Eriksson, 1998) with some minor modifications. The mean values calculated for each parameter per genotype were used to perform factor analysis and clustering of the genotypes. Morphological pair-wise distances of genotypes were measured using Ward's method (Anderberg, 1973). Data processing was performed using XLSTAT, 2008 software.

DNA extraction

Leaf samples were collected from genotypes in the beginning stage of growth, washed three times in sterile distilled water, frozen in liquid nitrogen and kept at -20℃ until used. Genomic DNA was extracted from leaves by large scale CTAB based method of Murry and Thompson (1998). The purity and quantity of the extracted genomic DNA were determined spectrophotometrically and confirmed by electrophoresis in 0.8% (w/v) agarose gel compared to known concentrations of lambda DNA.

Application of RAPD markers

Fourteen Operon 10-mer primers (Operon Technologies, Alameda, CA, USA), including some of which were previously reported to be polymorphic in walnut (Nicese et al., 1998), were used in this study. Polymerase chain reactions were carried out in 25 µl of final volume each containing 10 ng template DNA, 2.5 µl 10× PCR buffer, 1.75 mM $MgCl_2$, 200 µM each dNTPs, 0.2 µM 10-mer primer, and 1 Unit of *Taq* DNA polymerase (CinnaGen, Tehran, Iran). Amplification reactions were performed on a thermocycler (Icycler, Bio Rad, Hercules, CA, USA) programmed as follows: 94℃ for 4 min, followed by 5 cycles of 92℃ for 1 min, 39℃ for 1 min, 72℃ for 2 min and 30 cycles of 92℃ for 1 min, 37℃ for 1 min, 72℃ for 2 min and a final extension at 72℃ for 5 min. PCR-amplified products were separated by electrophoresis on 1.5% (w/v) agarose gels in 1X Tris-Borate-EDTA (TBE) buffer (pH:8.0), stained by 5 $\mu g \cdot L^{-1}$ ethidium bromide, destained in distilled water and photographed under UV light with a Gel Doc system (UVP, Bio Doc, Upland, CA, USA). A 100-bp

Table 1. Code, cultivar, and genotype, and collection site of walnuts used in this study.

No.	Code/Genotype/Cultivar	Collection site
1	CODK4	Shahrekord
2	CCGM1	Shahrekord
3	COKH5	Shahrekord
4	CCEM1	Shahrekord
5	CCHA2	Shahrekord
6	COYK5	Shahrekord
7	CCGM2	Shahrekord
8	NFE1	Neyriz
9	NFE2	Neyriz
10	NFE3	Neyriz
11	NFE4	Neyriz
12	NFE5	Neyriz
13	N FE6	Neyriz
14	NFE7	Neyriz
15	NFE8	Neyriz
16	NFE9	Neyriz
17	NFE10	Neyriz
18	BFE1	Bavanat
19	BFE2	Bavanat
20	BFE3	Bavanat
21	BFE4	Bavanat
22	BFE5	Bavanat
23	BFE6	Bavanat
24	JAHAN1	Karaj
25	JAHAN2	Karaj
26	SERR	Karaj
27	VINA	Karaj
28	Z30	Karaj
29	K72	Karaj
30	FRANQUETTE	Karaj
31	LARA	Karaj
32	JAHAD	Karaj
33	ZIAABAD	Karaj
34	DANESHKADEH	Karaj
35	MIANEH	Mianeh

DNA ladder was used as molecular-weight size marker in side lanes of each gel. All PCR reactions were repeated and only bright, repeatable and polymorphic bands with high resolution were scored for presence (1) or absence (0). Resolving power index (Rp=$\sum I_b$, where I_b=1-(2×| 0.5-p |) and p= the proportion of present (1) band among genotypes) were measured according to Prevost and Wilkinson (1999). Then, 0/1 data matrix was analyzed by NTSYS software (version 2.1) to estimate genotypes pair-wise Jaccard's similarity coefficients (Rohlf, 2000). The matrix of similarities was used to generate a dendrogram by the unweighted pair-group method with arithmetic average (*UPGMA*) and robustness of the clustering pattern was tested using 1000 resampling permutations using bootstrap software. The *COPH* module was applied to compute the co-phenetic matrix. Then, the *MXCOMP* subroutine was used to calculate a co-phenetic correlation coefficient (r) between the co-phenetic matrix and original matrix of similarity to measure their goodness-of-fit.

