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Genetic Diversity Evaluation of Some Walnut (*Juglans regia* L.) Genotypes in Meshkin-Shahr by ISSR Marker

Alireza Ghanbari[*1], Mohammad Faraji[1], Mahdi behnamian[1], Asgar Estaji[1], Adel Pyrayesh[2], Somayeh Fahim[1]

[1]*Department of Horticultural sciences, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran*

[2]*Horticultural Science Research Center, Agriculture Research Education and Extension Organization, Meshkin Shahr, Iran*

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ABSTRACT

Genetic resources are the most valuable national treasure of countries, being vital to identify, conserve and use them. The Persian Walnut (*Juglans regia*L.), a diploid species native to the mountainous regions of Central Asia, is the major walnut species cultivated for nut production and one of the most widespread tree nut species in the world. This research was conducted in Meshkinshahr in Ardabil Province. In this study, to determine the level of genetic diversity, a total of 31 walnut genotypes were analyzed using Inter Simple Sequence Repeats(ISSR) markers. Genotypes were evaluated using 10 ISSR markers and a total of 26 polymorphic alleles were identified.The average of the observed alleles was equal to 7 in each locus. The lowest and highest RP valueswere found ISSR5and ISSR9, respectivly.Cluster analysis based on Jacard similarity coefficient matrix using WARD method classified the genotypes into three main groups.The highest genetic distance was found between genotypes AK4 and RM2, and the lowest was among genotypes MZ4 and ES1.Both clustering and PCA divide genotypes with similar geographic origins.

Introduction

Walnut (*Juglans regia* L.) belongs to the family Juglandaceae. The genus Juglans consists of approximately 20 species having 32 chromosomes (Robert, 1930).

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. Persian walnut was probably domesticated Iran and Afghanistan and subsequently introduced to China, Russia and Eastern Europe by ancient tribes (Bayazit *et al*., 2007).

The Persian walnut (*Juglans regia* L.), also known as the English or common walnut, is native to the mountainous regions of Central Asia and distinguished from other walnut species by its dehiscent husk, thin shells and thin septum separating the kernel halves, traits which considerably facilitate kernel extraction. Annual worldwide Persian walnut production is estimated at 3.2 million MT. The leading producers are China (1.7 million MT) followed by Iran, the United States, and Turkey (FAO, 2014). Iran was ranked as the second largest walnut producer in 2012 by producing 450,000 t (FAO, 2015).

Several techniques have been developed to estimate genetic diversity in walnut, including morphological characteristics (Sharma & Sharma, 2001), and various molecular markers, such as random amplified polymorphic DNA (RAPD) (Nicese *et al.*, 1998), restriction fragment length polymorphism (RFLP) (Fjellstrom *et al.*, 1994), Inter-Simple Sequence Repeat (ISSR) (Potter *et al.*, 2002), simple sequence repeats or microsatellites (SSR) (Dangl *et*

[*]**Corresponding author**: Email address: ghanbari66@uma.ac.ir,ghanbari66@yahoo.com
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al., 2005; Foroni *et al.,* 2005; Victory *et al.,* 2006; Wang *et al.,* 2008), and amplified fragment length polymorphism (AFLP) (Bayazit *et al.,* 2007). However, RAPDs have low reproducibility, RFLPs are time-consuming and labor-intensive, SSRs require the knowledge of the flanking to develop species-specific primers and AFLPs have high cost (Reddy *et al.,* 2002). The ISSR markers are fast, inexpensive, hyper-variable, and appear to be suitable for genetic diversity studies (Reddy *et al.,* 2002). Moreover, within any plant or animal species, neutral mutations are well known to occur, and neutral genetic markers are maintained wile evolutionary forces acting on natural populations. Therefore, analyses of neutral markers, such as ISSRs can provide valuable information about genetic diversity and rates of evolutionary change within and between populations. ISSR markers have been successfully used for walnut cultivar identification and provided information on genetic relationships among cultivars (Potter *et al.,* 2002).ISSR markers have been successfully used in many tree species, including Walnut (*J. regia* L.) (Potter *et al.,* 2002; Pollegioni *et al.,* 2003), Olive (*Olea europaea* L.) (Terzopoulos *et al.,* 2005), Fig (*Ficus carica* L.) (Sahli-Hannachi *et al.,* 2005), Mulberry (*Morus* L.) (Vijayan *et al.,* 2006) and Plum (*Prunus domestica*) (Liu *et al.,* 2007).

