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Genetic Diversity of Persian Walnut from Iran as Revealed by Inter-Simple Sequence Repeat (ISSR) Markers

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Abstract

The relationships of 19 accessions and two cultivars of Persian walnut (*Juglans regia* L.) were investigated using ISSR analysis. Thirteen ISSR primers amplified a total of 208 fragments of which 182 were polymorphic (87.5%). Primer 841 produced the smallest number of bands (9) and primer 888 generated the largest number (23). Polymorphism percentage ranged from 69.2% (primer 828) to 100% (primer 868). Genetic similarity among genotypes ranged from 0.39 to 0.67. Cluster analysis was performed using NTSYSpc 2.02 software and revealed two main groups. ISSR markers could detect high polymorphism among studied accessions and identified DW and OR126 as having minimum similarity to KZ7 and KZ1, respectively. These accessions could be considered as good candidates for breeding programs.

Persian walnut (*Juglans regia* L.) is one of the most important cultivated species in temperate regions worldwide. It was domesticated in ancient Iran and subsequently introduced to the Carpathian Mountains of Eastern Europe, the Southern Caucasus, Turkey, China, India and other countries (1). According to the FAO (2007), Persian walnut grows in an area of about 70,000 ha. Seedling orchards in Iran, produce 170,000 tonnes of nuts in shell, ranking it third in production globally, after China and the United States of America (7, 13). The large number of seedling trees is the source of great variation and may offer genetic as well as breeding potential in Iran. A small number of breeding programs were started to identify and develop superior genotypes from the existing diverse genetic pool.

Traditional approaches to assess genetic variability in fruit crop species are time consuming and affected by environment. DNA-based markers can be used for fingerprinting and for direct comparison of genetic material independently of environmental influences (16). Estimation of genetic variation is important for effective conservation, management and utilization of germplasm (1, 21).

Molecular markers that have been used in walnut for assessing genetic diversity include Restriction Fragment Length Polymorphisms (RFLP) (9), Random Amplified Polymorphic DNA (RAPD) (16), Inter-Simple Sequence Repeat (ISSR) (4), microsatellite (1, 5, 10, 11, 12, 13, 17) and Amplified Fragment Length Polymorphism (AFLP) (3). The ISSR technique is a PCR-based method and involves the amplification of DNA fragments between two identical microsatellite repeat regions oriented in opposite directions (8). The advantage of the ISSR technique is that it is a combination of AFLP and microsatellite analysis with the universality of RAPDs (18). ISSR gives multi-locus patterns which are reproducible, abundant, polymorphic, highly informative and quick to use in plant genome studies (4). In the present study, our goal was to use the ISSR technique to investigate the genetic relationships and genotype identities among 19 accessions and two cultivars of Persian walnut.

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Table 1. Accessions used and their origin. The code used in Figure 1 is listed for each accession.

Code	Name	Original source	Code	Name	Original source
1	KZ1	Kahriz (Iran)	12	FA2	Fars (Iran)
2	KZ3	Kahriz (Iran)	13	KH53	Khorasan (Iran)
3	KZ4	Kahriz (Iran)	14	T26	Hamedan (Iran)
4	KZ6	Kahriz (Iran)	15	Alamot	Qazvin (Iran)
5	KZ7	Kahriz (Iran)	16	Z53	Karaj (Iran)
6	KZ8	Kahriz (Iran)	17	Pedro	France
7	KZ13	Kahriz (Iran)	18	Sebin	Turkey
8	KZ15	Kahriz (Iran)	19	Kaman	Turkey
9	OR126	Urmia (Iran)	20	Adilcevaz	Turkey
10	DW	Urmia (Iran)	21	Chandler	USA
11	OR60	Urmia (Iran)			

Materials and Methods

Plant material. The 19 walnut accessions and two cultivars were selected from the Kahriz Agriculture Research Station, located in the Northwest part of Iran (Table 1), based on a number of pomological and morphological traits associated with fruit quality.