Analysis of molecular variances (AMOVA) (Excoffier et al., 1992) was used to describe populations (different sampling locations) structure where the variance was divided between and within locations of sampling walnuts. AMOVA was performed with the AMOVA-PREP v. 1.01 (Miller, 1998) and WINAMOVA v.1.55 (Excoffier, 1993; Excoffier et al., 1992). To examine patterns of genetic differentiation among locations, Nei's genetic distance (Nei, 1978) of provinces was calculated using TFPGA, ver. 1.3 (Tools for Population Genetic Analyses) (Miller, 1997) among all possible pairs.

Result and Discussion

Fruit characteristics

Mean values of the morphological characters recorded among genotypes are presented in Table 2. Descriptive analysis of each morpho-pomological trait including mean value, maximum, minimum, range, and diversity index among genotypes

Table 2. Mean values of some important morphological traits of 35 evaluated walnuts.

No	Genotype	Nut shape (code:1-9)	Nut diameter (mm)	Nut length (mm)	Shell hardiness (code:1-7)	Shell seal (code:1-9)	Removal kernel (code:1-5)	Nut weight (g)	Kernel weight (g)	Kernel color (code:1-4)	Kernel percentage
1	CODK4	8.0	35.5	48.6	5.0	5.0	3.0	15.6	8.0	3.0	51.2
2	CCGM1	3.0	38.2	44.8	5.0	3.0	3.0	17.2	8.7	1.0	50.4
3	COKH5	8.0	33.4	50.8	1.0	5.0	1.0	13.5	8.2	1.0	60.8
4	CCEM1	5.0	35.8	41.8	5.0	3.0	1.0	15.6	8.8	1.0	56.6
5	CCHA2	1.0	36.5	40.8	5.0	5.0	1.0	12.4	5.1	3.0	40.9
6	COYYH5	7.0	33.3	39.0	7.0	3.0	7.0	14.9	5.8	3.0	38.8
7	CCGM2	5.0	37.0	41.3	1.0	5.0	1.0	14.0	8.3	1.0	59.1
8	NFE1	6.0	33.3	41.8	3.0	5.0	1.0	13.3	6.5	1.0	48.9
9	NFE2	1.0	33.8	37.8	3.0	1.0	1.0	12.0	6.8	3.0	56.5
10	NFE3	4.0	36.0	42.3	3.0	5.0	1.0	14.4	8.7	1.0	60.7
11	NFE4	6.0	36.3	38.3	3.0	3.0	1.0	14.5	7.5	3.0	52.0
12	NFE5	6.0	33.7	38.3	5.0	3.0	4.0	12.8	6.1	4.0	48.0
13	NFE6	5.0	34.3	37.8	5.0	3.0	3.0	12.8	6.2	4.0	48.1
14	NFE7	1.0	31.7	36.3	3.0	3.0	1.0	11.3	6.8	3.0	60.5
15	NFE8	6.0	37.3	44.3	3.0	1.0	1.0	14.5	7.2	3.0	49.7
16	NFE9	5.0	38.0	39.0	5.0	7.0	1.0	17.7	8.3	2.0	46.8
17	NFE10	6.0	35.3	40.3	3.0	1.0	5.0	14.3	9.3	3.0	65.1
18	BFE1	2.0	37.4	42.8	5.0	7.0	3.0	17.7	8.8	3.0	49.6
19	BFE2	2.0	38.8	44.6	5.0	7.0	3.0	15.5	7.7	2.0	50.1
20	BFE3	1.0	41.8	39.0	3.0	3.0	3.0	15.9	6.9	3.0	43.7
21	BFE4	1.0	40.8	44.9	3.0	9.0	3.0	16.8	8.0	3.0	47.7
22	BFE5	5.0	40.3	39.5	7.0	9.0	3.0	15.3	7.2	1.0	47.3
23	BFE6	6.0	39.6	41.9	5.0	7.0	3.0	15.2	8.4	1.0	54.9
24	JAHAN1	5.0	34.0	33.0	5.0	7.0	3.0	13.0	7.9	2.0	60.7
25	JAHAN2	6.0	35.0	39.0	3.0	7.0	3.0	10.3	5.7	4.0	55.7
26	SERR	4.0	32.5	36.7	3.0	5.0	3.0	10.2	6.8	4.0	66.9
27	VINA	7.0	31.7	37.2	3.0	3.0	5.0	9.9	4.6	1.0	47.0
28	Z30	5.0	36.2	36.8	3.0	5.0	3.0	14.7	9.8	2.0	67.0
29	K72	6.0	36.3	38.7	5.0	7.0	3.0	15.0	8.3	3.0	55.2
30	FRANQUETTE	1.0	34.0	34.0	5.0	7.0	5.0	11.2	5.3	1.0	47.3
31	LARA	5.0	36.0	33.3	7.0	7.0	5.0	12.5	6.6	4.0	52.4
32	JAHAD	1.0	35.5	36.1	3.0	1.0	5.0	13.1	7.8	2.0	59.8
33	ZIAABAD	1.0	30.5	31.3	3.0	3.0	3.0	7.5	4.0	1.0	53.5
34	DANESHKADEH	4.0	34.0	36.0	3.0	5.0	3.0	12.8	8.5	2.0	66.3
35	MIANEH	1.0	37.4	40.2	5.0	9.0	3.0	13.1	7.0	3.0	54.0

