Assessment of Genetic Relationship among Some Iraqi Walnut Genotypes (*Juglans regia* L.) in Sulaimani Region Using RAPD and SSR Molecular Markers

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ABSTRACT

Juglans regia L. is cultivated for both nuts and timber in Hawraman region in Iraq. To clarify the genetic variability in walnuts grown in this region, twelve walnut genotypes of diverse origins were analyzed with 10 selected Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeat (SSR) markers. RAPD primers produced 85 bands, out of which 36 were polymorphic ranging from 2 to 7 with an average of 3.6 per primer. The genotypes shared 42.35% of polymorphic bands. In SSR analysis, nine primers gave 26 bands, out of which 23 were polymorphic ranging from 2 to 5 with an average of 2.3 per primer. SSR primers shared 88.16% of polymorphic bands. Genetic similarities were calculated and ranged from 0.4 to 0.93 for RAPD data and 0.27 to 1.00 for SSR data. Cluster analysis by RAPD and SSR markers revealed clear distinct diversity between genotypes. The dendrogram realized from the RAPD and SSR markers grouped the 12 genotypes into four major clusters. Wazi and Kirmashan1 were closely related for RAPD or SSR data. Twana and Awesar revealed the highest dissimilarity as compared with the others genotypes. Combination of RAPD and SSR data shared that Twana showed the highest dissimilarity comparing with all other genotypes. A wide range of genomic diversity was observed among all genotypes, making them candidates for selective breeding for specific traits and broadening the genetic base. Based on these results, RAPD and SSR analysis can be used for the characterization and grouping of walnut genotypes.

Keywords: Walnut, Genetic Diversity, RAPD, SSR.

INTRODUCTION

The genus *Juglans* is characterized by monoecious and heterodichogamous habit, including about 20 species. Among those, *Juglans regia*, the English or Persian walnut, is the most economically important species. Its cultivars are grown primarily for nut production, as varietal clones (Mcgranahan and Leslie, 1990) Walnut grows well in areas with a temperate climate. Production is concentrated in China (29%), USA (17%) and Turkey (10%) (FAO, 2012). Walnut is a kind of precious nut and oil economic species. Thickness kernel rate, diameter kernel and weight are the main quality indicators of economic character.

In Hawraman of Iraq, walnut plantations were established historically for both fruits and timber production. The importance of intra- and inter-specific hybridization for the genetic improvement of forest trees has been evident for at least 50 years (Zobel and Talbert, 2003).

The knowledge of the genetic relationships among walnut genotypes and their pomological characteristics will be very useful in walnut cross-breeding programs. Several techniques have been used to examine genetic

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diversity and relationships among cultivars of Persian (English) walnut, including isozymes (Solar *et al*., 1994 and Malvolti *et al*., 1994) restriction fragment-length polymorphism (RFLP) (Fjellstrom *et al*., 1994), random amplified polymorphic DNA (RAPD) markers (Malvolti *et al*., 2001) and inter-simple sequence repeat (ISSR) markers (Potter *et al*., 2002). RAPD markers have been used to evaluate genotype characterization among walnuts (Francesca *et al*., 2010).

RAPD markers are not only important for the characterization of the germplasm but can also be used to evaluate the effects of selection over time and to aid in the development of crossing schemes in walnut improvement programs since this method allows the study of the genetic diversity of the available germplasm (Nicese *et al*., 1998).

Simple Sequence Repeat (SSR) markers also have proven useful in the repository setting (Mitchell *et al*., 1997) to examine potential redundancies and propagation errors within collections (Dangl *et al*., 2001 and Phippen *et al*., 1997).

The aim of this study was to determine the genetic relationships among some walnut genotypes from Hawraman region of Iraq, with RAPD and SSR markers.

