Journal of Nuts 5(2):21-31, 2014
ISSN: 2383 - 319x

Morphological and Molecular Evaluation of Persian Walnut Populations in Northern and Western Regions of Iran

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Received: 27 September 2014 Accepted: 10 December 2014

Abstract

In this study, morphological characteristics as well as ten of Simple Sequence Repeat (SSRs) loci were used to analyze the genetic diversity and relationships among 12 natural populations of Persian walnut (*Juglans regia* L.) in northern and western regions of Iran. The results showed that there was a high level of genetic diversity among the walnuts, both in terms of their SSRs loci as well as morphological traits. The nut weight ranged from 11.5 to 17.2 g, kernel weight from 3.2 to 6.3 g, and kernel percentage from 28 to 46.7%. In SSRs analysis, the number of alleles per locus ranged from 6 to 11, with a total of 83 alleles and average of 8.3 alleles and 4.9 effective alleles per locus. The expected heterozygosity (He) varied between 0.70 and 0.87, with an average of 0.79 per locus. The proportion of genetic differences among the walnut populations accounted for 19% of the total variation. The overall gene flow among populations equaled 1.10. The 12 walnut populations were separated into four main groups via the unweighted pair group method (UWPGM) with arithmetic mean cluster analyses based on Nei's unbiased genetic distances.

Keywords: Gene flow, Genetic diversity, *Juglans regia*, Microsatellite, Molecular marker

Introduction

The genus *Juglans* includes 21 species, of which the Persian walnut (*Juglans regia* L.) is the most economically important cultivated species in worldwide. Walnut stands are found in Kopet Dagh, the low land along the southern shore of the Caspian Sea, and in sporadic locations through the ranges of western and southern regions in Iran (Leslie and McGranahan, 1988). Walnuts were presumably domesticated in Iran and Afghanistan and subsequently introduced to China, Russia and Eastern Europe by ancient tribes (Vahdati 2000, Karimi *et al*., 2010). The major producers of this species are China, Iran, USA and Turkey (FAO, 2012). Recent research on the high nutritional value of walnut (Pereira *et al*., 2008), its beneficial effect on human health derived from its high antioxidant capacity (Anderson *et al*., 2001), as well as its high ω-3 fatty acid concentration (Ros and Mataix, 2006) have resulted in an increased world demand for walnuts (FAO, 2012). Genetics analysis can provide data on a variety of important evolutionary parameters, including genetic variation, the partitioning of variability among populations and the overall levels of inbreeding, selfing versus outcrossing rates. In addition to providing basic evolutionary insights, such analyses are also an important tool for developing effective

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management strategies for endangered and invasive species (Sakai *et al*., 2001). Since walnuts are 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 walnut populations (Fatahi *et al*., 2010).

Several techniques have been developed that can be used to estimate the genetic diversity in walnuts, including morphological characteristics (Atefi, 1997) and various molecular markers, such as isozymes (Fornari *et al*., 2001), restriction fragment length polymorphism (RFLP) (Fjellstrom and Parfitt, 1994), randomly amplified polymorphic DNA (RAPD) (Fatahi *et al*., 2010), inter simple sequence repeat (ISSR) markers (Potter *et al*., 2002) and simple sequence repeat (SSRs) markers (Wang *et al*., 2008; Karimi *et al*., 2010). In order to investigate the walnut population structure and to facilitate various programs in genetic improvement, forest restoration, conservation, and sustainable management of *J. regia*, suitable molecular markers are required. Simple sequence repeats (SSR) or microsatellites are hypervariable, codominant and highly informative, because they are ideal for studies in population and conservation genetics (Streiff *et al*., 1998) and for the analysis of variation between species and populations (Queller *et al*., 1993). The objectives of this study were to determine the level and distribution of genetic diversity among 12 populations of *J. regia* from western and northern regions of Iran using some morphological traits and 10 SSR primer pairs.