Table 3. Minimum, maximum, mean values, standard deviation and diversity index of twenty two traits observed in 35 walnuts. [Diversity index=(Standard deviation/Mean)×100].

Traits	Min.	Max.	Mean	Std. deviation	Diversity index
Fill kernel	4.33	7.00	5.61	0.88	15.76
Fleshy kernel	3.67	7.00	5.79	1.04	17.92
Kernel color	1.00	4.00	2.31	1.16	-
Kernel diameter	24.50	49.80	31.81	6.01	18.89
Kernel length	24.50	37.27	31.88	2.79	8.74
Kernel percentage	38.78	67.05	53.52	7.23	13.51
Kernel shrivel	1.00	3.00	0.20	0.58	-
Kernel spot	1.00	3.00	0.88	1.10	-
Kernel taste	1.00	3.00	1.07	0.34	-
Kernel veins	1.00	7.00	2.81	1.80	63.99
Kernel weight	4.02	9.83	7.30	1.37	18.71
Kernel removal	1.00	7.00	2.84	1.50	52.77
Seed weight	7.52	17.73	13.72	2.29	16.73
Seedshape	1.00	8.00	4.20	2.30	55.00
Shell color	1.00	7.00	4.45	1.31	29.37
Shell diameter	30.50	41.80	35.75	2.65	7.40
Shell length	31.33	50.80	39.67	4.20	10.58
Shell seal	1.00	9.00	4.81	2.30	47.77
Shell strength	1.00	7.00	4.18	1.50	35.88
Shell texture	1.00	7.00	4.66	1.42	30.56
Shell thickness	0.06	0.26	0.14	0.05	34.04
Tegument thickness	1.00	6.33	3.30	1.32	39.95

showed a relative high degree of variation (Table 3). Do to the high diversity in the measure traits, it is more probable that we can use those from the feuture breeding programs in order to obtaining superior genotype. The shape of seed showed the highest index of diversity (55%). The kernel percentage varied from 38.78 to 67.05%. The nut weight was recorded as between 7.52 and 17.72 g and kernel weight from 4.20 to 9.83 g. The mean values for nut and kernel weight were standard as were for those of length, width and thickness. Genotypes for morphological data were clustered according to Ward's method over standardized data (Anderberg, 1973). It tends to identify three main groups in which cluster *I* had 22 entries, cluster *II* four entries and cluster *III* 9 entries. Cluster *I* had lowest values for kernel weight and kernel percentage and on the contrary, presented highest value for shell length. Cluster *II* showed entries with highest weight of nuts and kernels and the highest percent of kernel was found in cluster *III*. All three clusters *I*, *II* and *III* showed intermediate mean values for several traits (Table 4).