Most of the results obtained by previous studies show that walnut has a high level of variation. Theobjective was to evaluate the genetic diversity of some walnut (*Juglans regia* L.) genotypes in the west of Meshkinshahr.Given that genetic diversity researches on the walnuts of Meshkinshahr region have been developed slowly in comparison with other regions in Iran .Therefore, research aim was to investigate genetic diversity walnut trees of this region. The implications of our study can be useful in future breeding programs of this plant species.

Materials and Methods

Plant Materials

The young leave samples were collected from non-grafted trees with the age of approximately 25–50 years from Ghasabehe, Majandeh and Andazgh regions in thewest of Meshkinshahr city. Meshkinshahr city is locatedin the central northern part of Ardabil, a province in the northwest of Iranwith altitude of 1490 m above sea level between longitudes 47° 190´and 48° 170´ East and latitudes 38° 570´ and 38° 130´ North.

DNA extraction

Genomic DNA was extracted from 100 mg of fresh leaves from each of the 31 cultivars by modified CTAB extraction method (Doyle & Doyle, 1987). Leaf material was ground to a fine powder in liquid nitrogen in a 2 ml tube. After adding 1 ml of extraction buffer [100 mM Tris-HCl, 2 M NaCl, 20 mM EDTA, 2% (w/v) PVP, pH 8], and 50 µl β-mercaptoethanol, the mixture was homogenized and incubated at 65˚C for 60 min and mix and thoroughly vortex. Following the incubation period, add an equal volume of 24:1 (v/v) mix of chloroform: isoamyl alcohol. After centrifugation at 11,000 g for 20 min, the supernatant was separated and mixed with 0.7 (v/v) volumes of cold isopropanol (-20˚C) and then, centrifugation at 10,000 g for 5 min the upper aqueous phase was separated. Precipitated DNA was washed in 70% (v/v) ethanol, and dried, and dissolved in 0.2 ml of double-distilled water. Quantities and qualitative of DNA samples were checked on the agarose gel and the 260 to 280 nm ratio. Genomic DNA samples were diluted to a final concentration of 10 ng/µl with 1 × TE (10 mM Tris-HCl, 0.1 mM EDTA, pH = 8.0) buffer and stored at -20ºC prior to the polymerase chain reaction (PCR) amplification.

ISSR analysis

PCR reactions were performed in a final volume of 15µl containing 7.5µlMaster Mix (Ampliqon,

Denmark), 0.625 µM primers,10 ng templates DNA and dH_2O up to the final volume. The PCR program was as follows: denaturation for 5min at 94 ◦C; then 35 cycles of 1minat 94 ◦C, 1min at annealing temperature of each primer, and 2min at 72 ◦C; with a final extension of5min at 72 ◦C. The PCR amplifications for all primers were processed using the same procedure. All amplified products were resolved by electrophoresis on 1.5% agarose gels.The DNA was visualised under a UV light, and the images were captured using a Kodak camera Gel Logic 1500 (Eastman Kodak, Rochester, NY, USA).

Data analysis

Each band was scored as present (1) or absent (0) and pairwise dissimilarity of genotypes with different dissimilarity functions was calculated by DARwin 5 (Dissimilarity Analysis and Representation for Windows) software Package Version 5.0.158 (Perrier *et al.*, 2003). Genetic distance and cluster analysis were calculated based on Jaccard similarity coefficient. The characterization of primers for their ability to differentiate the genotypes was assessed by calculating polymorphic information content (PIC), marker index (MI) and resolving power (Rp). PIC was calculated according to De Riek *et al*. (2001) as: PIC = 2i (1-fi), where 'f' is the band frequency in the data set. PIC for dominant markers was a maximum of 0.5 for 'f' = 0.5. PIC value of each primer was calculated as the averaged PIC values of its bands. MI of each primer was calculated according to Powell et al. (1996). PopGene software (Version 1.31; Yeh *et al.*, 1999) was used to calculate dissimilarity matrix and cluster analysis. Percentages of polymorphic loci (P), observed number of alleles (Na), effective number of alleles (Ne) and gene diversity (H) were analyzed.