Materials and Methods

Plant materials

Samples from 12 populations of *J. regia* were collected from northern and western regions of Iran. The study included five accessions from different parts of the northern province of Golestan: Galikesh (GK), Afratakhteh (AT), Kalaleh (KL), Kordkouy (KK) and Cheshmeh-Juzi (CJ), four accessions from different parts of the western province of Hamedan including Pary-Zanganeh (PZ), Simin (SM), Timijan (TN), and Golzar (GZ), and one accession from another western provinces of Lorestan (Ali-Gudarz or AG), Kermanshah (Sahneh or SH), and Kurdestan (Kamyaran or KY) (Table 1).

Table1. *Juglans regia* L. populations surveyed and their ecological and geographical

Population	Regions in Iran	Altitude (m)	Longitude (m)	Latitude (m)	Annual rainfall (mm)	Annual avgerage temperature (°C)
PZ	west	1729	48°52′	33°82′	305	14.05
SM	west	1626	48°23′	34°42′	300	10.90
TN	west	1780	48°01′	34°11′	302	11.52
GZ	west	1760	48°05′	34°26′	302	11.41
AG	west	1986	49°64′	32°81′	469	11.09
SH	west	1385	47°32′	34°11′	392	13.81
KY	west	1445	46°52′	34°63′	382	14.02
GK	north	750	55°47′	37°14′	450	18.40
CJ	north	1200	55°70′	36°50′	500	19.50
AT	north	1550	55°56′	36°57′	550	18.20
KK	north	250	54°60′	36°52′	800	18.70
KL	north	850	55°49′	37°31′	400	19.00

Populations consisted of old walnut trees from open-pollinated seedlings. Seven western (SM, PZ, AG, TN, GZ, SH and KY) and five northern populations (GK, CJ, KK, KL and AT) inhabit disjunctive mountainous areas with a relative narrow geographic range of latitude and longitude. The distribution of the sampled populations was from 250m to 1986m altitude. Populations were surveyed and their ecological and geographical traits are presented in Table 1. Walnut genotypes within 10 km of each other were considered to belong to the same deme.

Morphological analysis

In the study, 204 genotypes from 12 native populations were evaluated based on 32 qualitative and quantitative traits. During harvest season, 15–20 nuts from each genotype were randomly collected and

evaluated according to the walnut international descriptor (IPGRI; Eriksson, 1998). Our sampling was limited to 15-24 genotypes per population, each genotype separated by a distance of 100m.

Molecular analysis

DNA extraction

For each mother tree, at least ten young leaves were collected in early morning hours during the summer. The leaves were used for DNA extractions. A 150- to 200-mg sample was placed in 2-mL Eppendorf tubes with 1800 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.

Polymerase chain reaction amplification and electrophoresis

Ten 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; Dangle *et al*., 2005) were used to amplify the genomic DNA of each individual plant from the 12 populations in order to identify polymorphic SSR loci (Table 3). Polymerase chain reaction (PCR) reactions were performed according to the protocol optimized by Dangle *et al.* (2005). Amplification reactions were performed in a volume of 15 ml containing 1x PCR buffer, 25 ng genomic DNA, 200 mM dNTPs, 0.2 mM of each primer, 2 mM $MgCl_2$, and 0.5 unit of Taq DNA polymerase (CinnaGen, Iran). PCR amplifications were carried out in a thermocycler (Ependroph, Germany) using the following temperature cycles:(1) one cycle of five minutes at 94°C; 30 cycles of one minute at 94° C, 40s at the appropriate primer annealing temperature at 72° C; and (3) a one cycle of two minutes for final elongation at 72°C, (Table 3). PCR products were denatured by adding 5µLformamide loading dye (80% deionized formamide, 10mM EDTA pH: 8, 0.05%xylene cyanol, 0.05% bromophenol blue), heated for five minutes at 94◦C. Then, 5µL of denatured preparations were loaded on a pre-warmed (50◦C) 6% polyacrylamide sequencing gel (Bio Rad, Sequi-Gen GT). Gels were run for 1–1.5 hours at 60W and the DNA bands were visualized by silver staining as described by Bassam *et al*. (1991). In all cases, PCR reactions were performed at least twice to ensure that the allele sizes were consistent. Allele sizing and scoring was done using a 100bp DNA ladder (MBI-Fermentas, Vilnius, Lithuania) as the length reference.