Materials and Methods

Twelve genotypes of walnut have been examined including seven from Iraq (Kore, Hoshe, Wazi, Horaman, Chama, Awesar and Hawar), four from Iran (Twana, Dana, Kirmashan2 and Kirmashan1) and one from Italy (Hana). All genotypes were obtained from Sulaimani (Hawraman) region in Iraq.

Genomic DNA extraction and purification

Young leaf samples (three leaves) were collected from individual trees, stored in an ice box and transported to the laboratory where DNA was extracted in the same day. Total DNA was extracted following the CTAB procedure (Mestav and Dalkilic, 2007). Leaves were collected and frozen in liquid nitrogen. Leaf material was then crushed to make a fine powder. One hundred milligrams of fine powder was used for DNA extraction. Five hundred microliters of CTAB buffer (1.4 M NaCl, 100 mM Tris, 20 mM EDTA, pH 8. 2% CTAB) was added to each eppendorf tube containing the crushed leaf material and was thoroughly mixed by pipetting. The mixture was incubated for 60 min at 60 °C. Samples were then centrifuged at 10285 g for 7 min in a bench centrifuge.

In order to remove RNA, the Supernatant was treated with 40 µg RNAase-A at 37 °C for 1 h. An equal volume (500 µl) of chloroform was added and tubes were then shaken until a homogeneous mixture was obtained. Samples were then centrifuged at 10285 g for 7 min in a bench centrifuge. The aqueous phase was transferred to a fresh tube. Ammonium acetate (0.08 volumes of 7.5 M) and cold isopropanol (0.54 volumes) were added into the tube and were mixed gently to precipitate the DNA at -20 °C for 1 h. Samples were centrifuged at 10285 g for 7 min to pellet the DNA. After discarding the supernatant, the pellet was washed 3 times with 70% ethanol. The pellet was dried at room temperature for 1 h and re-suspended in 40 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Then a 1:5 dilution of DNA was made in double distilled deionized and autoclaved water for use in polymerase chain reaction (PCR).

RAPD reaction and electrophoresis conditions

Ten primers (S 177, S 156, S 139, S 135, S 21, S 280, S 35, S 151, S 418 and S 124) were used in this research, which were published by Erturk and Dalkilic (2011). All primers gave the products. The reaction mixture (25 µl) contained 1× reaction buffer (PCR buffer) , 2.5 mM $MgCl_2$, 400 µM dNTP's (Fermantas), 5

pmoles of primer, 100 ng template DNA and 1 U of Taq DNA Polymerase (Fermantas). Amplification was carried out in a thermo-cycler (Master cycler) for 40 cycles, each consisting of a denaturation step at 94°C for 1 min, annealing at 38°C for 50 second and an extension step at 72°C for 2 min. An initial denaturation step at 94°C for 5 min, and a final synthesis step of 6 min at 72°C were also included. Amplified PCR products were mixed with 1/5 volume of loading buffer and separated on a 1.5% (w/v) agarose gel (to which ethidium bromide (BioBasic) was added) in 1× TBE at 70 V for 2 h30 (BioBasic). The DNA fragments were visualized under UV light. A 1 kb ladder (BioBasic) was used to estimate the approximate molecular weight of the amplified products.

SSR reaction and electrophoresis conditions

Ten microsatellite loci (WGA1, WGA4, WGA9, WGA69, WGA89, WGA118, WGA202, WGA276, WGA321, WGA331) used for the preliminary genetic characterization of Walnut (Pollegioni *et al*., 2009). All primers were amplified in all of the samples except the primer WGA321. The amplification reaction contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM of each primer, 1 U Taq polymerase and 40 ng template DNA. Amplifications were performed in a Mastercycler thermocycler with the following cycling profile: an initial denaturation at 94°C for 5 min, followed by 32 cycles of 30 s at 94°C, 1 min at 58°C, and 40 s at 72°C, with a final extension for 5 min at 72°C. PCR products were mixed with 1/5 volume of loading buffer and separated on a 1.5% (w/v) agarose gel (to which ethidium bromide (BioBasic) was added) in 1× TBE at 70 V for 2 h30. The DNA fragments were visualized under UV light. A 1 kb ladder (BioBasic) was used to estimate the approximate molecular weight of the amplified products.