Results

ISSR analysis

Seventy fragments were obtained from 10ISSR primer combinations ranging from 200 to 1480 bp, and were used for analysis in this study. The number of bands per primer ranged from 2 to 7 with a mean value of 7. The maximum number of fragments was found in ISSR9, and the highest level of polymorphism was found in ISSR9,ISSR6 and ISSR3,respectively. Ratio of polymorphic ISSR markers was 61.44%. The PIC values for the 10 primer combinations ranged from 0.2 (ISSR10) to 0.48 (ISSR3), with a mean of 0.31 (Table 1).Primers' MI values ranged from2 to 7, with primer ISSR10having the highest MI (Table 1). The primer resolving power (Rp) varied from 0.32to 3.29. Primer ISSR9showed the highest Rp value, whereas primer ISSR5had the lowest (Table 1).

Table 1. Some information on amplified fragments of ISSR primers used in this study

Primer	Locus	Primer sequence (5´→3´)	TotalFragments	Polymorphic bands	%Polymorphic	PIC	MI	Rp
ISSR1	UBC810	GAGAGAGAGAGAGAGAT	6	2	50	0.34	4	0.96
ISSR2	UBC811	GAGAGAGAGAGAGAGAC	6	2	50	0.31	3	0.7
ISSR3	UBC814	CTCTCTCTCTCTCTCTA	4	2	100	0.48	2	0.45
ISSR4	UBC834	AGAGAGAGAGAGAGAGYT	4	1	33	0.43	3	0.7
ISSR5	UBC823	TCTCTCTCTCTCTCTCC	3	1	50	0.23	2	0.32
ISSR6	UBC860	TGTGTGTGTGTGTGTGRA	6	3	100	0.35	3	0.51
ISSR7	UBC826	ACACACACACACACACC	6	1	20	0.3	4	0.45
ISSR8	UBC855	ACACACACACACACACYT	9	3	50	0.24	5	0.64
ISSR9	UBC817	CACACACACACACACA	14	7	100	0.23	6	3.29
ISSR10	UBC827	ACACACACACACACACG	12	4	50	0.2	7	2.06
Mean					61.44	0.31	3.09	1.008

The observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H) and Shannon's information (I) were 7, 5.10, 1.05 and 0.18, respectively.The number of observed alleles varied from3 (ISSR5) to 14 (ISSR9), with a mean value of 7.

The number of effective alleles ranged from 2.18 for ISSR5to 9.27 for ISSR10, with an average value of 5.10 for all primers studied. The Nei's gene diversity for primer ranged from 0.08 for ISSR6and ISSR2 to 0.21 for ISSR9, with an average of 1.05 (Table 2). The Shannon's Information Index for primer loci ranged from 0.07 for ISSR7to 0.36 for ISSR9, with an average of 0.16.

Table 2. Genetic parameters for 10 ISSR loci 31 genotypes of walnut

Primer	Na	Ne	H	I
ISSR1	6	5.07	0.15	0.22
ISSR2	6	4.41	0.08	0.15
ISSR3	4	2.24	0.1	0.22
ISSR4	4	3.92	0.16	0.22
ISSR5	3	2.18	0.07	0.14
ISSR6	6	3.28	0.08	0.17
ISSR7	6	5.26	0.04	0.07
ISSR8	9	6.38	0.05	0.09
ISSR9	14	9.02	0.21	0.36
ISSR10	12	9.27	0.11	0.19
Mean	7	5.10	1.05	0.16

Na, observed number of alleles; Ne, effective number of alleles (Kimura & Crow, 1964)(H) Nei's gene diversity; (I) Shannon's Information Index.

Genetic similarity

Genetic similarity is an important index to estimate of genetic similarities among genotypes. Based on Jaccard coefficient, the genetic similarity for each pair of genotypes was calculated. The minimum coefficient (0.02) was observed between MZ4 and ES1 genotypes and AH1 and AH2genotypes. The maximum coefficient was observed between AK4 andRM2.

Cluster analysis

The data obtained from ISSR analyses were used to perform the genetic similarity analysis among the 31 walnutgenotypes. Cophenetic correlation between the tree ultra-metric similarities and the similarity matrix was r=0.99 with $P < 0.001$. All genotypes used in this study were distinguished. Based on cluster diagram and Jaccard similarity coefficient, the 31 genotypes were divided by the genetic distance of 0.02 into three groups (Fig. 1). Group A consisted of 14genotypes including AH7, AH6, AH4, AA, MZ3, JM1, RM2, JM2, RM3, RM1, SS, UM, RB and AB; Group B included fourgenotypes, AH12, AH11, AH10 and AK4; and Group C included 10genotypes, NM, AH5, AH9, AH2, AH1, AH3, AK2, MZ4, AK1, ES1, ES2, AH8 and YS (Fig. 1).