Data analysis

Analyses of morphological traits were carried out using SAS software package (SAS Institute Inc., Cary, NC, USA Version 9.2). The mean values for each parameter of a given genotype were used to perform statistical analysis of morphological traits. The polymorphic SSRs allele data were also fed in the form of single-individual genotypes. The following parameters of genetic variation were assessed for each population: the mean number of alleles per locus (A), the effective number of alleles (Ae), percentage of polymorphic loci (P), expected heterozygosity (He) (Nei, 1978), and observed heterozygosity (Ho) by using POPGENE Version 1.32. Departures from the Hardy-Weinberg (H-W) equilibrium were assessed at each locus for every population and per locus across all populations using F-statistics of Wright (1978). The significance of the deviations was evaluated using a chi-square test following the method of Workman and Niswander (1970). Spatial genetic structure was further investigated using Wright's analysis of hierarchical F-statistics (Wright, 1978). Nei's (1978) unbiased genetic distances were calculated for all population pairs and used to construct a phylogenetic tree [unweighted pair group method (UPGMA) with arithmetic mean]. All of these calculations were performed using POPGENE Version 1.32 (Yeh *et al*., 1997). An analysis of molecular variance (AMOVA) was conducted separately for each population using GenAlEx 6 software (Peakall and Smouse, 2006).

Results

Morphological analysis

Morphological analysis was carried out on the basis of some main qualitative and quantitative traits. A high morphological variability was observed within 12 populations (Table 2). The mean nut weight of 11.5 g (Cheshmeh-Juzi) to 17.2 g (Galikesh) was e over 13 g in 6 populations and over 15 g and 17 g in four populations. In terms of kernel weight, all of the populations had kernel weights of more than 4 g, except the Cheshmeh-Juzi and Ali-Gudarz populations. Timijan, Afratakhteh and Kalaleh ranked first (6.3, 5.7 and 5.7 g, respectively), followed by Golzar, Pari-Zanganeh (5.3 g) and Galikesh, Kordkouy (5 g). The highest and lowest kernel weight variability was found in the Kordkouy (9.5- 26 g) and Sahneh (12.5-20.2 g) populations, respectively (Data not shown). Kernel percentage varied from 28% (Cheshme-Juzi) to 46.7% (Timijan). Kalaleh (40.5%), Golzar (38.7%) and Afratakhteh (38.1%) populations had the highest kernel percentage (Table 2).

Table 2.Variability in phenological and pomological traits among studied walnut populations.

population	Number of individual	Flowering period*	Kind of dichogamy**	Maturity time***	Nut characteristics(mean ± SD)				
					Nut weight (gr)	Nut length (mm)	Nut width (mm)	Kernel weight (gr)	Kernel percentage (%)
PZ	20	20	PR	16	15.81±0.38	35.85±0.45	30.66±0.70	5.44±0.23	34.20±0.82
SM	20	23	HG	20	14.53±0.43	34.97±0.56	31.45±0.44	4.95±0.30	33.73±0.39
TN	20	23	PR	20	13.50±0.49	33.85±0.67	29.68±0.54	6.33±0.43	46.74±0.48
GZ	20	23	PR	20	13.74±0.29	32.86±0.82	29.63±0.53	5.34±0.44	38.79±0.70
AG	20	29	PG	16	11.94±0.72	31.43±0.34	27.93±0.38	3.51±0.39	29.43±0.48
SH	20	17	HG	16	14.57±0.51	33.97±0.45	30.45±0.46	4.90±0.49	33.83±0.59
KY	20	17	HG	16	13.61±0.33	33.54±0.44	29.24±0.63	4.83±0.52	36.14±0.34
GK	24	30	HG	06	17.28±0.21	35.10±0.34	32.33±0.40	5.05±.0.27	29.89±0.54
CJ	20	10	HG	06	11.52±0.34	32.33±0.41	27.20±0.66	3.24±0.32	28.08±0.54
AT	20	10	PR	16	15.33±0.32	35.81±0.58	29.56±0.54	5.75±0.36	38.16±0.52
KK	15	30	PR	16	15.01±0.39	32.67±0.49	28.83±0.47	5.03±0.40	35.31±0.44
KL	20	30	PR	16	14.44±0.54	34.88±0.44	29.93±0.49	5.75±0.32	40.50±0.45