Data analysis

The data obtained by the RAPD and SSR techniques were scored in binary form as the presence or absence (1/0) of bands for each sample. IBM-SPSS version 19 Software was used to calculate Jaccard similarity coefficient and variance. The similarity matrices were converted into distances matrices and used to generate dendrograms by unweighted pair group method with arithmetic mean (UPGMA) analysis.

Results and Discussion

RAPD amplified results

The introduction of molecular markers in plant breeding has presented a valuable tool for the characterization of genetic materials. Among these, the RAPD markers have been successfully used in walnut genotypes evaluation because of their many advantages. Ten primers were selected to produce distinct, reproductible, polymeric profiles. A total of 85 bands were apmlified by ten primers among 12 genotypes. Electrophoresis of PCR products revealed different degrees of polymorphism for different primers (Figure 1). In total, 36 polymorphic bands were observed. The polymorphic bands produced fragments ranging from about 250 bp to 1500 bp in size. The minimum number of polymorphic bands was 2 and the maximum number of polymorphic bands observed was 7 with an average of 3.6 per primer. The genotypes shared 42.35% polymorphic bands.

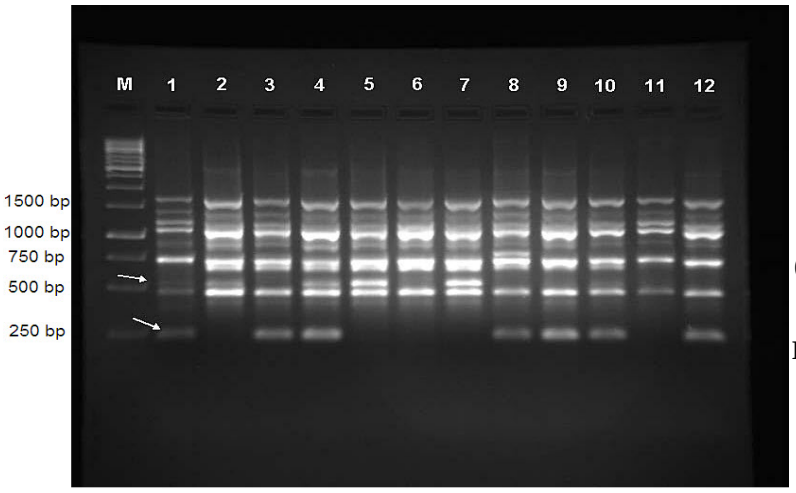

Figure 1. An example of an RAPD banding pattern obtained from primer S 21 in 12 genotypes of walnut. M = Marker (DNA ladder 1 kbp), 1= Twana, 2= Dana, 3= Kore, 4=Kirmashan2, 5= Kirmashan1, 6= Hoshe, 7= Wazi, 8= Hana, 9= Horaman, 10= Chama, 11= Awesar, 12= Hawar

The generated RAPDs were used to determine the genetic distances between the walnut genotypes. The relationship of these genotypes, as identified by the classification, has been represented as a dendrogram (Figure 2). The similarity matrix based on all possible pairs of genotypes ranged from 0.4 to 0.93 (Table 1). The lowest pair-wise similarity matrix value was between Twana and Awesar (0.4). This reveals a relatively high degree of genetic variability within the species. The highest pair-wise similarity was between Wazi and Kirmashan1 (0.93). The reason for this higher similarity is that the two varieties may have the same parents.