Factor analysis was used based on principle components to provide a reduced dimension model indicating differences measured among groups. The first PCA represented a total variance of 23.30% while the second and third PCs contributed each to total variances of 13.72 and 11.40%, respectively. The loading factors for traits in PC_1 showed that seed weight (0.655), shell length (0.849), kernel length (0.788) and kernel diameter (0.799) were major discriminators. The second PC variant was dominated by shell thickness (0.816), shell strength (0.768) and kernel percentage (-0.88). The third variant (PC_3) revealed that shell texture (0.771), kernel (0.552) and shell color (0.874) had the highest loading factors (Table 5). These results are in agreement with the results reported by Arzani et al. (2008)

RAPD analysis

A total of 180 scoreable and reproducible RAPD bands were generated by applying 14 decamer primers across the 31 Iranian genotypes and four foreign cultivars of *J. regia*, of which 95% were polymorphic. The band sizes ranged from 300 to 3000 bp. Figure 1 shows the RAPD profile of individuals amplified with primer opAA18. Number of bands produced per primer was between 8 (opF12) to 16 (opF14 and opK04) with an average of 12.8 (Table 6) that was higher than the 3.9 polymorphic bands generated by ISSRs markers (Potter et al., 2002) reported on walnuts. Nicese et al. (1998) by using 18 primers on walnut genotypes observed only 23 polymorphic fragments with an average of 1.3 bands per primer. Kafkas et al. (2005) by using six primer combinations of AFLP and two SAMPLs observed 230 DNA fragments with an average of 14.5 polymorphic segments per primer combination. The walnut genotypes characterized in this study

Table 4. Cluster means for 22 quantitative and qualitative traits estimated in 3 morphological sub-clusters.

	Cluster		
Trait	I	II	III
Fill kernel	5.306	6.168	6.111
Fleshy kernel	5.500	7.000	5.952
Kernel color	2.517	1.500	2.149
Kernel diameter	30.174	45.510	29.721
Kernel length	31.520	34.723	31.502
Kernel percentage	49.452	54.760	62.897
Kernel shrivel	0.144	0.000	0.412
Kernel spot	0.866	1.500	0.619
Kernel taste	1.109	1.000	1.000
Kernel veins	3.109	3.000	2.000
Kernel weight	6.728	8.418	8.218
Kernel removal	2.988	2.000	2.852
Seed weight	13.661	15.463	13.073
Seed shape	5.000	4.000	6.000
Shell color	4.527	4.165	4.407
Shell diameter	36.192	35.738	34.681
Shell lenghth	39.254	46.510	37.661
Shell seal	5.221	4.168	4.100
Shell strength	4.664	4.000	3.074
Shell texture	4.721	4.500	4.566
Shell thickness	0.155	0.155	0.104
Tegument thickness	3.445	2.500	3.297

Table 5. Eigen values, variance, cumulative variance for three first factors and loading factor in 22 morphological traits.