DNA extraction. Young leaves were collected in the spring, ground in liquid nitrogen and stored at - 80˚C. The extraction was performed according to Doyle and Doyle (1987) with some modifications. In summary, 200 mg of frozen ground leaves were added to 500 µl of cetyltrimethyl-ammonium bromide (CTAB) buffer and incubated at 55˚C for 15 min. After centrifugation at 12,000 g for 5 min, the aqueous solution was extracted with 250 µl of chloroform-isoamyl alcohol (24:1) by centrifugation for 1 min at 13,000 rpm. The upper aqueous phase was transferred to a new tube followed by the addition of 50 µl 7.5 M ammonium acetate and 500 µl ice-cold absolute ethanol. The pellet was resuspended in 500 µl of 65˚C preheated H_2O with 1.25 µl RNase (10 mg·ml^{-1}) for 20 min. The DNA was precipitated, washed, dried and finally resuspended in 100 µl H_2O (6). The concentration and quality of the extracted DNA were determined using a Biophotometer (Eppendorf) and by 1% agarose gel electrophoresis, respectively.

ISSR amplification. ISSR primers were purchased from Cinnagen Inc, Tehran, Iran. Thirteen out of 22 ISSR primers produced a large number of reproducible, clear and scorable bands (Table 2). Polymerase chain reaction (PCR) was performed in 25 µl containing 1.5 µl DNA template (30 ng), 12.5 µl master mix (Cinnagen, Tehran) including 10X buffer, $MgCl_2$, dNTP and Taq DNA polymerase, 0.3 µl primer (100 pmol·µl^{-1}) and 10.7 µl deionized water. PCR reactions were carried out in a Veriti 96-well Thermal Cycler (Applied Biosystem) with the following steps: an initial denaturation at 94˚C for 3 min; 35 cycles of 94˚C for 30 s, 45 s optimum annealing temperature for each primer (Table 2) and 72˚C for 2 min; and then a final extension step at 72˚C for 10 min. The amplified fragments were separated by 2% agarose gel electrophoresis in 0.5X Tris-Borate-EDTA (TBE) buffer and examined under UV light. In all cases, two replicate experiments were carried out for each ISSR primer. To estimate the size of the amplified ISSR fragments, Gene Ruler 100 - 3000 bp (Fermentas) was used as the length reference.

Data analyses. The polymorphic ISSR bands in each gel were scored as present (1) or absent (0). Genetic similarity was calculated according to Jaccard's similarity index, using the similarity of qualitative data (simqual) routine in the NTSYSpc 2.02 software (20). Cluster analysis was performed using the complete linkage. The cophenetic correlation coefficient was calculated and a Mantel test performed to check the goodness of the fit between the similarity matrix and the dendrogram (14).

Table 2. List of ISSR primers, sequences, number of amplified fragments, number of polymorphic bands, percentage of polymorphism and optimum annealing temperatures (T_a).

Primers	Sequence[a] (5′-3′)	Amplified fragments	Polymorphic bands	Polymorphism percentage (%)	T_a (°C)
827	(AC)8G	16	15	93.7	47
814	(CT)8A	18	16	88.9	45
818	(CA)8G	17	16	94.0	50
811	(GA)8C	12	9	75.0	50
836	(AG)8YG	16	15	93.7	54
841	(GA)8YC	9	7	77.8	53
868	(GAA)6	15	15	100.0	53
825	(AC)8T	16	14	87.5	53
826	(AC)8C	18	16	88.8	53
828	(TG)8 A	13	9	69.2	53
830	(TG)8 G	15	12	80.0	56
848	(CA)8AGC	20	17	85.0	57
888	GAG(CA)7	23	21	91.3	58
Total		208	182	87.5	

[a]In the primer sequences, Y= C or T.

Results and Discussion

Thirteen selected ISSR primers generated 208 fragments that ranged in size between 280 to 3000 bp (Fig. 1). Of these, 26 bands were monomorphic and 182 were polymorphic in the 21 individuals. The number of amplified bands per primer varied from 9 in primer 841 to 23 in primer 888, with an average of 16. The number of polymorphic bands ranged from 7 to 21 with an average of 14 per primer. The percentage of polymorphic bands per primer ranged from 69.2% (828) to 100% (868) with an average of 87.5% (Table 2). A unique DNA fragment was amplified by primers 888, 827 and 826 in genotypes KZ1, DW and KZ4, respectively. Genetic similarity for each pair of accessions was calculated based on Jaccard's similarity coefficient. The lowest similarity value was obtained between accessions DW and KZ7 (0.39) and between accessions OR126 and KZ1 (0.40). Accessions T26 and FA2 had the highest similarity value (0.67). The complete linkage dendrogram revealed two main clusters (Fig. 2). In cluster I, DW did not group with KZ1 and Adilcevaz which clustered together. Cluster II consisted of two subclusters. The first subcluster contained KZ3, OR60, KZ6, 'Pedro', 'Sebin', FA2, T26, KZ13 and 'Kaman' while the second subcluster consisted of KZ4, KZ8, KZ15, Alamot, Z53,