The dendrograms were constructed to express the similarity among the genotypes based on the RAPD. The position of the genotypes in different clusters is presented in Figure 2. The dendrogram divided the walnut varieties into six groups: group 1 includes Twana, while group 2 includes Dana, Kirmashan1, Wazi and Hoshe, group 3 contains Kore and Chama, group 4 contains Kirmashan2, Horaman and Hana, group 5 includes Hawar and group 6 includes Awesar. The genotypes Wazi and Kirmashan1 showed high similarity. The most distant genotypes in the dendrogram were Awsar and Twana.

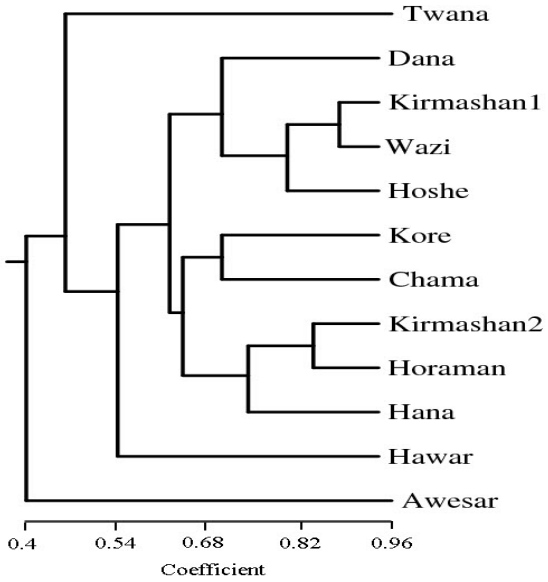

Figure 2. Dendrogram of walnut genotypes showing the genetic similarity based on RAPD data by UPGMA cluster analysis.

Table 1. Similarity matrix computed with Jaccard coefficient showing the relationship among the walnut genotypes based on RAPD data.

Genotypes	Twana	Dana	Kore	Kirmashan2	Kirmashan1	Hoshe	Wazi	Hana	Horaman	Chama	Awesar	Hawar
Twana	1.00	0.61	0.47	0.47	0.53	0.53	0.49	0.54	0.46	0.51	0.40	0.47
Dana		1.00	0.63	0.70	0.77	0.74	0.76	0.67	0.68	0.65	0.47	0.56
Kore			1.00	0.70	0.72	0.63	0.67	0.67	0.63	0.76	0.67	0.50
Kirmashan2				1.00	0.77	0.75	0.85	0.81	0.90	0.71	0.46	0.64
Kirmashan1					1.00	0.89	0.93	0.65	0.76	0.73	0.52	0.55
Hoshe						1.00	0.82	0.62	0.73	0.65	0.44	0.56
Wazi							1.00	0.74	0.79	0.68	0.47	0.61
Hana								1.00	0.79	0.68	0.53	0.61
Horaman									1.00	0.83	0.56	0.71
Chama										1.00	0.69	0.68
Awesar											1.00	0.45
Hawar												1.00

Some questions have been raised about the reliability of RAPD data due to their variable nature under different experimental conditions and by the fact that comigrating bands from different individuals do not necessarily represent homologous amplification products (Newbury and Ford-Lloyd, 1993 and Bachmann, 1996). However, the fragment size can be considered a reliable predictor of homology among closely related individuals, like the case of this study, although this is not necessarily true at higher taxonomic levels (Rieseberg, 1996). The high level of polymorphism probably reflects the outcrossing nature of walnut since similar results have been obtained with RAPDs in order to outcrossing of fruit and nut tree species such as pistachio (Hormaza *et al*., 1994) or olive (Fabbri *et al*., 1995). Comparisons of genetic distances obtained with molecular markers and theoretical data based on pedigree information have been already made in different herbaceous species and generally molecular marker-based measures of genetic distance agree with pedigree information (Dudley, 1994). Since pedigree and passport data are often unknown or incomplete for many fruit and nut tree species (Warburton and Bliss, 1996). RAPDs can be a useful tool to assess the degree of similarity of accessions or cultivars in these woody species in order to select the best parents to obtain new genetic combinations; this is especially important if we consider the long generation times of most fruit and nut tree species and, consequently, the length of the breeding process.