*Day after reference standard (for maturity time, reference standard was considered 1,Sep., 2011).
** PR: protandrous; PG: protogenous; HG: homogamous.
*** Day after reference standard (for Flowering period, reference standard was considered 25, Mar., 2011)

Molecular analysis

Based on the SSR analysis, a total of 83 alleles were scored with the sizes ranging from 160 to 275 bp (Table 3). The number of alleles per locus varied from 6 to 11, with an average of 8.3 alleles per locus. All 10 loci were polymorphic across all 12 populations sampled. Forty of the alleles had an overall frequency greater than 0.1; the remaining 43 alleles were rare with a frequency less than 0.1. No private alleles (alleles present only in a single population) were detected. Forty alleles were common to the majority of the populations (at least ten populations), whereas the other alleles were shared among fewer populations. A summary of the loci and their genetic diversity parameters are presented in Table 3.

Table 3. Walnut SSR loci used in the present study and their Genetic diversity parameters*.

SSR Locus	Primer pair sequences	Ta (°C)	Size range (bp)	Na	Ne	Ho	He
WGA1	TTGGAAGGGAAGGGAAATG CGCGCACATACGTAAATCAC	56	176-210	6	3.70	0.672	0.732
WGA9	CATCAAAGCAAGCAATGGG CCATTGCTCTGTGATTGGG	56	230-275	9	5.34	0.627	0.815
WGA3	CTCGGTAAGCCACACCAATT ACGGGCAGTGTATGCATGTA	57	160-204	8	6.30	0.642	0.843
WGA7	ACCCGAGAGATTTCTGGGAT GGACCCAGCTCCTCTTCTCT	57	210-230	8	4.22	0.602	0.765
WGA8	ACCCATCTTTCACGTGTGTG TGCCTAATTAGCAATTTCCA	60	210-234	7	4.00	0.642	0.752
WGA2	CCCATCTACCGTTGCACTTT GCTGGTGGTTCTATCATGGG	59	178-198	11	4.29	0.786	0.769
WGA2	CTCACTTTCTCGGCTCTTCC GGTCTTATGTGGGCAGTCGT	56	190-230	11	7.54	0.781	0.870
WGA3	TCCAATCGAAACTCCAAAGG GTCCAAAGACGATGATGGA	58	224-262	10	5.80	0.710	0.830
WGA3	ACGTCGTTCTGCACTCCTCT GCCACAGGAACGAGTGCT	56	210-240	6	3.35	0.413	0.703
WGA3	TGGCGAAAGTTTATTTTTTGC ACAAATGCACAGCAGCAAAC	55	186-210	7	4.90	0.864	0.669
Mean	-	-	-	8.3	4.94	0.655	0.788

Primers developed by Woeste et al. (2002). Ta= annealing temperature Na = number of alleles. Ne = number of effective alleles.Ho = observed heterozygosity;He = expected heterozygosity

Genetic diversity parameters within the walnut populations based on allelic frequencies are shown in Table 4. In individual populations, the mean number of alleles per locus (A) varied from 3.60 to 5.40 with an average of 4.30, whereas the effective number of alleles per locus (Ae) varied from 2.53 to 3.66 with an average of 3.04. The observed heterozygosities (Ho) ranged from 0.525 to 0.713 with an average of 0.659. The average expected heterozygosity (He) was 0.657 and ranged from 0.593 to 0.725.A comparison of the genetic diversity of *J. regia* was performed among the 12 populations. The highest levels of diversity existed in population KL (He = 0.725), whereas the lowest values were in population TN (He =0.593).

Table 4. Genetic variation within *J. regia* L. populations based on 10 microsatellite loci*.