Factors	1	2	3
Eigen value	**5.01**	**3.1**	**2.5**
Variance (%)	**23.61**	**13.72**	**11.40**
Cumulative variance (%)	**23.61**	**37.33**	**48.73**
Traits		Factor loading value	
Fill kernel	0.416	-0.391	-6.15E-02
Fleshy kernel	**0.528**	-0.255	4.29E-02
Kernel color	-0.113	0.246	**0.552**
Kernel diameter	**0.799**	5.46E-02	-0.13
Kernel length	**0.788**	-0.104	0.145
Kernel percentage	-0.155	-0.88	2.69E-02
Kernel shrivel	**-0.538**	-0.125	-9.59E-02
Kernel spot	9.84E-02	-7.41E-02	-0.156
Kernel taste	4.74E-02	5.35E-02	0.25
Kernel viens	-2.40E-03	0.217	0.317
Kernel weight	0.479	-0.48	0.335
Removal kernel	-0.166	0.232	-6.32E-02
Seed weight	**0.655**	0.169	0.377
Shell color	0.165	-3.05E-02	0.874
Shell diameter	0.433	0.139	0.377
Shell length	**0.849**	-1.73E-03	5.22E-02
Shell seal	-3.37E-02	0.154	-6.35E-02
Shell shape	0.163	0.118	5.27E-02
Shell strength	-0.134	**0.768**	-3.35E-02
Shell texture	1.94E-02	-0.111	**0.771**
Shell thickness	4.92E-02	**0.816**	8.18E-02
Tegument thickness	-0.283	0.156	0.142

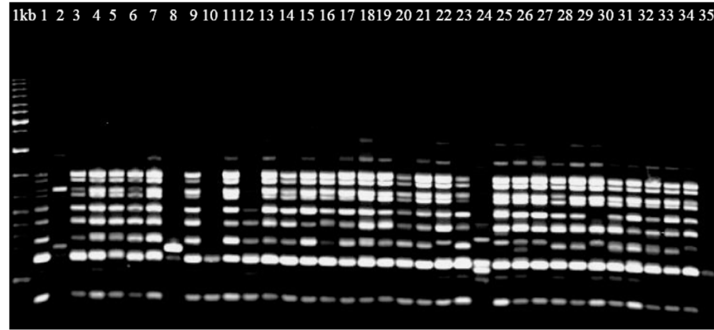

Fig. 1. RAPD profile of 35 *Juglans regia* accessions amplified by opA18. The numbers represent different walnut accessions according to Table 1.

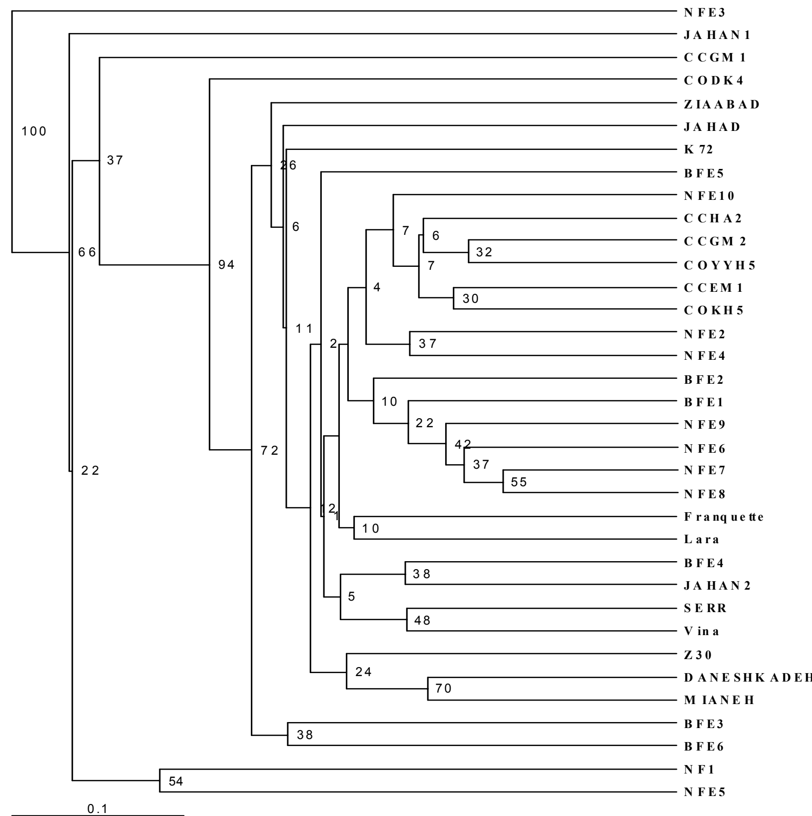

Fig. 2. Cluster analysis of 35 walnut genotypes (31 Iranian and 4 introduced cultivars) based on similarities, UPGMA method and bootstrap resampling replicates.