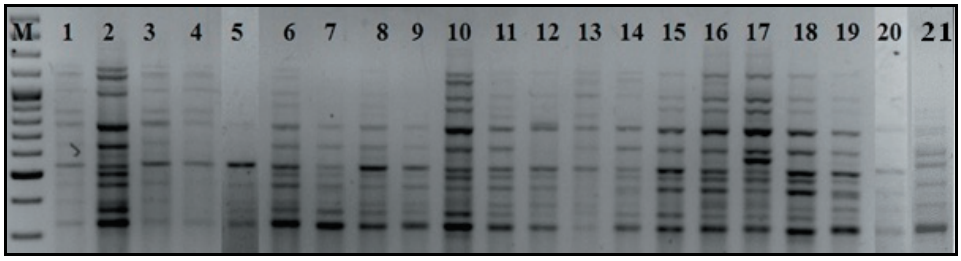


Fig. 1. ISSR profiles of 21 walnut genotypes with primer 811. M: 100bp-3000bp ladder. Lanes 1 through 21 correspond to the accessions listed in Table 1.

KH53, KZ7, OR126 and 'Chandler'. The cophenetic correlation coefficient between Jaccard's similarity matrix and the plotted dendrogram was 0.7.

ISSR markers were reported to be a useful and reliable method for determining genetic diversity among plant species (2). In this study, the percentage of polymorphism was as high as 87.5%, which probably reflects the outcrossing nature of walnut. Similar results were obtained in other outcrossing fruit and nut tree species such as *Prunus* (21) and olive (15). In general, primers with (AG), (GA), (CT), (CA) and (AC) repeats have been reported to detect higher levels of polymorphism than primers with other di-, tri- or tetra nucleotide repeats (19). Differences in the percentage of polymorphism among studies can be due to the use of different primers and the diversity of germplasm being studied. The occurrence of unique bands could be explained by the formation of hetroduplex molecules between alternate alleles, mutations, or recombination within the primer binding sites inside the amplified fragments (16). The level of similarity found between FA2 and T26 indicates gene flow among these genotypes or homoplasy. This is true for 'Pedro' and 'Sebin', KZ8 and KZ15, Alamot and Z53; and KZ7 and OR126. Gene flow among walnut genotypes may be partially explained by the high level of outcrossing that occurs in all *Juglans* species as a result of dichogamy (10, 12). The presence of commercial cultivars ('Chandler' and 'Pedro') in the same cluster with Iranian genotypes could indicate genetic relatedness.

There are reports indicating that the probable source of Persian walnut is ancient Iran (3). *J.regia* was distributed from Iran to other countries including Turkey, Greece, Spain, USA, China, India, and other countries located in the vicinity of traditional markets associated with the Silk Road (1). The high cophenetic correlation coefficient (r=0.7) between Jaccard's similarity data matrix and the dendrogram (Fig. 2) indicates a good fit between the dendrogram clusters and the similarity matrix. ISSR appears to be a useful tool for assessing the degree of similarity among accessions or cultivars in woody species in order to select the best parents for maximizing genetic diversity. This is especially important in woody fruit and nut species due to the long juvenile period which affects the efficiency of breeding programs (16). Most of the studies in Iran have focused on using morphological characters to identify the most diverse genotypes.

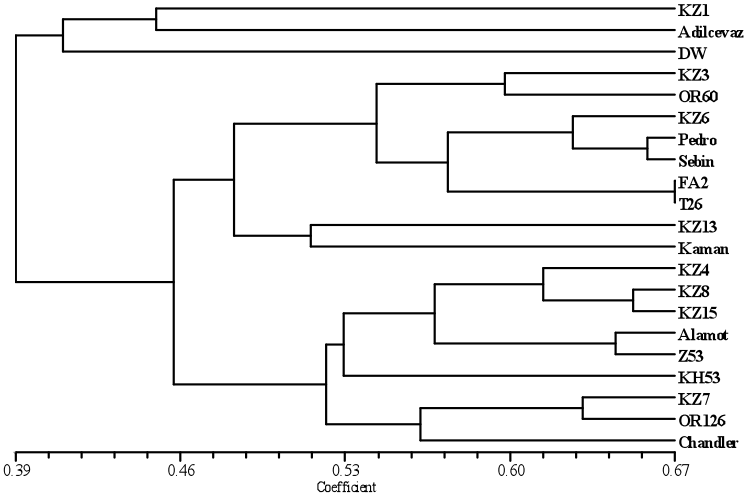


Fig. 2. Dendrogram based on the complete linkage method showing the relationships among the 21 walnut accessions tested.