Population	N	A	Ae	Ho	He
PZ	15	3.80	2.62	0.713	0.627
SM	15	4.30	3.05	0.713	0.678
TN	15	3.90	2.53	0.647	0.593
GZ	15	4.20	3.00	0.700	0.643
AG	15	3.60	2.56	0.671	0.611
SH	15	4.40	3.07	0.700	0.678
KY	15	3.80	2.74	0.613	0.625
GK	24	5.40	3.66	0.642	0.696
CJ	20	5.00	3.20	0.675	0.670
AT	20	5.10	3.54	0.525	0.704
KK	15	3.90	2.87	0.675	0.632
KL	20	5.30	3.64	0.635	0.725
Mean	17	4.30	3.04	0.659	0.657

*N = sample size;A = mean number of alleles; Ae = effective number of alleles per locus;Ho = observed heterozygosity; He = expected heterozygosity.

The genetic analyses revealed high levels of differentiation among populations. The coefficient of hierarchical FST (Table 5), estimated according to Wright (1978), ranged from 0.091 for locus WGA1 to 0.256 for locus WGA332 with the average value equaling 0.186. The highest FST value was found in

WGA332, WGA89 and WGA321 loci. However, significant departures from the H-W equilibrium were observed in WGA276, WGA202, and WGA321 loci. The overall gene flow (Nm) among populations was 1.10. The mean FIT value of 0.156. Within populations, five loci showed a surplus of heterozygotes (FIS ranged from –0.012 to 0.717).

Table 5. Relative measurements of genetic differentiation and the estimates of gene flow among populations of *J. regia.**

SSR Locus	F_{IS}	F_{IT}	F_{ST}	Nm
WGA1	0.005	0.095	0.091	2.49
WGA9	0.024	0.223	0.205	0.97
WGA32	0.034	0.225	0.199	1.06
WGA71	0.717	0.181	0.118	1.86
WGA89	-0.012	0.138	0.231	0.83
WGA202	-0.255	-0.029	0.180	1.14
WGA276	-0.783	0.105	0.170	1.23
WGA321	-0.145	0.124	0.235	0.81
WGA332	0.145	0.364	0.256	0.73
WGA349	-0.028	0.148	0.171	1.21
Mean	-0.036	0.156	0.186	1.10

*F_{IS} = fixation index within populations; F_{IT} = fixation index with respect to total populations; F_{ST} =fixation index among populations; Nm= Gene flow estimated from $Nm = 0.25 (1 – F_{ST})/F_{ST}$.

Genetic distances were calculated for each pair of populations to estimate the extent of their divergence (Table 6). The average genetic distance among populations equaled 0.452. The lowest genetic distance (0.094) was found between populations TN and GZ. The greatest genetic distance (0.731) was found between populations KY and KK. The UPGMA cluster analyses based on Nei's unbiased genetic distances was performed to show the genetic relationships among the populations (Fig.1). The dendrogram separated the 12 populations into four main groups. The first group consisted of the populations PZ and KY. The second group consisted of the populations SM, TN, GZ, AG and SH. In this group, populations TN and GZ as well as AG and SH clustered together while population SM differs considerably from the others. Within the third group, the population KK was distinct from the others (GK, CJ). The populations AT and KL were the most similar and were clustered in the fourth group of the present study.

Table 6. Nei's unbiased measures of genetic distances among 12 Persian walnut populations (Nei, 1978)

Population	PZ	SM	TN	GZ	AG	SH	KY	GK	CJ	AT	KK	KL
PZ	0.000											
SM	0.300	0.000										
TN	0.501	0.170	0.000									
GZ	0.441	0.155	0.094	0.000								
AG	0.381	0.238	0.283	0.147	0.000							
SH	0.222	0.244	0.331	0.258	0.143	0.000						
KY	0.295	0.338	0.359	0.362	0.368	0.234	0.000					
GK	0.505	0.507	0.604	0.550	0.475	0.520	0.580	0.000				
CJ	0586	0.622	0.667	0.647	0.631	0.605	0.661	0.147	0.000			
AT	0.557	0.550	0.565	0.5811	0.647	0.583	0.605	0.397	0.302	0.000		
KK	0.593	0.672	0.667	0.634	0.678	0.679	0.731	0.277	0.241	0.405	0.000	
KL	0.590	0.536	0.578	0.522	0.575	0.594	0.660	0.329	0.354	0.268	0.279	0.000