This result indicated that 10 random primers gave a better understanding of genetic relationships between walnut genotypes. Nicese *et al*. (1998) found the correlation coefficient 0.65 for 19 walnut genotypes with 23 random primers. Morali and Nabulski (2003) studied the genetic diversity of 19 almond cultivars and found that 10 random primers were sufficient of the genetic relationships between almond cultivars. Qianwen *et al*. (2010) estimated heterosis by RAPD markers. The researchers showed that different cross combination has different genetic similarity index and genetic distance. They also found that genetic distance has a negative relationship with thickness but has a positive relationship with kernel rate.

SSR amplified results

In order to increase the confidence level of the fragments included in the matrices, using this approach, it is possible to lose more than one useful information, but the aim was to obtain reproducible and clear data. Electrophoresis of PCR products (Figure 3) revealed different degrees of polymorphism for different primers. In the SSR analysis, ten primers pairs were used with all genotypes in the first screening of the experiment. In total, 9 pairs out of 10 gave good and reproducible polymorphic band. These primers produced fragments ranging from about 250 to 750 bp in size. Twenty three polymorphic fragments were obtained from 26 amplified fragments derived from the use of 9 primers on the studied walnut genotypes.

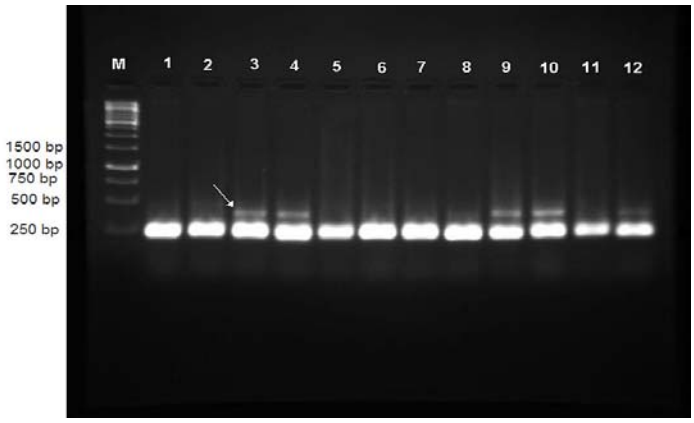

Figure 3. An example of an SSR banding pattern obtained from primer WGA069 in 12 genotypes of walnut. M = Marker (DNA ladder 1 kb), 1= Twana, 2= Dana, 3= Kore, 4=Kirmashan2, 5= Kirmashan1, 6= Hoshe, 7= Wazi, 8= Hana, 9= Horaman, 10= Chama, 11= Awesar, 12= Hawar

These primers ranged in their amplification fragments between 1 and 5 with an average number of 2.3 polymorphic fragments per primer. Figure 3 shows the amplification patterns resulted from primer (WGA069) that produced thirteen polymorphic bands. The relative number of polymorphic fragments to the total number of amplified fragments is 88.46%. The maximum numbers of fragments were produced by the primer WGA069 while lower frequencies of fragments were produced by the primer WGA001.

Dendrograms are efficient means of summarizing microsatellite data and can reveal relationships, including individuals with identical genotypes (Dangl *et al*., 2001). The similarity matrix based on all possible pairs of genotypes ranged from 0.27 to 1.00 (Table 2). The lowest pairwise similarity matrix value was found between Chama and Kirmashan1 and between Chama and Wazi (0.27). The reason for this lower similarity matrix was that the varieties might have different original parents. The highest pairwise similarity was found between Wazi and Chirmashan1 (1.00). The reason for this higher similarity was that the two genotypes may have the same parents. UPGMA cluster analysis separated the walnut genotypes included in this study into four clusters (Figure 4) that represented a wide genetic diversity pattern among all the genotypes. The first group included Twana and Awesar. The second cluster included Dana, Hana, Kore, Horaman, Kirmashan2 and Hawar. The third group contained Chama and group 4 Kirmashan1, Wazi and Hoshe. Two of the genotypes, Wazi and Kirmashan1, were closely grouped, indicating their genetic similarities. The results show that it is possible both to classify the genetic diversity of genotypes and to achieve the highest genetic diversity using SSRs, as indicated by cluster analysis. Among all tested genotypes, Twana and Awesar appeared to be the most distantly related to others.

