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ORIGINAL ARTICLE

Genetic diversity and gene flow of some Persian walnut populations in southeast of Iran revealed by SSR markers

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Abstract Iran is reported to be a center of diversity for *Juglans regia* and wild walnut trees are found in virtually every corner of the country. Thus Iran is considered a rich natural pool of walnut germplasm for developing improved genotypes. Kerman province is the most important Iranian province for walnut culture and has the largest walnut plantations in Iran. Genetic structure and gene flow were analyzed in six walnut populations of this province using 17 microsatellite loci. The number of alleles per locus ranged from 4 to 11, with a total of 147 alleles and 5.16 effective alleles per locus. The polymorphism information content for the loci ranged from 0.56 to 0.82. The expected heterozygosity (H_e) for the populations ranged from 0.65 to 0.87. There were differences between populations regarding the number of effective alleles and Shannon's information index (I). In all populations, observed heterozygosity (H_o) was lower than expected, but diversity within the populations was high ($I = 1.5$) and many of the private alleles were present at relatively high frequency. The average F_{st} value was 0.08. The level of gene flow based on F_{st} was high ($N_m = 3.01$), which meant that the high level of genetic diversity maintained within each population was less susceptible to genetic drift. The geographical proximity of the populations was not correlated with their level of genetic relatedness. These results imply the high potential of walnut populations of Kerman province for breeding programs.

Keywords Genetic structure · Gene flow · *Juglans regia* · Microsatellites · Polymorphism

Introduction

Persian walnut, *Juglans regia* L., is the most economically important member of its genus. It is cultivated for its timber and edible nuts throughout temperate regions of the world (Vahdati 2000; Bayazit et al. 2007). Persian walnut

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was probably domesticated in the region of Iran and Afghanistan and subsequently introduced to China, Russia and Eastern Europe by ancient tribes (Bayazit et al. 2007). According to the FAO (2011), Persian walnut grows on 64,000 ha in Iran, producing 165,508 tons of nuts in shell, ranking second globally. Kerman province is the leading area for walnut production in Iran, with about 17,095 ha under cultivation. This province, with varied eco-geographical regions, is one of the major centers for Persian walnut diversity, and walnut populations are widely scattered in this region (Vahdati 2000).

Genetic variation among walnut populations has been studied using a large number of marker systems, including morphological indices (Malvolti et al. 1994), isozymes (Arulsekar et al. 1986; Malvolti et al. 1993; Solar et al. 1994; Fornari et al. 1999), restriction fragment length polymorphisms (RFLPs) (Fjellstrom et al. 1994; Fornari et al. 2001), randomly amplified polymorphic DNA (RAPD) (Nicese et al. 1998), amplified fragment length polymorphisms (AFLPs) (Bayazit et al. 2007) and inter-simple sequence repeats (ISSRs) (Potter et al. 2002). These studies reflect the need to identify cultivars accurately (genetic fingerprints) and verify paternity and genealogy. Woeste et al. (2002) developed a panel of 30 nuclear microsatellites (SSR) for a wide range of genetic investigations in *Juglans*, including clonal identification (Dangl et al. 2005; Foroni et al. 2007), a broad-scale study of the genetic structure of *J. nigra* populations in the Central Hardwood Region of the United States of America (Victory et al. 2006) and the identification of hybridogenic walnut plants (Pollegioni et al. 2009). In a recent study, Gunn et al. (2010) evaluated 220 walnut trees from six Tibetan villages in China using 14 SSR markers.

Microsatellites or simple sequence repeats (SSRs) have proved to be suitable markers for variety characterization, and a few have already been developed for *J. regia* (Dangl et al. 2005; Foroni et al. 2007; Pollegioni et al. 2011). Microsatellites are ideal markers for characterizing relationships among individuals because of the co-dominant inheritance, hyper-variability, high information content and the reproducibility of genotyping results among laboratories (Foroni et al. 2006). The published data on genetic diversity and structure for Persian walnut are mostly from breeding populations and European populations, and there are no data for the species in either its native range or in the region of its domestication (Malvolti et al. 1993, 2010; Fjellstrom and Parfitt 1994; Nicese et al. 1998; Fornari et al. 2001; Dangl et al. 2005).