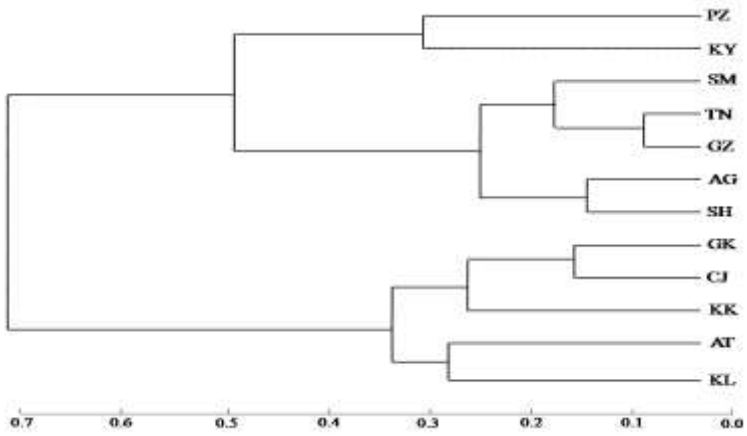

Fig.1. UPGMA dendrogram of 12 *Juglans regia* L. populations in west and north of Iran based on Nei's unbiased genetic distances.

Discussion

In northern and western Iran, we observed a high genetic diversity in 12 walnut populations based on both morphological traites and SSR primer paires. Kernel weight and kernel percentage for some the populations (Timijan, Kordkouy and Kalaleh) were even higher than those reported for some Iran commercial cultivars (Haghjuyan 2001). The variability found in the present study is in agreement with that reported for the Eurasian walnut distribution range from Iran (Atefi, 1997), India (Sharma and Sharma, 2001) and Turkey (Akccedila and Sen, 2001).The investigated populations also differed in their bud breaking time. The time of walnut bud breaking did not only depend on the general climatic conditions of the area, but also on the specific weather conditions in different years. The phenomenon of variation in bud breaking time in early genotypes is also reported by Solar et al., (2002). The proportion between early and late genotypes found in this study is in agreement with those reported by Atefi (1997). Some of the evaluated morphological traits such as nut size, flowering period, nut maturity time and phenological characteristics for each population are provided in Table 2. Persian walnut is a species with a very wide distribution range. Populations and individuals located in different ecological niches have differentiated adaptive traits due to natural selection, limited gene flow and limited phenotypic plasticity (Eriksson, 1998). Native walnut populations in the present study conserve as the core of future germplasm collections due to their high variability in nut production and forestry traits.

Previous studies have shown that SSR analysis were useful for discriminating the variations within walnut populations (Wang *et al*., 2008; Karimi *et al*., 2010). Wang *et al*. (2008) applied some of these SSRs markers on different walnut populations in China and observed a total of 73 alleles with the sizes ranging from 153 to 306 bp. In the present study, the observed numbers of alleles were lower than that those reported in *J. nigra* population (Victory *et al*., 2006), the species from which the markers were originally developed (Woeste *et al*., 2002). This may be because of the large number of samples and populations analyzed in previous *J. nigra* studies, or because SSRs may have shown a decrease in allele number when applied in related species (Ana *et al*., 2000).

The observed heterozygosity within the walnut populations (mean Ho= 0.66) was higher than that detected in *J. regia* population based on SSR (mean He = 0.63) (wang *et al*., 2008). However, amounts lower than the heterozygosity of 0.80 was reported in wild populations of *Juglans nigra* (Victory *et al*., 2006), a species that is also highly outcrossed. According to results, 18.6% of the total genetic diversity existed among populations and 81.4% within populations. This result was consistent with the

variation models reported in an emophilous pines (Yeh and Layton, 1997). The high level of inter population differentiation might result from the differentiation of habitats, such as climate, temperature, annual rainfall, or landform features (Wang *et al*., 2008). The FST value detected here in *J. regia* was higher than the differentiation among populations observed in *J. nigra* (FST = 0.058) based on microsatellite markers (Victory *et al*., 2006) and is much closer to the value FST = 0.186 observed by Wang *et al*. (2008) using SSR on different walnut populations in China. This means that there are some processes that isolate the populations, and that they may have unique genetic qualities that can be applied in breeding. It could indicate that there are selection pressures operating in each population that are different from the other populations.