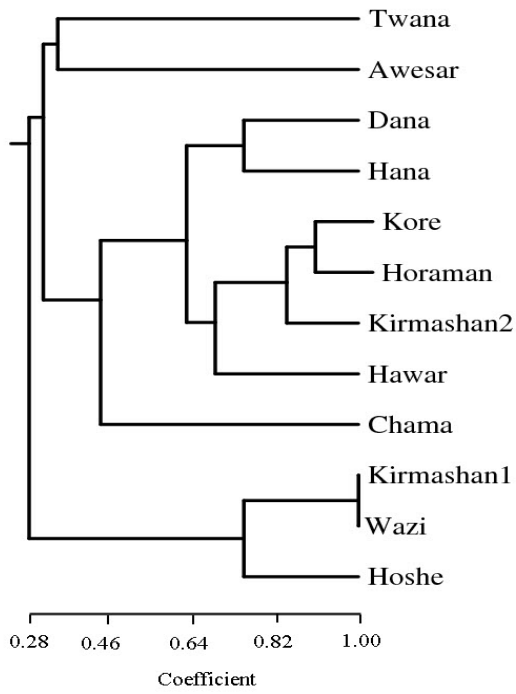

Figure 4. Dendrogram of walnut genotypes showing the genetic similarity based on SSR data by UPGMA cluster analysis.

Table 2. Similarity matrix computed with Jaccard coefficient showing the relationship among the walnut genotypes based on SSR data.

	Twana	**Dana**	**Kore**	**Kirmashan2**	**Kirmashan1**	**Hoshe**	**Wazi**	**Hana**	**Horaman**	**Chama**	**Awesar**	**Hawar**
Twana	1.00	0.67	0.72	0.69	0.44	0.61	0.44	0.50	0.64	0.44	0.53	0.58
Dana		1.00	0.88	0.77	0.52	0.70	0.52	0.83	0.80	0.61	0.42	0.75
Kore			1.00	0.89	0.58	0.75	0.58	0.72	0.92	0.58	0.50	0.72
Kirmashan2				1.00	0.64	0.80	0.64	0.69	0.89	0.64	0.57	0.85
Kirmashan1					1.00	0.82	1.00	0.61	0.58	0.27	0.33	0.44
Hoshe						1.00	0.82	0.61	0.75	0.46	0.56	0.61
Wazi							1.00	0.61	0.58	0.27	0.33	0.44
Hana								1.00	0.80	0.52	0.32	0.67
Horaman									1.00	0.58	0.50	0.72
Chama										1.00	0.56	0.70
Awesar											1.00	0.53
Hawar												1.00

No prior information is available regarding the level of polymorphism of these loci in *J. nigra*. The average of 3.6 alleles per locus is lower than other clonally propagated crops (Bowers *et al*., 1999). Others have observed a decrease in polymorphism when loci cloned from one species are used in related species (Aldrich *et al*., 2003), but the low number of alleles in this study is best attributed to the narrow germplasm in the study group.

