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Genetic diversity among *Juglans regia* L. genotypes assessed by morphological traits and microsatellite markers

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Abstract

In this study, genetic diversity was assayed among 16 accessions and five cultivars of Persian walnut (*Juglans regia* L.) using morphological traits and nine simple sequence repeat (SSR) markers. Samples were collected from Agriculture Research Center of Urmia city (North West Iran). Study on important morphological traits revealed genetic similarity of –0.6 to 0.99 based on CORR coefficient. The microsatellite marker system produced 34 alleles in range of 160-290 bp. The minimum (2) and maximum (7) number of alleles were obtained from WGA71 and WGA202 genetic loci, respectively. The mean number of alleles per locus was 4.25. Jaccard's similarity coefficient ranged from 0.13 to 0.76. The results of this paper indicate high diversity among these genotypes which could be used for breeding management.

Additional key words: breeding programs; cluster analysis; SSR markers; walnut.

Introduction

The genus *Juglans* includes 21 species of long lived, deciduous trees producing large, woody and shelled nuts (Manning, 1978). Persian walnut (*Juglans regia* L.) is one of the most economically important cultivated species for its timber and nutritious nuts (Bayazit *et al*., 2007). Furthermore, the plant has medicinal importance for human health which is derived from its high antioxidant capacity (Rahimipanah *et al*., 2010) and ω-3 fatty acid concentration (Ros & Matrix, 2006). The species grows in areas with a temperate climate and has been naturally distributed from east of Turkey to north of Iran, Afghanistan, Pakistan, mountains of Nepal and Central Asia (McGranahan *et al*., 1998). According to the FAO statistics, Iran produces 10% of world walnut production but only owns less than 1% of international export (Anonymous, 2010). In order to increase the walnut production and exportation, new cultivars need to be introduced and old cultivars to be conserved in gene banks (Francesca *et al*., 2010). To achieve this goal, the first step is identification of superior genotypes and subsequently characterization of their genetic variety. Morphological characteristics often do not result in a clear diagnosis due to effects of different environmental conditions. Hence, utilization of both morphological traits and molecular markers are recommended to investigate diversity in walnut due to its highly divergence (Ebrahimi *et al*., 2011). The ability to distinguish cultivars could be greatly enhanced by using appropriate molecular markers (Lezzoni & Brettin, 1998). These techniques directly measure variations at the DNA level and are not affected by environmental factors (Nicese *et al*., 1998). The DNA markers are useful in studies of evolution, domestication, ecology, phylogeny, genetic mapping and gene cloning (Nazaré Oliveira Ribeiro *et al*., 2011). There are reports concerning assessment of genetic diversity among walnut genotypes using restriction fragment length polymorphism (RFLP) (Fjellstrom & Parfitt, 1994), random amplified polymorphic DNA (RAPD) (Nicese *et al*., 1998), inter-simple sequence repeat (ISSR) markers (Christopoulos *et al*., 2010; Mahmoodi *et al*., 2012), microsatellite markers (Dangle

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Abbreviations used: AFLP (amplified fragment length polymorphism); He (expected heterozygosity); Ho (observed heterozygosity); ISSR (inter-simple sequence repeat); Na (number of alleles); Ne (number of effective alleles); PCR (polymerase chain reaction); PIC (polymorphic information content); RAPD (random amplified polymorphic DNA); RFLP (restriction fragment length polymorphism); SSR (simple sequence repeat).

et al., 2005; Foroni *et al*., 2005, 2006, 2007; Aradhya *et al*., 2010; Ebrahimi *et al*., 2011) and amplified fragment length polymorphism (AFLP) (Bayazit *et al*., 2007). Among DNA markers, microsatellites are abundant, uniformly distributed, hyper variable, codominant and highly reproducible. Therefore, they are powerful and informative to study genetic relationships and genetic identity (Foroni *et al*., 2006). Ultimately, cultivars and genotypes of good quality could be used in crossing programs to produce new commercial cultivars. Therefore, we aimed to use morphological traits and power of simple sequence repeat (SSR) technique to investigate the genetic relationships and identity among 16 accessions and five cultivars of walnut in Iran.

Material and methods

Plant material and morphological traits

A total of sixteen accessions and five cultivars of Persian walnut (*Juglans regia* L.) were studied. The samples were collected from the Kahriz Agriculture Research Station located in the northwest part of Iran (Table 1 & Fig. 1). These genotypes were selected based on a number of important pomological and morphological traits including growth habit, fruit bearing habit, tree vigor, bud break, nut weight (g), kernel percentage (nut %) and kernel color (Table 1). These traits were evaluated during harvest season according to IPGRI standards (IPGRI, 1994).