were mainly sampled from different provinces of Iran as well as few cultivars introduced from California and France. Nevertheless, genotypes selected in this study, having many promising and suitable horticultural traits, showed high divergence in RAPD loci. Markers with high value of resolving power (Rp) are more efficient and useful both in terms of producing more polymorphic bands and also having more equal proportion of present/absent bands among genotypes. The Rp of the primers (Table 6) used in this study ranged between 2.17 (opAD16) and 8.4 (opAA19), with an average of 5.04 and a total Rp of 70.6. Kafkas et al. (2005) reported a Rp equal to 96 for AFLP and 29.8 for SAMPLs by using six combinations of AFLP primers and two SAMPLs.

Genetic relatedness among walnut genotypes

The Jaccard's pair-wise genetic similarities (GS) were calculated between genotypes and cultivars. The lowest GS (0.27) was between 'NFE3' and 'Vina' and the highest value (0.87) between genotypes 'NFE8' and 'NFE9'. According to GS values, five genotypes namely 'CCGM2', 'COYK5', 'CCHA2', 'CCGM1', and 'COKH5' were closely related. A high co-phenetic correlation coefficient (r=0.94) measured between the similarity matrix and co-phenetic matrix originated from dendrogram indicated a good fit. According to the dendrogram (Fig. 2), at

Table 6. List and the results of polymorphic RAPD primers used on 35 walnut genotypes.

Primer	Sequence (5'-3')	Total bands (a)	Polymorphism band (b)	Polymorphic% (b/a ×100)	Rp [z]
opAC11	CCTGGGTCAG	10	7	70	2.74
opAD16	AACGGGCGTC	9	9	100	2.17
opAE10	CTGAAGCGCA	8	6	75	2.17
opA18	AGGTGACCGT	15	15	100	4.51
opAA19	TGAGGCGTGT	13	13	100	8.4
opD15	GTGTGCCCCA	10	9	90	3.54
opF14	TGCTGCAGGT	16	16	100	4.69
opF12	ACGGTACCAG	8	7	87.5	4.29
opK04	CCGCCCAAAC	16	16	100	8
opK19	CACAGGCGGA	15	15	100	6.45
opMG16	GAAGAACCGC	11	11	100	5.6
opMG01	AGCGCCGACG	14	14	100	7.71
opZ10	CCGACAAACC	15	14	93	5.49
opZ6	GTGCCGTTCA	12	12	100	4.4
Total	-	180	174	-	70.61

[z] Resolving power was calculated by $Rp = \sum I_b$, $I_b = 1-(2\times|0.5-p|)$.

Table 7. Summary of analysis of molecular variances (AMOVA) for *Juglans regia* 35 accessions from four Iranian locations with 4 introduce foreign samples.

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance component	% of total variance	Probability of significances
Among locations	4	176.68	44.171	2.69	9.48	< 0.001
Within locations	34	771.371	25.712	25.712	90.52	< 0.001