Walnut has a long juvenile period; therefore, it is important to identify desired genotypes as early as possible. DNA fingerprinting can be used to validate the identity of scion sources, and to test for propagation errors, reducing the risk of mixing valuable genotypes with less valuable ones. SSR analysis is a powerful and informative method to fingerprint cultivars and study genetic relationships. SSR is abundant in most genomes, are generally distributed across the whole genome, and are hyper variable, co-dominant, and highly reproducible. Their multiallelic nature makes them especially useful for analysis of heterozygous, allogamous species, permitting the development of SSR fingerprints for each genotype (Powell *et al*., 1996). Here, it is demonstrated that SSR developed in *J. nigra.* Woeste *et al*. (2002) proved to be effective for fingerprinting of *J. nigra.*

RAPD and SSR combination

To decrease the inaccuracies of the independent techniques, a dendrogram was developed by pooling the data of both RAPD and SSR. Two major clusters were observed in this dendrogram (Figure 5). The Similarity matrix of walnut genotypes varied from 0.42 to 0.95 (Table 3), whereas the maximum value of similarity shared by Wazi and Kirmashan1, whereas Wazi and Awesar revealed the minimum values.

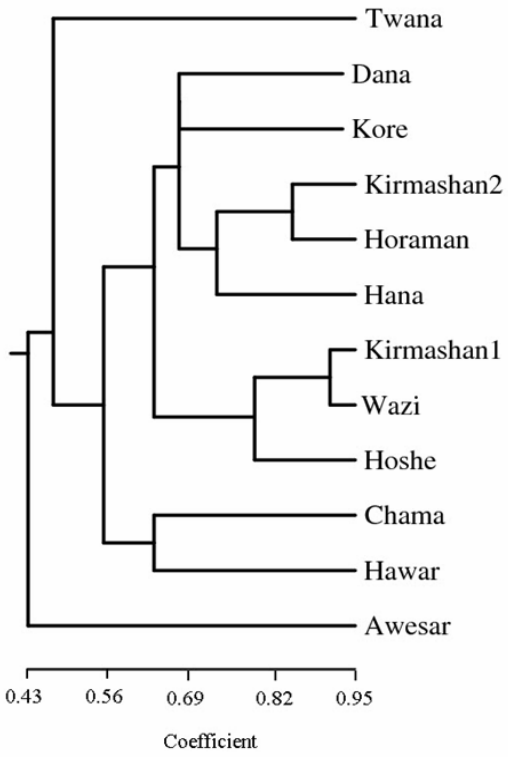

Figure 5. Dendrogram of walnut genotypes showing the genetic similarity based on RAPD and SSR data by UPGMA cluster analysis.

Table 3. Similarity matrix computed with Jaccard coefficient showing the relationship among the walnut genotypes based on RAPD and SSR data.

Genotypes	Twana	Dana	Kore	Kirmashan2	Kirmashan1	Hoshe	Wazi	Hana	Horaman	Chama	Awesar	Hawar
Twana	1.00	0.63	0.58	0.56	0.49	0.57	0.47	0.53	0.53	0.48	0.46	0.52
Dana		1.00	0.73	0.73	0.68	0.72	0.68	0.73	0.73	0.64	0.45	0.64
Kore			1.00	0.78	0.67	0.68	0.64	0.69	0.75	0.69	0.60	0.59
Kirmashan2				1.00	0.73	0.77	0.78	0.77	0.90	0.69	0.50	0.71
Kirmashan1					1.00	0.86	0.95	0.64	0.70	0.57	0.45	0.51
Hoshe						1.00	0.82	0.61	0.74	0.58	0.49	0.58
Wazi							1.00	0.70	0.72	0.55	0.42	0.55
Hana								1.00	0.80	0.63	0.45	0.63
Horaman									1.00	0.74	0.54	0.71
Chama										1.00	0.64	0.69
Awesar											1.00	0.48
Hawar												1.00

Cluster analysis defined five main groups: group 1 consisted of Tawana. Groupe 2 consisted of Dana, Kore, Kirmashan2, Horaman and Hana genotypes, showing close similarity between them. In this group, two further subgroups were formed. First, a further cluster was formed by Dana and Kore, a second cluster formed by Kirmashan2, Horaman and Hana. Group 3 consisted of three genotypes, namely Kirmashan1, Wazi and Hoshe.