DNA extraction and SSR amplification

Young leaves of genotypes were collected in spring, frozen in liquid nitrogen and kept in –80°C. Genomic DNA was extracted and purified using Accuprep® GMO DNA Extraction Kit, according to the manufacturer's instructions (Bioneer, Inc). DNA quality and quantity were assessed using 1% agarose gel and Biophotometer (Eppendorf, Germany), respectively. Nine primer pairs, described by Woeste *et al.* (2002), were used to amplify genomic DNA (Table 2). Polymerase chain reaction (PCR) performed in a final volume of 25 µL contained 1 µL DNA template (30 ng), 0.3 µL

Table 1. Genotypes, their origin and studied morphological traits

Genotypes	Original source	Growth habit	Bearing habit	Tree vigor	Bud break	Nut weight (g)	Kernel (%nut)	Kernel color
Accessions								
Alamot	Qazvin (Iran)	Spreading	Lateral	Weak	Early	12.5	56.8	Light
DW	Urmia (Iran)	Spreading	Lateral	Very weak	Early	13.0	48.5	Light
KH53	Khorasan (Iran)	Semi spreading	Intermediate	Weak	Mid early	11.3	46.3	Light
KZ1	Kahriz (Urmia, Iran)	Semi spreading	Intermediate	Medium	Early	12.6	53.9	Light amber
KZ3	Kahriz (Urmia, Iran)	Semi erect	Lateral	Weak	Early	13.3	52.5	Light
KZ4	Kahriz (Urmia, Iran)	Erect	Terminal	Vigorous	Early	14.5	50.4	Light
KZ6	Kahriz (Urmia, Iran)	Spreading	Lateral	Weak	Early	13.6	54.5	Light
KZ7	Kahriz (Urmia, Iran)	Spreading	Lateral	Weak	Early	11.4	62.7	Very light
KZ8	Kahriz (Urmia, Iran)	Spreading	Lateral	Weak	Early	14.6	51.7	Light
KZ13	Kahriz (Urmia, Iran)	Semi erect	Lateral	Medium	Mid early	9.6	66.8	Light
KZ15	Kahriz (Urmia, Iran)	Semi spreading	Lateral	Medium	Mid early	13.5	63.5	Very light
OR60	Urmia (Iran)	Spreading	Lateral	Medium	Early	11.6	47.4	Light
OR126	Urmia (Iran)	Spreading	Lateral	Weak	Mid early	12.6	53.9	Very light
T26	Hamedan (Iran)	Spreading	Intermediate	Weak	Early	12.9	51.5	Light
Z53	Karaj (Iran)	Semi erect	Lateral	Medium	Early	13.5	54.0	Light
Cultivars								
Adilcevaz	Turkey	Semi erect	Lateral	Medium	Late	16.0	50.0	Light
Chandler	USA	Spreading	Lateral	Medium	Late	9.7	46.6	Light amber
Kaman	Turkey	Erect	Terminal	Medium	Early	16.0	38.0	Light
Pedro	France	Spreading	Lateral	Medium	Mid early	10.5	44.3	Light amber
Sebin	Turkey	Spreading	Lateral	Medium	Late	12.0	60.0	Light amber

Figure 1. Location of 16 walnut accessions used in this study.

of each forward and reverse primers (100 pmol μL^{-1}), 10.9 µL deionized water and 12.5 µL 2X PCR Master Mix (Cinnagen, Tehran) including dNTP, $MgCl_2$, 10X PCR buffer and Taq DNA polymerase. Reactions were performed in a Veriti™ 96 well thermal cycler (Applied Biosystem, USA) according to the following procedure: an initial denaturation at 94°C for 3 min, followed by 35 cycles of 30 s at 94°C, 30 s at the optimum annealing temperature for each pair of primers, and 20 s at 72°C, then a final extension step at 72°C for 5 min. The amplified fragments were run on 10% acrylamide gel in 0.5X TBE buffer and stained with ethidium bromide (0.5 µg mL^{-1}). DNA bands were visualized under UV light using gel documentation system (Carestream Gel Pro 212 Imaging System, USA). In all cases, two replicate experiments were carried out for each SSR primer. To determine the size of amplified SSR fragments 50 bp DNA ladder (Gene Ruler 50-1000 bp, Fermentas) was used as the length reference. All allele sizes were compared to ladder by gel scanner (Carestream Gel Pro 212 Imager, USA).