The objective of the present study was to use microsatellite markers to determine genetic structure and gene flow among six landraces of *J. regia* from different valleys of Iran's Kerman province, which is home to one of the world's largest populations of Persian walnut.

Materials and methods

Plant materials

Six populations of walnut, namely Hanza (HA, lat. 57°11′N), Gogher (GO, lat. 56°24′N), Kiskan (KI, lat. 56°38′N), Rayen (RI, lat. 57°19′N), Bidkhan (BI, lat. 56°30′N) and Bezenjan (BE, lat. 56°41′N), located in different valleys of Kerman province in southeastern Iran, were selected for study (Fig. 1). The sampled populations inhabit disjunctive mountainous areas with a narrow geographic range of longitude (29°14′–29°37′E). Populations consisted of old walnut trees from open pollinated seedlings (70- to 150-year-old trees with terminal or lateral bearing habit). Summary of morphological and phenological traits in studied populations are shown in Table 1. Plants within 15 km of each other were considered to belong to the same population (Malvolti et al. 1993). The number of samples collected per site ranged from 6 to 18 based on the density of the plants found at each site. In total, 66 genotypes in six populations were analyzed.

The trees cultivated in these populations represent local populations (seedlings) that were randomly planted by humans or birds (mostly crows) and now grow across wide areas at a spacing of about 4–8 m. Moreover, the number of individuals collected is not same in each population because of the different plant density found in each valley. This is the reason of the low number of trees sampled in some populations. The sampled populations, located in different valleys of Kerman province, were separated by 14–100 km (approximately 45 km in average).

DNA extraction

For each mother tree, at least six young leaves were collected in summer and used for DNA extractions. A 150- to

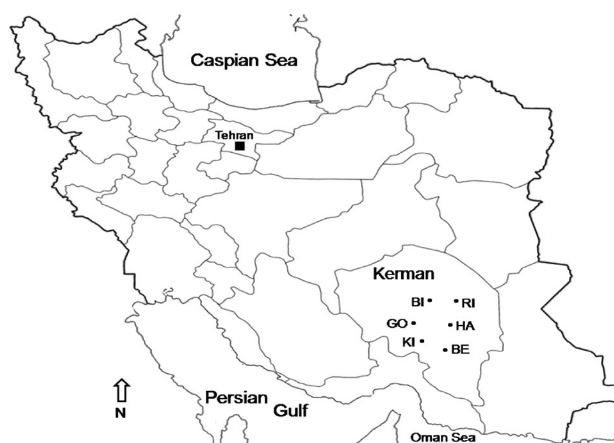

Fig. 1 Location of the studied walnut populations in Kerman province, Iran

Table 1 Comparison of some morphological and phenological traits of the studied walnut (*Juglans regia*) population

Population	Number of samples	Tree vigor	Growth habit	Time of leaf bud burst	Type of dichogamy	Bearing habit	Maturity time	Nut weight (g)	Kernel weight (g)	Kernel color
Hanza (HA)	18	Strong to very strong	Spreading	Early to medium	Mainly protandrous	Terminal	Early to medium	13.4	6.28	Dark
Gogher (GO)	10	Medium to strong	Spreading to medium	Early to medium	Protandrous	Intermediate	Early to medium	11.5	5.14	Light to medium
Kiskan (KI)	14	Medium	Spreading	Medium	Homogamy	Lateral	Early to medium	10.5	5.03	Medium
Rayen (RI)	6	Medium to strong	Spreading	Early to medium	Homogamy	Lateral	Early to medium	11.2	5.27	Medium to dark
Bidkhan (BI)	8	Medium	Spreading	Early to medium	Mainly protandrous	Intermediate	Early to medium	11.9	5.16	Medium
Bezenjan (BE)	10	Medium	Spreading to medium	Early to medium	Homogamy	Intermediate	Early to medium	10.2	4.92	Medium

Data are means of all sampled trees in each population. The traits were recorded according to UPOV and IPGRI descriptors

200-mg sample was ground in 2-mL Eppendorf tubes with 1,800 mL of extraction buffer (2 % CTAB, 100 mM Tris, 1.4 M NaCl, and 20 mM EDTA, pH 8.3). Then DNA was extracted following the CTAB method (Doyle and Doyle 1987). DNA quantity and concentrations were determined spectrophotometrically at 260 and 280 nm and by electrophoresis on 0.8 % (w/v) agarose gel. The agarose gel was stained by ethidium bromide and visualized with UV light.