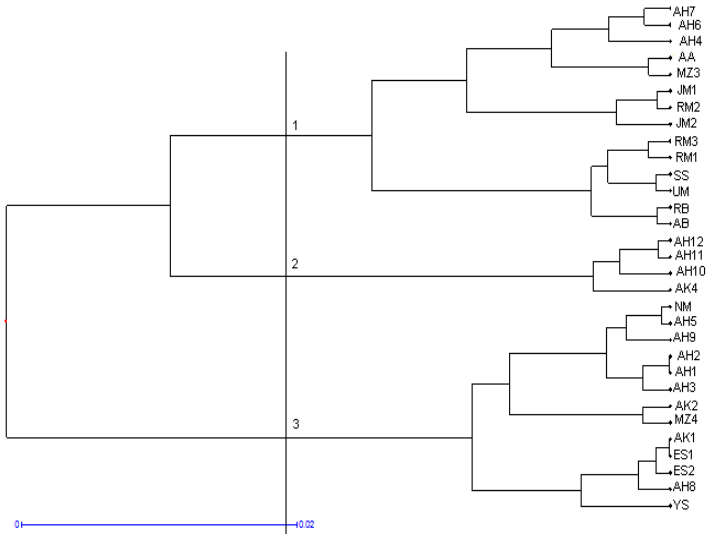

Fig. 1. Dendrogram of 31 walnut genotypes based on Ward's similarity coefficient.

PCA analysis

PCA was performed to reduce the number of effective traits in group characterization.Principal component analysis was performed based on the genetic similarity matrix to better understand the relationships between genotypes.The results were in accordance with the WARD cluster analysis with groupings among 31 walnutgenotypes. The percentages ofthe total variation in the similarities explained by PC1 and PC2 were 20.03% and 16.35%, respectively. The genotypes belonging to Group Awere AH7, AH4, AA, JM1, RM2, JM2, RM3, RM1, SS, UM, RB and AB(as inferred by WARD clustering),and they were mainly distributed in the right upright position of the resulting plot.The genotypes associated with Group Bwere AH12, AH11, AH10, AK4 and IM2 they werein the left and bottom position of the plot.The genotypesbelonging to the GroupCwere , NM, AH5, AH9, AH2, AH1, AH3, AK2, MZ4, AK1, ES1, ES2, AH8 and YS; they werein the right and bottom position of the plot (Fig. 2).

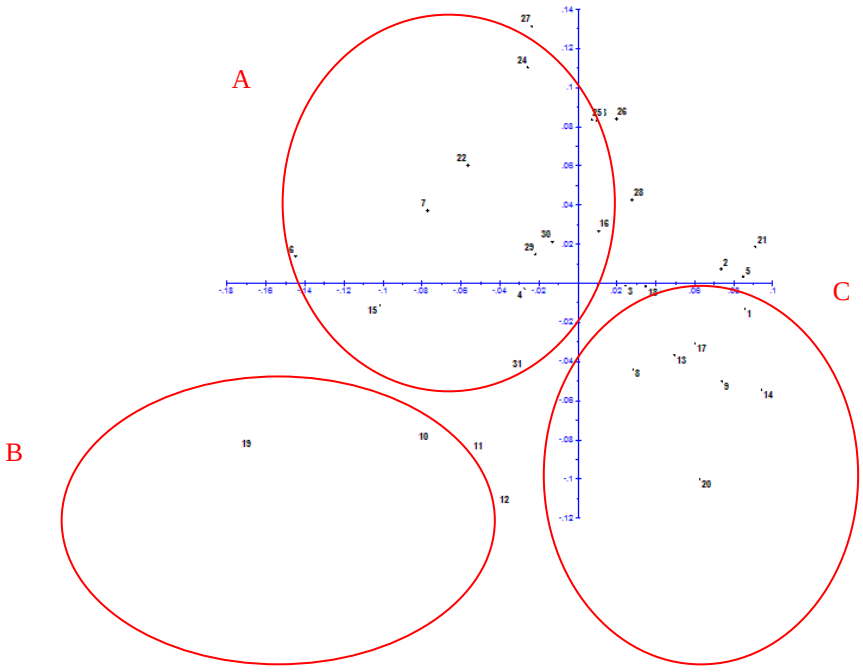

Fig. 2. Principal component analysis (PCA) score plot of 31 walnut genotypes based ISSR markers
1:AH1, AH2:2, AH3:3, AH4:4, AH5:5, AH6:6, AH7:7, AH8:8, AH9:9, AH10:10, AH11:11, AH12:12, ES2:13, ES1:14, MZ3 : 15, MZ4:16, AK1:17, AK2:18, AK4:19, YS:20, NM1:21, RM1:22, RM2:23, RM3:24 UM:25, AB:26, RB:27, SS:28, AA:29, JM1:30, JM2:31