Data analysis

Morphological data analysis was carried out using NTSYS pc 2.02 software (Rohlf, 1998) and similarity matrix obtained by CORR coefficient. The dendrogram was plotted by help of UPGMA method in SAHN program. The SSR amplified products were scored as presence (1) or absence (0) of band. The numbers of alleles (Na), effective number of alleles (Ne), observed and expected heterozygosity were estimated using the GenAlEX (vers. 6) software (Peakall & Smouse, 2006). Polymorphic information content (PIC) value was obtained using formula, $PIC = 1 - \Sigma p_i^2$ (where p_i is the frequency of each allele per primer). Genetic similarity was estimated according to Jaccard's similarity index and dendrogram constructed using UPGMA method by NTSYS pc 2.02 software (Rohlf, 1998).

Results

Genetic similarity among individuals was calculated using CORR coefficient based on seven qualitative and quantitative traits (data not shown). Genetic similarity coefficient ranged from –0.6 to 0.99. The lowest genetic similarity was observed between 'Chandler' and KZ4 & 'Kaman' and 'Chandler' (–0.6). The KZ6 and KZ8 genotypes were most similar ones with coefficient of 0.99. The average similarity value was 0.13. The UPGMA clustering divided 21 walnut genotypes into two main clusters; cluster I and cluster II. Cluster I was

Table 2. Walnut SSRs loci used to study 21 walnut genotypes

Locus	Primers sequences 5'-3'	Tm (°C)	Allele size (bp)	Na	Ne	Ho	He	PIC
WGA1	ATTGGAAGGGAAGGGAAATG CGCGCACATACGTAAATCAC	48	185-207	3	2.27	0.33	0.56	0.56
WGA5	CAGTTTGTCCCACACCTCCT AACCCATGGTGAGAGAGTGAGC	47	202-283	5	2.86	0.95	0.65	0.65
WGA32	CTCGGTAAGCCACACCAATT ACGGGCAGTGTATGCATGTA	51	160-195	4	2.76	0.95	0.63	0.63
WGA71	ACCCGAGAGATTTCTGGGAT GGACCCAGCTCCTCTTCTCT	47	202-216	2	1.89	0.57	0.47	0.49
WGA89	ACCCATCTTTCACGTGTG TGCCTAATTAGCAATTTCCA	45	211-290	4	3.18	0.71	0.68	0.70
WGA118	TGTGCTCTGATCTGCCTCCC GGGTGGGTGAAAAGTAGCA	53	185-220	5	3.69	0.47	0.72	0.72
WGA202	CCCATCTACCGTTGCACTTT GCTGGTGGTTCTATCATGGG	62	193-282	7	4.06	0.76	0.75	0.75
WGA276	CTCACTTTCTCGGCTCTTCC GGTCTTATGTGGGCAGTCGT	56	180-212	4	2.69	0.23	0.62	0.64
WGA27	AACCCTACAACGCCTTGATG TGCTCAGGCTCCACTTCC	53	—	—	—	—	—	—
Mean		55	—	4.25	2.92	0.62	0.63	0.64

Na: number of alleles, Ne: number of effective alleles, Ho: observed heterozygosity, He: expected heterozygosity, PIC: polymorphism information content.

further divided into two sub clusters. KZ1, T26, KZ3, KZ15, KZ6, KZ8, DW, OR60, Alamot, Z53, KZ7, OR126, 'Pedro', 'Chandler', 'Sebin' and KZ13 were grouped in the first sub cluster and second sub cluster consisted of FA2. Cluster II comprised of KZ4, 'Kaman', KH53 and 'Adil cevaz' (Fig. 2a). Correlation coefficient of similarity matrix and dendrogram was calculated 0.76. Within cluster I, KZ6 and KZ8 showed highest similarity. In addition, OR60 and Alamot & 'Pedro' and 'Chandler' revealed to be close to each other. In cluster II, KZ4 and 'Kaman' showed close relationship.

From nine primers used in this study, WGA27 primer showed monomorphic results among all genotypes. A total of 34 alleles were amplified in range of 160-290 bp. The number of alleles per primer ranged from two (WGA71) to seven (WGA202) with a mean of 4.25. The number of effective alleles varied from 1.89 (WGA71) to 4.06 (WGA202), with an average of 2.92 alleles per primer (Table 2). The WGA276 primer revealed the lowest (0.23) observed heterozygosity and the highest (0.95) was produced by WGA5 and WGA32 primers. The mean of 0.62 was obtained for observed heterozygosity. The expected heterozygosity ranged from 0.47 to 0.75 with an average of 0.63 (Table 2). The lowest (0.49) and highest (0.75) PIC values were estimated for WGA71 and WGA202 loci, respectively, with an average of 0.64 (Table 2). The lowest and highest allele frequency was related to WGA202 (0.024) and WGA71 primers (0.61), respectively (data not shown). Genetic similarity among individuals was calculated based on Jaccard's coefficient (data not shown). The lowest genetic similarity was observed between KH53 and KZ3 & between KH53 and KZ4 accessions (0.13), followed by 0.14 between Alamot and KZ3 & between KH53 and KZ1. The T26 and Alamot accessions were most similar ones with coefficient of 0.76. The average similarity value was 0.36.