SSR primers and PCR amplification

Seventeen SSR paired primers with the prefix WGA designed from the sequence of clones from an enriched $(GA/CA)_n$ library of black walnut (*J. nigra*) (Woeste et al. 2002; Dangl et al. 2005), were used to amplify genomic DNA of each individual plant from the six populations in order to identify polymorphic SSR loci (Table 2). Polymerase chain reaction (PCR) amplifications were performed in 20 µL reaction solution containing 2 µL 10× buffer (100 mM Tris–HCl, pH 8.0 and 500 mM KCl), 2 mM $MgCl_2$, 0.2 mM deoxyribonucleoside triphosphate (dNTP), 50 ng of each forward and reverse primer, 1 unit *Taq* polymerase (Cinnagene,Tehran, Iran) and 60 ng template DNA. Reactions were performed in a Biorad MJ Mini thermocycler according to the following procedure: an initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, an annealing step at 57 °C for 40 s and an extension at 72 °C for 1 min, with a final extension step at 72 °C for 10 min; the final product was kept at 4 °C. A negative control reaction without DNA template was included in each amplification. The product was run on a 1-mm-thick, 6 % non-denaturing polyacrylamide gel. Gels were pre-run at 1,500 V for 30 min. The samples were loaded and run at 1,500 V for about 1 h. Fragments were visualized by silver staining (Bassam et al. 1991). Each gel had a 100–1000-bp DNA ladder (Cinnagene, Tehran, Iran) and a standard sample to estimate molecular weight and control gel-to-gel variation. In all cases, PCR reactions were performed at least twice to ensure that allele sizes were consistent.

Evaluation of polymorphisms and statistical analysis

Polymorphic alleles were scored as AA, BB, CC, etc. for homozygous individuals and AB, AC, BC, etc. for heterozygous individuals. For each locus, genotypes showing one and two bands were scored as homozygous and heterozygous, respectively. Observed heterozygosity (H_o) was calculated as the ratio between the number of heterozygous individuals and the total number of genotypes per locus. Expected heterozygosity (H_e) was estimated according to the formula $H_e = 1 - \Sigma p_i^2$, where p_i is the frequency of the *i*th allele for the studied locus (Levene 1949). Population structure was analyzed using Wright's (Wright 1978), *F*-statistics (F_{IT}, F_{IS} and F_{ST}), where F_{IT} represents the overall inbreeding coefficient, F_{IS} represents the level of inbreeding due to nonrandom mating within populations, and F_{ST} represents the population subdivision. Polymorphism information content PIC and *I* values are frequently used to estimate the genetic diversity of genotypes (Shannon and Weaver 1949). The POPGENE (ver. 1.31) software (Yeh et al. 1999), was used to determine percentage of polymorphic loci, observed number of alleles (N_a), effective number of alleles (N_e), Shannon's information index (*I*), Wright's (Wright 1978), *F*-statistics (F_{IT}, F_{IS} and F_{ST}) and Nei's (Nei 1978). The H_e, H_o and *I* values for each locus and population

were compared separately by analysis of variance using a completely randomized design by SAS software (version 9.0, SAS Institute, Inc., Cary, North Carolina, USA). The Neighbor-Joining dendrogram using genetic distance measures on SSR markers data among the populations was computed with NTSYSpc (ver 2.02) (Rohlf 1998) software.

Results and discussion

Genetic diversity

All the tested primers produced satisfactory amplification products and were multiallelic (Table 2). The total number of alleles per locus ranged from 4 (in WGA225) to 11 (in WGA001, WGA009, WGA071, WGA069 and WGA202), with a total of 147 alleles for all loci.