Discussion

In general, ISSR markers are known to be useful in studies concerned with genetic diversity, phylogeny, gene tagging, genomic mapping and evolutionary biology (Reddy *et al.,* 2002). The study described in this article shows that ISSR markers are a good and reliable molecular tool to analyze genetic diversity and relationship in walnutgenotypes. In this respect, 31 walnutgenotypes from the west of Meshkinshahr were studied using 10 ISSR markers. A total of 70 fragments were obtained, of which26 ones showed polymorphism.

In the present study, 61.44% of polymorphism was detected by 10 primers and the number of polymorphic bands per primer ranged from 1 to 7 with an average of2.6.The highest level of polymorphism was observed in ISSR9 and ISSR6, ISSR3 (100%), therefore these primers with the highest level of polymorphism rather than another had more affect to determine genetic distance. The level of polymorphism (61.44%) was higher than RAPD (21.54%) (Albuin *et al.,* 2002) and ISSR (55%) (Pollegiono *et al.,* 2003). The source of polymorphism may include deletion of a priming site and insertion or deletion causing changes to the DNA fragment size (Pollegiono *et al.,* 2003).

Prevost and Wilkinson (1999) reported a strong and linear relationship between the ability of a primer to distinguish genotypes and Rp. Rp is the best index to choose effective primers, since it is also impacted by individuals with band and number of alleles (Zeinodini *et al.,* 2012). However, they also found that a high Rp was not the only prerequisite to distinguish many cultivars in certain cases. Therefore, primers Rp and MI based on band in formativeness and diversity index, are the parameters used identify primers with high discrimination ability (Zhi-Hui *et al.,* 2014).In the present study, the average Rp value obtained for the ISSR primers used was 1.008 and ISSR9, and ISSR10 had the highest Rp (3.29, 2.06) and MI (7, 6).Correlations between Rp and MI which indicate the ability of primers to distinguish genotypes (Zhi-Hui *et al.,* 2014).PIC can be determined based on both the number and frequency of the amplified fragments. Although the average PIC (0.31) in this study is low, PIC still confirms the good discriminatory capacity of the primers as a maximum PIC value of 0.5 for dominant markers (De Riek *et al.,* 2001).

To better understand the relationships among the genotypes, PCA results were conducted using the genetic similarities data set. PCA was congruent with the assignments generated by WARD clustering. The genotypes belonging to Group A (as inferred by WARD clustering)were mainly distributed in the right portion of the resulting plot. The genotypes belonging to the Group C were mainly distributed in the above and bottom left portion of the resulting plot. Both clustering and PCA divided genotypes with similar geographic origins and, PCA were comparable with WARD clustering.

To assess the relationships between genotypes, genetic distance was calculated. Genetic similarity matrix based on the WARD method, showed a narrow level of genetic diversity among walnut genotypes. For breeding programs, parents having a high genetic distance with each other, they would help to design breeding program. Genetic distance is one of the valuable yardstick to select parents in hybrid breeding. but in this study, most of the walnut genotypes had short genetic distance. The minimum genetic distance was observed between MZ4 and ES1 genotypes and AH1 and AH2 genotypes, It can be that close geographic distance between those. So thatthese genotypes were also located on one side of the slope, and these genotypes close together by pollination, accordingly genetic similarity increased over time. However, genotypesAK4 and RM2 had maximum genetic distance (0.30) between other genotypes. The genetic distance between the two genotypes was also due to the geographical distance between them. The geographical distance between AK4 and RM2 was more than 10 km and with natural obstacle. Most of

the variations observed were due to genotypes per geographic group.

In conclusion, ISSR marker technology is an inexpensive, easy and satisfactory method to assess genetic relationships and evaluate genetic diversity among walnut populations.

In this study, the main goal was genetic characterization of walnutgenotypes in the west of Meshkinshahr. This information can be used to generate of a core collection of walnut by eliminating of redundant genotypes and for walnut breeding, by identifying useful lines. In addition to collection management, our information on diversity and relationship in walnut genotypes will bealso useful for plant breeders to select germplasm samples with maximum diversity, to choose desirable parents for high yield, quality and resistance to biotic and abiotic stress conditions.

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