Genotypes can be selected for high diversity by molecular marker analyses.

The DW genotype did not group closely with other accessions in this study. This genotype is very dwarf with a cluster-bearing habit which makes it an ideal choice for ease of fruit harvesting and intensive orchard establishments. DW and OR126 showed minimum similarity to KZ7 and KZ1, respectively. All genotypes selected for this study have good fruit quality, although they are diverse in traits such as: growth habit, bearing habit, bud break, nut weight, kernel percentage and kernel color. For example, KZ7 genotype has highest kernel percentage among all genotypes with a very light kernel color. OR126 and KZ1 have high nut weight and kernel percentage but are different in growth habit, bearing habit, kernel color and bud break. Therefore, genotypes with smallest similarity and valuable agro-morphological traits could be considered as good candidates for breeding programs. We hope our findings assist walnut breeders to maximize genetic diversity of Persian walnut in Iran.

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Comparison of irrigation scheduling strategies for achieving water use efficiency in highbush blueberry

A glasshouse experiment was carried out to assess the feasibility of applying partial rootzone drying (PRD) to highbush blueberries (*Vaccinium corymbosum*). A subsequent field experiment was established to assess four irrigation strategies with the aim of improving water use efficiency in blueberry production. Applying PRD to plants during a glasshouse experiment reduced stomatal conductance without reducing plant water potential. Hindered by high rainfall, a physiological response to PRD was not repeated in field grown plants. However, irrigation scheduled using a K_c (crop coefficient) curve constructed from Food and Agriculture Organization (FAO) 56 guidelines and post-harvest regulated deficit irrigation (RDI) delivered annual water savings of 0.8 ML ha^{-1} and 1.3 ML ha^{-1}, respectively, compared with a total 3.6 ML ha^{-1} applied using a 'rule-of-thumb' approach commonly adopted by Australian blueberry growers. These savings were achieved without reducing berry yield or quality. This study is the first to report on the feasibility of applying FAO 56 guidelines, RDI and PRD as strategies to maximize water use efficiency in highbush blueberry production. Abstract from B. Keen and P. Slavich, 2012. New Zealand Journal of Crop and Horticultural Science 40(1): 3-20.

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Enhancing budbreak and axillary shoot development of 'M.9' apple rootstock during the early period of stool-bed establishment

During the early period of establishment, productivity of newly planted stool beds is often limited because a low proportion of axillary buds break along the rootstock stem, despite these buds being anatomically well developed. Using newly established stool beds of the apple rootstock 'M.9', we applied thidiazuron (TDZ; 1000 mg L^{-1}) on to the rootstock stem at budbreak (on 15 September 2005) or 2 weeks later to ascertain whether axillary budbreak along the rootstock stem could be enhanced. Subsequently, foliar sprays of gibberellins (GA_{4+7}; 400 mg L^{-1}) were applied to determine whether elongation of the resultant axillary shoots could be increased. TDZ applied at budbreak or 2 weeks later increased the percentage of axillary buds that broke to form shoots, with more than 80% of buds breaking on TDZ-treated rootstocks compared with 41% for the untreated control. The elongation growth of newly produced shoots in response to foliar application of GA_{4+7} was pronounced for the TDZ-treated rootstock stems. TDZ painted on to the rootstock stems at budbreak or 2 weeks later increased the yield of stool shoots per metre length of new stool bed from approximately 18 in the control to c.30 with GA_{4+7}. Paint application of TDZ (1000 mg L^{-1}) followed by foliar application of GA_{4+7} (400 mg L^{-1}) can increase stool shoot yield in newly planted stool beds. Abstract from G.A. Dayatilake, J.W. Palmer and D.S. Tustin, 2011. New Zealand Journal of Crop and Horticultural Science 39(4): 263-270.