The average N_a and N_e per locus were 8.65 and 5.16, respectively (Table 3). Allele numbers were relatively high compared to the levels of variability detected in 15 European walnut populations (Fornari et al. 1999), 48 *J. regia* cultivars (Dangl et al. 2005) and five *J. regia* populations from central and southwestern China (Wang and Pei 2008). Although descriptive data for WGA69 included in Table 3, this locus showed significant deviations from the other loci and was not included in subsequent analyses.

The peculiar results of WGA069 for *Juglans* have been observed in several studies. Lewontin and Krakauer (1973)

Table 2 Properties of the microsatellite loci used to characterize the studied walnut populations in Kerman province, Iran

Locus	Primer sequences (5′–3′)	Tm[A] (°C)	Size range (bp)	Size of private alleles (bp) for each population
WGA001	ATTGGAAGGGAAGGGAAATG CGCGCACATACGTAAATCAC	56.5	198–265	198(BZ)–215(BI)–245(KI)–265(KI)
WGA004	TGTTGCATTGACCCACTTGT TAAGCCAACATGGTATGCCA	56	220–270	245(BZ)
WGA009	CATCAAAGCAAGCAATGGG CCATTGCTCTGTGATTGGG	56	230–290	235(H)–265(H)
WGA069	TTAGTTAGCAAACCCACCCG AGATGCACAGACCAACCCTC	59	175–240	–
WGA089	ACCCATCTTTCACGTGTGTG TGCCTAATTAGCAATTTCCA	56.5	220–270	220(H)–230(BI)
WGA118	TGTGCTCTGATCTGCCTCC GGGTGGGTGAAAAGTAGCAA	62.5	190–260	–
WGA202	CCCATCTACCGTTGCACTTT GCTGGTGGTTCTATCATGGG	62	200–330	260(G)–270(H)
WGA225	AATCCCTCTCCTGGGCAG TGTTCCACTGACCACTTCCA	60	200–230	230(G)
WGA276	CTCACTTTCTCGGCTCTTCC GGTCTTATGTGGGCAGTCGT	62.5	180–235	–
WGA321	TCCAATCGAAACTCCAAAGG GTCCAAAGACGATGATGGA	56.5	240–265	–
WGA331	TCCCCCTGAAATCTTCTCCT CGGTGGTGTAAGGCAAATG	60	250–315	315(BZ)
WGA332	ACGTCGTTCTGCACTCCTCT GCCACAGGAACGAGTGCT	56	230–300	230(H)–275(BI)
WGA349	GTGGCGAAAGTTTATTTTTTGC ACAAATGCACAGCAGCAAAC	56.5	250–290	–
WGA376	GCCCTCAAAGTGATGAACGT TCATCCATATTTACCCCTTTCG	59	230–300	265(KI)–285(BI)
WGA027	AACCCTACAACGCCTTGATG TGCTCAGGCTCCACTTCC	56	225–260	–
WGA032	CTCGGTAAGCCACACCAATT ACGGGCAGTGTATGCATGTA	55.5	185–205	197(KI)–205(BZ)
WGA071	ACCCGAGAGATTTCTGGGAT GGACCCAGCTCCTCTTCTCT	56	180–230	–

[A] Melting temperature

Table 3 Genetic diversity, genetic differentiation and gene flow of the studied walnut populations in Kerman province, Iran

Locus	N_e^A	N_a^B	H_e^C	H_o^D	I^E	PIC[F]	Ar^G	N_m^H
WGA032	2.28	6	0.65	0.14	1.22	0.56	3.49	1.83
WGA332	3.90	9	0.74	0.23	1.68	0.70	5.02	4.13
WGA001	5.65	11	0.83	0.03	2.00	0.77	6.05	3.34
WGA009	6.92	11	0.86	0.15	2.13	0.76	6.54	2.29
WGA027	4.09	8	0.76	0.00	1.68	0.68	5.05	2.34
WGA071	7.98	11	0.87	0.00	2.22	0.81	6.85	3.18
WGA004	6.65	10	0.85	0.27	2.10	0.79	6.48	3.69
WGA069	5.68	11	0.82	0.85	2.03	0.79	6.19	6.10
WGA089	4.46	9	0.78	0.12	1.75	0.74	5.18	5.52
WGA118	7.84	10	0.87	0.05	2.18	0.82	6.77	3.89
WGA202	6.91	11	0.86	0.44	2.07	0.78	6.27	2.79
WGA225	3.13	4	0.68	0.14	1.18	0.61	3.23	2.43
WGA276	5.06	8	0.80	0.58	1.84	0.76	5.58	4.16
WGA321	3.59	6	0.72	0.5	1.45	0.67	4.23	4.68
WGA331	3.14	7	0.68	0.21	1.40	0.62	4.14	2.48
WGA349	4.42	5	0.77	0.06	1.54	0.69	4.47	2.02
WGA376	5.52	10	0.82	0.18	1.96	0.71	5.91	2.19
Average	5.16	8.65	0.79	0.23	1.79	0.72	5.38	3.03