The SSR dendrogram, resulting from the UPGMA cluster analysis, grouped the 21 genotypes into two clusters, cluster I and cluster II (Fig. 2b). Cluster I was further divided into two sub clusters. Three Kahriz genotypes including KZ1, KZ3 and KZ4 together with 'Sebin', 'Kaman' and 'Adil cevaz' from Turkey were grouped in the first sub cluster and second sub cluster consisted of KZ6 (Kahriz) and 'Chandler' (commercial cultivar of USA). Cluster II was further divided into two sub clusters, too. First sub cluster included KZ7, KZ13, KZ15, OR60, 'Pedro', KZ8, Z53, OR126, DW and FA2 accessions. Second sub cluster comprised of

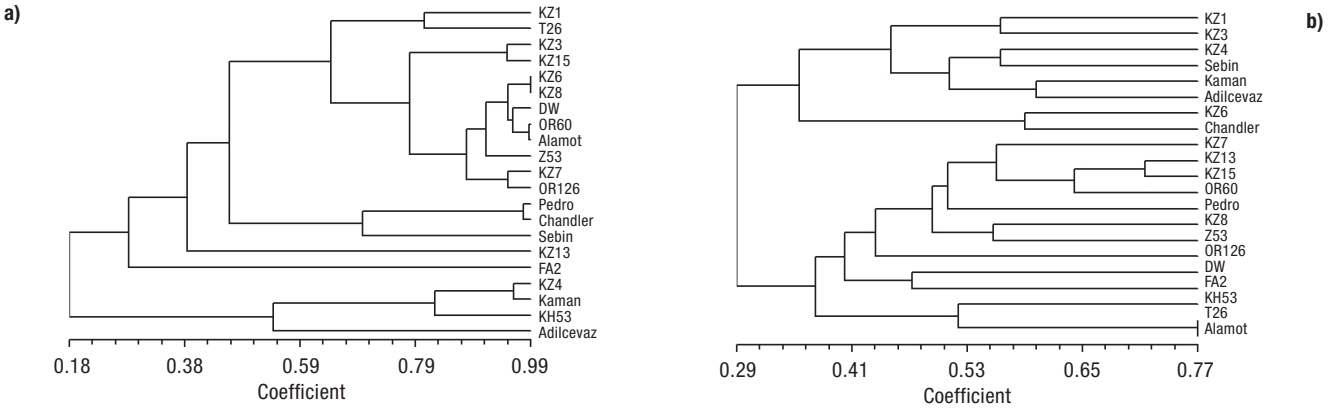

Figure 2. Dendrogram of the 21 walnut genotypes based on a) morphological traits using CORR coefficient and b) SSR markers using Jaccard's similarity coefficient.

KH53, T26 and Alamot. Correlation coefficient of similarity matrix and dendrogram was calculated 0.71. The KZ4, 'Kaman' and 'Adil cevaz' were grouped in the same cluster based on results generated by morphological and molecular dendrograms. Based on SSR dendrogram, the KZ7, KZ13, KZ15, OR60, 'Pedro', KZ8, Z53, OR126, DW, FA2, T26 and Alamot genotypes were grouped in one cluster. These genotypes were grouped together in one cluster according to morphological dendrogram, too. Moreover, the KZ1, KZ3, KZ6, 'Sebin' and 'Chandler' were clustered in one group in both dendrograms.

Discussion

Based on CORR morphological similarity and obtained dendrogram, KZ6 and KZ8 showed the highest similarity among studied genotypes. These accessions showed the same qualitative traits with highest similarity in quantitative traits. In cluster I, OR60 and Alamot genotypes also showed high similarity. In addition, 'Chandler' and 'Pedro' cultivars appeared to be most similar, too. OR60 and Alamot posses' similar traits including growth habit, bearing habit, bud break and kernel color. The 'Chandler' and 'Pedro' cultivars also bear similar traits like growth habit, bearing habit, tree vigor and kernel color. In cluster II, KZ4 and 'Kaman' shared the same growth habit, bearing habit, bud break and kernel color traits. According to similarity coefficient 'Chandler' and KZ4 & 'Chandler' and 'Kaman' showed the lowest similarity. In fact, 'Chandler' and KZ4 revealed differential quantitative and qualitative traits. In fact, 'Kaman' and 'Chandler' cultivars showed differences in all traits except tree vigor.