similarity level of 50%, the genotypes were divided into five main clusters. Each genotype of 'NFE3', 'NFE1', 'NFE5', 'CCGM1', and 'JAHAN1' was grouped individually, and the remaining thirty genotypes located in one group. 'NFE3' genotype, a seedling selection from Neyriz of Fars province had some interesting characteristics including late leafing date, lateral fruiting habit, high yielding, high weight and percentage of kernel, and bright kernels. 'CCGM1', a genotype from Shahrekord province was grouped alone and located far from other genotypes of this province. The results of morphological data showed that both 'NFE3' and 'CCGM1' had nut weight of more than 13 g., white kernels, and 60% kernel. The Neyriz genotypes were also placed in different groups. The major cluster contained 30 genotypes; the most related ones were between NFE8 and NFE8 from Neyriz (0.72 similarity value). Genotypes from different locations with variation in nut characteristics were placed in one group. The two cultivars from USA, 'Vina' and 'Serr', showed genetic similarity of 48%. Nicese et al. (1998) reported that both 'PI-159568' from Iran plateau and 'Serr' cultivar from California placed in one group. McGranahan et al. (1998) confirmed the 'Serr' cultivar to be a hybrid between 'Payne' and 'PI-159568'. These cultivars have been traditionally grown in California over the past decades. 'Vina' is also a hybrid from controlled crossing of 'Payne' and 'Franquette' (Lonnie et al., 1998; Nicese et al., 1998). Molecular study by Nicese et al. (1998) also put these two cultivars in one group. 'Franquette' and 'Lara' from France and USA showed high similarities with most Iranian genotypes indicating their close genetic relatedness. The relatively high similarity of foreign cultivars with Iranian genotypes shown in this study is in agreement with the origin of Persian walnut distribution. It has been reported that the origin of Persian walnut is somewhere from Iran through Afghanistan, then it moved to other areas toward China, Russia and southern European districts (Bayazit et al., 2007). The variance components of within and between sampled locations detected with AMOVA were 90.52% and 9.48% of the total variance, respectively, which were both significant ($p<0.001$) (Table 7). It was in approximate agreement with the results derived from Shannon's index, in which variances within and between sampled locations was 78.9% and 21.1%, respectively (Miller, 1997). Likewise, Bartlett's test for the homogeneity of the RAPD variance components indicated a significant difference (79.5%, $P<0.001$) in the amount of genetic variability within the five locations.

A matrix of Nie's (1978) distance values between locations are presented in Table 8. Values of Nei's distance ranged from 0.0658 (between samples from Karaj and Neyriz) to 0.1357 (between foreign and Shahrekord samples). This indicates

Table 8. Nei's genetic similarities among studied locations that walnut sampled from Iran and introduced cultivars.

Location	Shahrekord	Neyriz Fars	Bavanat Fars	Karaj	Foreign
Shahrekord	-				
Neyriz Fars	0.0694	-			
Bavanat Fars	0.1032	0.0759	-		
Karaj	0.1021	0.0658	0.0907	-	
Foreign	0.1357	0.1253	0.0870	0.1242	-