[A] Effective number of alleles
[B] Observed number of alleles
[C] Expected heterozygosity
[D] Observed heterozygosity
[E] Shannon's information index
[F] Polymorphism information content
[G] Allelic richness based on sample of six
[H] Gene flow estimated from $F_{st} = 0.25(1-F_{st})/F_{st}$

and Luikart et al. (2003) observed that selection and mutation have locus-specific effects while genetic drift and gene flow act at a genome-wide scale. By analyzing the number of alleles per locus and the large number of alleles in common between *J. nigra* and *J. regia*, Pollegioni et al. (2009) postulated a low mutation rate at locus WGA69. Several studies report that the interruption of perfect microsatellites is related to DNA stability in the region (Taylor et al. 1999). These authors suggested that the purity of a repeat region influences its mutation rate and consequently, the level of polymorphism in SSR loci. Interrupted microsatellites, such as WGA69, appear to have lower mutation rates than pure microsatellites. As reported by Cornuet and Luikart (1996), this feature makes WGA69 a useful marker for detecting a bottleneck. Storz (2005) also indicated that the risk of detecting false positives is high using Beaumont and Nichols (1996) because bottlenecks can produce effects similar to natural selection. Pollegioni et al. (2011) in study of genetic structure of Italian walnut by SSR markers suggested that the atypical behavior of WGA69 may be a consequence of its low rate of mutation and a human-mediated domestication bottleneck.

The alleles present with the highest frequency (0.50) were the 190- and 300-bp alleles at the WGA032 and WGA331 loci, respectively, whereas the alleles with the lowest frequency (0.07) were the 260- and 270-bp alleles at the WGA202 locus (data not shown). The H_e values ranged from 0.65 for WGA032 to 0.87 for WGA071 and WGA118, with an average of 0.79 for all loci (Table 3). The analysis of variance showed that H_e differed significantly among loci ($F = 3.45$, $P \leq 0.01$). The H_o values ranged from 0.00 for WGA027 and WGA071 to 0.85 for WGA069, with an average of 0.23 for all loci. The H_o values differed significantly among loci ($F = 18.64$, $P \leq 0.01$). The difference between H_e and H_o was high that it can be result from selection of the best genotypes by growers and increase inbreeding coefficient in populations. The average number of alleles per locus was 8.05, much higher than the 1.3, 3.9 and 5.5 detected in *J. regia* with RAPDs (Nicese et al. 1998), ISSRs (Potter et al. 2002), and SSRs (Foroni et al. 2007), respectively. Dangl et al. (2005) also observed 3–8 alleles per locus in their genetic analysis and cultivar identification of walnut using 14 SSR markers. In the present study, the number of alleles for WGA032 and WGA279 was similar to results published by Foroni et al. (2006) and Dangl et al. (2005), respectively.

In the present study, at the level of populations, the PIC and I of the populations averaged 72 % and 1.79, respectively. PIC values provide an estimate of the discriminatory power of any locus by considering the number of alleles per locus and their relative frequencies in the population (i.e. gene diversity values, essentially) (Rongwen et al. 1995). The PIC values for the populations in the present study ranged from 0.56 to 0.82 and classified six loci as informative markers (PIC >0.5) and eleven loci as suitable for mapping (PIC >0.7) (Table 3). These results indicate that all of the markers could contribute substantial information to walnut genetics and breeding research. The results also show that the walnut populations of Kerman province have relatively high levels of neutral genetic diversity. Pollegioni et al. (2011) observed that except WGA004 (0.355) and WGA331 (0.382), all markers had PIC >0.50. The I values for loci were highly variable: they averaged 1.79, with the maximum (2.22) for WGA071 and the minimum (1.18) for WGA225. The PIC and I values differed significantly among loci and populations (data not shown).