In the present study, a wide range of variation was observed in nut and kernel characteristics as well as in other traits. The average morphological similarity value was 0.13 which is indicative of high morphological diversity among studied genotypes. A high variability in morphological traits has been reported for walnut trees from different studies (Malvolti *et al*., 1994; Balci *et al*., 2001). Our studied collection originated from different provinces in seedling form and existence of high diversity among walnut trees could be explained by outcrossing nature and ultimate seed propagation (Foroni *et al*., 2005). Correlation coefficient of similarity matrix and dendrogram was 0.76, which is indicative of good relationship between the result of similarity matrix and morphological cluster analysis. In SSR analysis, the average number of each microsatellite allele and its frequency indicates the appropriateness of each locus for estimating genetic variability. Hence, primers with more alleles and low frequency are fit to study genetic diversity. The WGA202 primer with 0.024 frequency and seven alleles created the most number of alleles. In the study of Ehteshamnia *et al*. (2009), Aradahya *et al*. (2010) and Ebrahimi *et al*. (2011), the most number of alleles were generated by WGA202 primer, too. The existence of the variability in allele frequencies, reported by other studies could be due to difference in selected genotypes and primers (Ehteshamnia *et al*., 2009). Our selected genotypes have shown high degree of heterozygosity (0.64) which has been developed through outcrossing. However, this observation is less than 0.8, obtained through the study of wild population of *Juglans nigra* by SSR marker (Victory *et al.,* 2006), which could be explained by more outcrossing nature of wild black walnut. Markers with high PIC value are more informative

due to production of more alleles and low frequency (Ebrahimi *et al*., 2010). Hence, WGA202, WGA118 and WGA89 with high PIC (> 0.7) are suggested for analysis of other walnut germplasms. The lowest genetic similarity was observed between KH53 and KZ3 & between KH53 and KZ4 accessions (0.13), followed by 0.14 between Alamot and KZ3 & KH53 and KZ1. This observation is in support of morphological data, since KH53 genotype has low nut weight and kernel percentage than KZ1, KZ3, KZ4 and Alamot genotypes. The bud break trait is mid early in KH53 and early in other four genotypes. According to the result of SSR dendrogram and matrix similarity, genotypes with lowest similarity including KH53 and KZ3 (0.13), KH53 and KZ4 (0.13), KH53 and KZ1 (0.14), KZ3 and Alamot (0.14) are recommended in breeding programs as parents. Based on SSR analysis, T26 and Alamot genotypes were most similar ones with coefficient of 0.76. These two genotypes are similar in all morphological traits except bearing habit. In molecular part average similarity value of 0.36, indicates the high variability in genetic resources of walnut in Iran. Correlation coefficient of similarity matrix and SSR dendrogram was calculated (0.71) which shows good relationship between the result of similarity matrix and cluster analysis. According to our SSR and morphological analyses commercial cultivars ('Chandler', 'Pedro', 'Kaman', 'Sebin' and 'Adil cevaz') were grouped together with Iranian accessions in the same cluster which could be because of their close genetic relationships. There are reports indicating that the probable source of Persian walnut is ancient Iran and subsequently distributed to other countries (Bayazit *et al*., 2007; Ebrahimi *et al*., 2011; Mahmoodi *et al*., 2012). Based on our previous study using ISSR molecular marker and its generated dendrogram, 'Pedro', FA2 ,T26, KZ13, KZ15, KZ8, KZ7, OR126, Alamot, Z53 and OR60 were placed together in the same cluster (Mahmoodi *et al*., 2012). Theses genotypes were grouped in the same cluster according to SSR and morphological analyses, too.

In brief, our data analyses based on SSR genetic markers and morphological traits are highly in support of ISSR analysis. The comparison of genotypes by ISSR marker, as previously reported (Mahmoodi *et al*., 2012), showed genetic similarity ranging from 0.39 to 0.67. In the present study, based on SSRs markers, the similarity among genotypes ranged from 0.13 to 0.76. The SSRs results showed higher genetic diversity compared to ISSR marker due to specific and codominant nature of SSR markers which represent higher resolution. These findings provide useful information for breeding programs and conservation of walnut germplasm management.

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