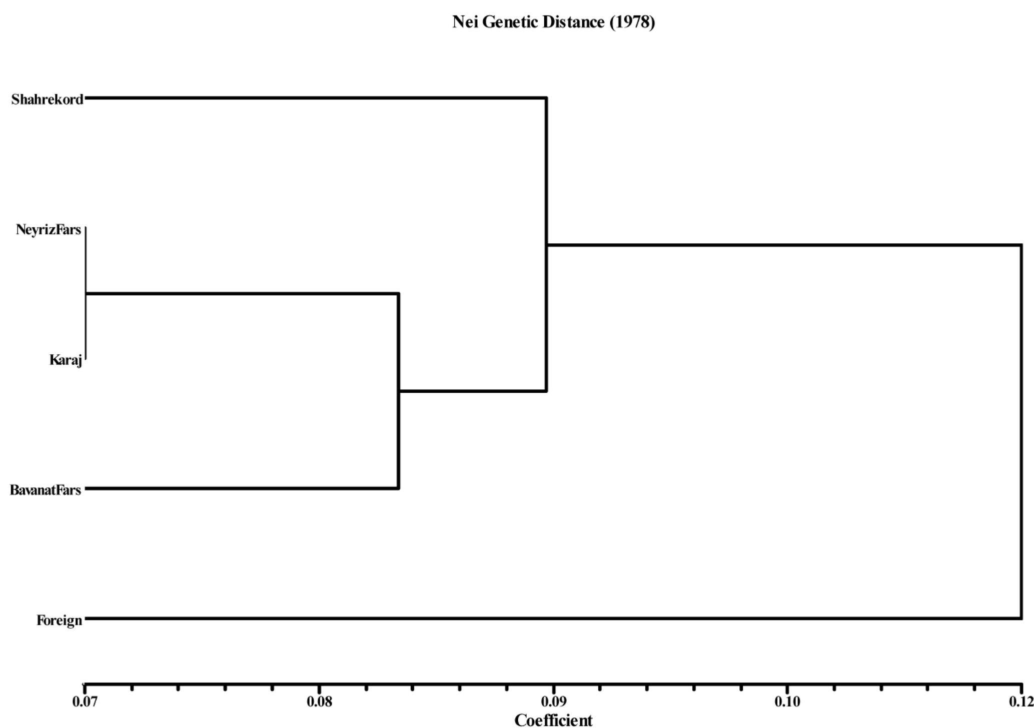

Fig. 3. Dendrogram of 5 locations that walnuts sampled based on Nei's (1978) genetic distances and UPGMA method.

that all locations may be considered different in genetic structure each other. Differentiation between locations was also revealed partially in the clustering analysis (Fig 3).

Discussion

Persian walnut, *Juglans regia*, is a species with a very wide distribution range from east to west. In the present study, a wide range of variation was observed in nut and kernel characteristics as well as in other studied traits. Genetic variation among individuals and also genetic structure of walnut from different locations of Iran compared to 4 introduced foreign cultivars was detected by RAPD technology in combination with morphological characters . The correlation between the Euclidian distance matrices based on morphological traits and RAPD data was low and not significant (r=0.32). Similarly weak correlation coefficients between RAPD markers and morphological characteristics were also reported in banana (Uma et al., 2004), pomegranate (Zamani et al., 2007) and Italian olive cultivars (Hagidimtriou et al., 2005). These similar results in different fruit crops support the view that morpho-pomological characteristics are not necessarily reliable for estimating genetic relationships among cultivars, especially in the case of using correlated traits belonging to a part of plant like fruit characters. [Lack of fit between morphological and RAPD data is also the results of the effects of environmental conditions on the morphological characteristics. It is postulated that RAPD markers mainly cover the non-coding region of the genome, while the phenotypic characters are the results of expressing (coding) region of the genome (Zamani et al., 2010)]. Morphological traits should be used mainly for cultivars phenotypic discrimination that is very important in fruit marketing and also varietal distinctness. However, genetic diversity in some Damask rosa genotypes, which were analyzed by RAPD, SSRs and morphological characteristics (Kiani et al., 2008) and some Rosa genotypes analyzed by RAPD, AFLP and morphological characteristics (Wen et al., 2004) showed a high significant correlation between all pairs of different marker systems. More and detailed morphological characters, which are less affected by environmental conditions and dis-

tributed to different parts of the plant such as fruit, leaves, flowers, growth habits as well as phenological traits, might provide more reliable results due to presenting larger parts of the genome. The germplasm of Persian walnut (*J. regia*) in Iran seems to be highly divergent since it has been sexually propagated by the seed during past thousands of years. Furthermore, this region is considered as a major center of diversity for the *regia* species (Bayazit, et al., 2007; McGranahan et al., 1998). Closely grouping of four foreign cultivars of walnut with most Iranian genotypes indicates their genetic relatedness. This confirms the hypothesis of historical migration of walnut from Iran and Afghanistan to other areas (Bayazit et al., 2007). The presence of broad genetic diversity encourages exploration, collection, conservation and utilization of genetically distinct genotypes for future use of germplasm in achieving desirable breeding objectives for walnut improvement.

To the best of our knowledge, this report is the first assessment on the genetic diversity of Iranian walnut genotypes sampled from diverse locations based on combined morphological data and RAPD molecular markers. According to present results, it is recommended that both morphological and molecular assays should be used in describing the diversity in the walnut genotypes. Further studies with more widely sampling of locations, populations and individuals and with more advanced molecular techniques such as AFLP, SSRs and SNPs are needed to reveal comprehensively the genetic structure of different walnut populations and genotypes.

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