On the other hand, gene flow is the movement of genes within and between populations (Grant 1991). In this study, the levels of gene flow (N_m) ranged from 1.83 to 6.10 with a mean of 3.03 for all loci which can be calculated indirectly by F_{ST} (Table 3), which meant that the high level of genetic diversity maintained within each population was

less susceptible to genetic drift. Seeds and pollen are the two main vectors of gene flow for seed plants (Hamrick et al. 1991). Seeds of wild walnut trees spread mainly by gravity and by animal movement over short distances. In long distance, seed dispersal by animal movement is rare, but dispersal of pollen is likely a main way of gene flow between populations. Another possibility is seed movement by growers in order to selection of the best genotypes or increasing cultivation areas. Dispersal of pollen is likely the main mechanism of gene flow among the studied populations, a conclusion that was also reached in other studies of similar large-seeded species (Victory et al. 2006). The high amount of gene flow ($N_m = 3.03$, Table 3) would reduce the disjunction between these populations. In study of genetic variation in Korean populations of *J. sinensis*, gene flow among populations was reported relatively low ($N_m = 1.80$, Lee and Lee 1997). The number and frequency of private alleles can provide insight into levels of gene flow among populations. The present study found that, among the Persian walnuts in Kerman province, 14 % of all detected alleles were private (19/136 alleles; locus WGA69 was excluded from this analysis), a figure that is not a great departure from the number of private alleles observed in a large study of *J. nigra* populations in the U.S. (about 10 % private; Victory et al. 2006) (Data not shown). The HA, GO and KI populations had the largest number of private alleles, with six, five and six, respectively (data not shown); RI and BE had no private alleles, but this was probably because of our small sample size from this population. It was surprising that several of the private alleles were present at relatively high frequencies, in three cases exceeding 10 %. Because the detection of rare and private alleles is highly dependent on sample sizes, which were small in this study, few conclusions can be drawn from these data. F_{ST} statistics at the loci assayed ranged between 0.04 and 0.12, with the average 0.08. Positive values of F_{ST} are usually interpreted as indicators of inbreeding and in general of assertive mating. The observed negative figures could be ascribed to the combination of a high degree of outcrossing and to some mechanism of heterozygote advantage (Hartel and Clark 2007). The positive values of F_{ST} were in agreement with high level of gene flow (N_m) between the populations. Because seed movement would increase the similarity of the genotypes, then sib-mating or inbreeding would enlarge in the populations. In wild populations of *J. sinensis* in Korea, F_{ST} was 0.122 (Lee and Lee 1997); among *J. nigra* populations in the U.S., F_{ST} was 0.017 (Victory et al. 2006); and among Asian and European populations of *J. regia*, F_{ST} was 0.108 (Fornari et al. 2001).

There were differences between the six studied *J. regia* populations regarding H_e (genetic diversity), N_e and I (Table 4). The average I value for all populations was

Table 4 Genetic diversity of the studied walnut populations in Kerman province, Iran

Population	N^A	N_e^B	N_a^C	H_e^D	H_o^E	I^F
Hanza (HA)	18	6.17	6.94	0.76	0.24	1.67
Gogher (GO)	10	4.37	5.64	0.74	0.20	1.53
Kiskan (KI)	14	4.37	6.3	0.75	0.25	1.59
Rayen (RI)	6	3.19	4.23	0.63	0.22	1.21
Bidkhan (BI)	8	4.23	5.65	0.74	0.25	1.52
Bezenjan (BE)	10	3.86	5.41	0.71	0.22	1.44
Average	66	4.36	5.69	0.72	0.23	1.49

[A] Sample size
[B] Effective number of alleles
[C] Observed number of alleles
[D] Expected heterozygosity
[E] Observed heterozygosity
[F] Shannon's information index