The loci WGA332, WGA89 and WGA321 possessed the highest FST value. These values indicated the important role of these three loci in inter population differentiation. In individual populations, tests for the departure from the H-W equilibrium showed significant deviations for at least one locus in every population. Most of the loci followed the H-W equilibrium in the majority of populations. However, significant departures from the H-W equilibrium were observed in WGA276, WGA202, and WGA321 loci. The deviations were primarily the result of the surplus of heterozygotes. The overall gene flow (Nm) among populations was 1.10, which gave an estimate of the average number of migrants between all studied populations per generation. The observed values indicated that gene exchange between populations was low.

FIT is the overall inbreeding coefficient of an individual relative to the whole set of populations, whereas FIS is the inbreeding coefficient of an individual relative to its own population. The mean FIT value of 0.156 indicates that the sampled populations of *J. regia* had a slight deficiency of heterozygotes and that there was inbreeding within the populations. Five loci showed a surplus of heterozygotes (FIS ranged from –0.012 to 0.717). The average FIS value equaled –0.036, which was significantly different from zero (Table 5). This means that there was a small heterozygote excess among the sampled trees. However, it is possible to get an excess of heterozygotes if stutter bands are erroneously scored as alleles. The negative values of FIS in the genetic diversity suggests an excess of heterozygosity present in the populations of walnut, which could be attributed to walnut's mating system (Fornari *et al*., 2001)It could also be caused by selection or disassortative mating.

Genetic distances were calculated for each pair of populations to estimate the extent of their divergence. The highest levels of diversity existed in population KL (He =0.725) because of high plant density, isolation, and lack of human selection, whereas the lowest value was observed in population TN (He =0.593). The relatively low diversity level in population TN is attributable to the human propagation manner, leading to the selection of trees with the most suitable traits, negatively affecting genetic variability (Fornari *et al*., 1999). Populations TN and GZ were closely located geographically in Hamedan province and were closely related (D = 0.094). The genetic parameters estimated from the microsatellite data indicated that there were substantial levels of genetic diversity in all sampled populations. This result is in accordance with the common observation of high levels of variation detected in wind-pollinated, long-lived tree species (Streiff *et al*., 1998; Victory *et al*., 2006). The 12 natural populations of *J. regia* studied here occupy fragmented habitats, as the populations were confined to islands that varied in size and degree of isolation within the northwestern Kurdestan province and southeastern Lorestan province and northern Golestan province.

Conclusions

Using morphological traits and 10 SSR markers, this study assessed the patterns of genetic diversity

and structure of 12 Persian walnut populations from northern and western Iran. The standard UPOV (1999) guidelines were used to distinguish walnut populations. A wide range of variation was observed in nut and kernel characteristics as well as in other traits such as phenological characteristics. The variations indicated that there was a high level of morphological diversity across all populations of *J. regia*, which are attributable to its reproductive strategies based on outcrossing to prevent self-pollination and inbreeding (Fornari *et al*., 1999). Molecular information on the nature and the degree of genetic diversity present in walnut populations could help identifying elite trees for genetic improvement through hybridization.

Briefly stated, we observed: 1) considerable genetic diversity across populations of *J. regia* in Iran, both within and among populations; 2) the relatively high inter population differentiation of *J. regia*, possibly resulting from factors such as restricted gene flow between populations and human disturbances; 3) the capability of *J. regia* to maintain a noticeable intra population differentiation, even in the presence of selection pressures, which might be attributable to its reproductive strategies based on outcrossing and 4) microsatellite markers, which are powerful tools for monitoring the genetic structure and diversity of *J. regia* populations. Future and further research in *J. regia* can be performed more extensive sampling and more intensive sampling with microsatellite markers to help describe the population genetic structures of Persian walnut in Iran in greater detail.

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