1.49, with the maximum (1.67) for the HA population and the minimum (1.21) for the RI population. The HA ($N = 18$) and KI ($N = 14$) populations also had the highest number of alleles (both N_a and N_e) and RI population ($N = 6$) had the lowest, reflecting correlation allele number with the sample size of each population. The mean number of alleles per polymorphic locus ($N_a = 5.69$) was higher than the typical values reported for widespread plant species ($N_a = 3.70$) and European and Asian walnut populations ($N_a = 2.27$) (10, 16). In all the studied Iranian populations, H_o was low, but diversity within the populations was high ($I = 1.5$). The maximum H_o was found in the KI and BI populations ($H_o = 0.25$) (Table 4), and there were significant differences among populations ($F = 3.45$, $P \leq 0.01$); the average H_o in the samples in the present study was 0.23, lower than the averages in the *J. regia* populations studied by ($H_o = 0.50$, Malvolti et al. 1994), ($H_o = 0.698$, Foroni et al. 2007), ($H_o = 0.585$, Foroni et al. 2006) and Dangl et al. (2005) ($H_o = 0.597$, Pollegioni et al. 2011) and in the *J. nigra* populations studied by ($H_o = 0.781$, Victory et al. 2006) based on SSR marker data. The H_o observed in the present study was closer to that of European and Asian populations of *J. regia* ($H_o = 0.39$, Fornari et al. 2001) and populations of *J. nigra* ($H_o = 0.31$; Hamrick et al. 1991) based on isozyme markers. The H_e ranged from 0.63 (RI) to 0.76 (HA). In study of Pollegioni et al. (2011) the value of H_e in populations was 0.644. The levels of H_o was significantly lower than H_e. This result is probably due to sample size, Wahlund effect or inbreeding.

Population variation

As mentioned previously, the F_{ST} estimates for each locus assayed ranged between 0.04 and 0.12, with an average of

Table 5 Statistics of genetic structure for the 17 polymorphic loci in six walnut populations in Kerman province, Iran

Locus	F_{IS}^{A}	F_{IT}^{B}	F_{ST}^{C}
WGA032	0.76	0.79	0.12
WGA332	0.68	0.69	0.06
WGA001	0.96	0.96	0.06
WGA009	0.83	0.84	0.10
WGA027	1.00	1.00	0.10
WGA071	1.00	1.00	0.07
WGA004	0.67	0.69	0.06
WGA069	−0.09	−0.05	0.04
WGA089	0.86	0.86	0.04
WGA118	0.95	0.95	0.06
WGA202	0.45	0.49	0.08
WGA225	0.75	0.78	0.09
WGA276	0.27	0.31	0.06
WGA321	0.23	0.21	0.05
WGA331	0.64	0.68	0.09
WGA349	0.93	0.94	0.11
WGA376	0.76	0.79	0.10
Average	0.68	0.71	0.08

[A] Level of inbreeding due to nonrandom mating within populations

[B] Overall inbreeding coefficient

[C] Population subdivision

0.08 (Table 5). Positive F_{ST} values are usually interpreted as an indicator of divergence among subpopulations or spatial genetic structure that could be the result of drift, assortative mating and natural selection, processes reinforced by restriction of gene flow among populations. In most of the wind-pollinated tree species studied, F_{ST} values tend to be lower than 0.10, demonstrating that more than 90 % of the neutral genetic variation is maintained within populations (Malvolti et al. 1993). The inbreeding coefficient (F_{IS} = 0.68, Table 5) indicated that all of the polymorphism loci showed significant heterozygote deficiency. This phenomenon was attributed to high inbreeding rate at each of the examined locus. In addition, the overall inbreeding coefficient (F_{IT}=0.71, Table 5) is greater than that within populations (F_{IS} = 0.68). The result implies that the mean genetic variability were under-estimated due to the Wahlund effect. Wahlund effect occurs when populations are subdivided due to restrict gene flow (Hartel and Clark 2007). F_{IS} and F_{IT} values are unusually high for a wind-pollinated species and indicate a considerable amount of inbreeding. The large degree of inter-population differentiation and the high within population variability could be attributed to the homogenizing effect of wind pollination or more in general of efficient out crossing (Hamrick et al. 1991). On the other hand, these stands are human origin, and anthropogenic effects may be relevant as

Table 6 Nei's (1978) similarity measures (S_{ij}, above diagonal) for the six walnut populations based on 17 simple sequence repeats

Population	Hanza	Gogher	Kiskan	Rayen	Bidkhan	Bezenjan
Hanza (HA)	****	0.77	0.82	0.67	0.85	0.81
Gogher (GO)		****	0.80	0.71	0.81	0.75
Kiskan (KI)			****	0.69	0.81	0.81
Rayen (RI)				****	0.68	0.62
Bidkhan (BI)					****	0.73
Bezenjan (BE)						****

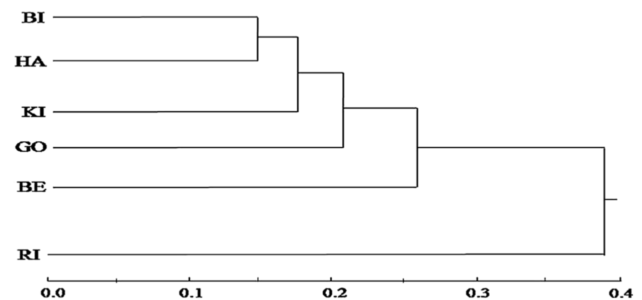

Fig. 2 Neighbor-Joining dendrogram showing genetic distance of the studied populations of Persian walnut based on SSR markers

well. The average F_{ST} value is 0.08, indicating that 92 % of genetic variability occurred among the six populations studied (Table 5). According to (Wright 1978), F_{ST} values of 0.05–0.15 indicate moderate differentiation among populations. The relatively low H_o within Persian walnut populations in Kerman province valleys could be explained by unusual levels of self-pollination or sib-mating (or anthropic effect). In a study of the genetic variation in Italian populations of *J. regia*, the means of F_{IS}, F_{IT} and F_{ST} were −0.004, 0.08 and 0.085, respectively (Malvolti et al. 1993). In another study, which analyzed the genetic structure of 21 stands of walnut in central Italy, F_{IS}, F_{IT} and F_{ST} were −0.22, −0.6 and 0.15, respectively (Malvolti et al. 1994). The F_{ST}, F_{IS} and F_{IT} values in *J. sinensis* in Korea were 0.122, 0.156 and 0.258, respectively (Lee and Lee 1997). In study of Pollegioni et al. (2011) the means of F_{IS}, F_{IT} and F_{ST} were 0.022, 0.074 and 0.054 respectively.

Genetic distance among populations

Analysis of the pairwise genetic similarity of the populations (Table 6) showed that the range of S (similarity) as defined by Nei (1978), was from 0.62 to 0.85. The RI population was, on average, the most genetically distant from the other populations (S = 0.67), and KI was the least distant (S = 0.78). The neighbor-joining dendrogram using genetic distance measures on SSR markers data among the populations (Fig. 2) showed that the RI population lay relatively far from the HA (S = 0.67) and BE (S = 0.62)

populations (Table 6). Furthermore, the HA, BI, KI and GO populations were in the same group, the BE and RI populations lay in the second and third group, respectively (Fig. 2). The most genetic similarity was observed between BI and HA populations ($S = 0.85$) (Table 6). Because HA is an ancient walnut-growing region that contains trees about 2,500 years (as the growers mention), it is possible that walnut growers of the BI valley obtained walnut seeds from this area.

The topology of the dendrogram suggests geographical isolation. This isolation could be due to the presence of hills or mountains as geographical barriers between the valleys of the area. This could be the case in the HA population, which is geographically close but genetically distant from the RI population, moreover small sample size of RI population may be reason of these results. Consequently, any correlation was not between genetic distance and geographical distribution of populations from Kerman. In study of genetic variation of *J. sinensis* in Korea, there was little relationship between geographic and genetic distance between pair populations (Lee and Lee 1997).

It is possible that selection based on unexamined ecological or environmental factors (e.g. rainfall, soil type or also manipulation) has contributed to the divergence and similarity of the populations that were sampled.

The results of this study illustrate the effectiveness of SSR markers for discriminating among genotypes. The results also show that the populations of the valleys of Kerman province have a high level of diversity that can be useful for future breeding programs. Additional collections and further analysis could improve and confirm the above results.

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