The main group, 3, consisted of 3 walnut genotypes, namely Kirmashan1, Wazi and Hoshe. Group 4 contained Chama and Hawar. Group 5 included only Awesar. Wazi and Kirmashan1 grouped together into one major cluster. Twana and Awesar showed the highest dissimilarity comparing with other of genotypes which demonstrates genetic divergence from the other genotypes. The reason for this lower similarity matrix may be that the genotypes had different original parents.

Conclusion

In this study, the described markers were able to distinguish some genotypes. The differences present among these walnut genotypes suggest that they originated from genetically divergent parents or have adaptation to their respective micro-climatic regions. On the other hand, early-bearing trait of walnuts is important for cross breeding. Screening markers linked to walnut early-bearing character by RAPD technique have been reported (Yang *et al*., 2002 and Wang *et al*., 2004). So the work for the next step is to use diverse phenotypes of walnut, recover, clone and sequence the remaining markers and convert these RAPDs and SSR into sequence characterized amplified region (SCAR) markers, which will establish the foundation for constructing genetic linkage map linked to walnut early-bearing character. These works are significant for further walnut breeding program and development of the walnut crop in Iraq.

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تقييم العلاقة الوراثية بين بعض التراكيب الوراثية للجوز العراقي (*Juglans regia* L.) في منطقة هورامان باستخدام وسمات جزيئية RAPD و SSR

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ملخـص

يزرع الجوز لكل من المكسرات والأخشاب في منطقة هورامان في العراق. لتوضيح التباين الوراثي في الجوز المزروع في هذه المنطقة، تم تحليل 12 تركيبا وراثيا من الجوز من أصول متنوعة مع 10 بادئات باستخدام طريقة تقنية وسمات متعددة الشكل المضخمة عشوائياً (RAPD) و التحليلات المعتمدة على التكرار المتعدد البسيط (SSR) . أظهرت نتائج التحليلات 85 قطعة من الحمض النووى منقوص الاكسجين منها 36 كانت متعددة الأشكال تتراوح من 2 إلى 7 مع متوسط 3.6 لكل بادئ. وكانت النسبة المئوية لعدد القطع التباين بين التراكيب الوراثية 42.35% فى طريقة تقنية وسمات متعددة الشكل المضخمة عشوائياً (RAPD). وفى الطريقة المعتمدة على التكرار المتعدد البسيط (SSR)، وجدت 26 قطعة من الحمض النووي منقوص الاكسجين منها 23 كانت متعددة الأشكال تتراوح من 2 إلى 5 مع متوسط 2.3 لكل بادئ. كما وجد الاختلاف الوراثي بنسبة 88.16%. والتشابة الوراثي بين التراكيب الوراثية يتراوح بين 0.4-0.93 فى (RAPD) و 0.27-1.00 فى (SSR). وتم استخدام التشابة الوراثي لتكوين شجرة التنوع الوراثي. واستنادا إلى نتائج شجرة التنوع الوراثي، قسم الجوز الى المجاميع الرئيسة، لوحظ ان اعلى نسبة من التشابة كانت بين وازى و كرمانشان1. واظهرت اعلى نسبة من الاختلاف الوراثى بين اويسر و تولنا. وفى تجميع البيانات (RAPD و SSR)، أظهرت النتائج أن تركيب الوراثي تولنا اكثر تبايناً مقارنة مع غيرها من التراكيب الوراثية. ولوحظ وجود مجموعة واسعة من التنوع الجيني بين جميع التراكيب الوراثية، مما يجعلها المرشحات لتربية السمات المحددة انتقائياً وتوسيع القاعدة الوراثية. و بناء على هذه النتائج، يمكن استخدام RAPD و SSR للوصف والتجميع للتراكيب الوراثية للجوز.

الكلمات الدالة: الجوز، الاختلاف الوراثي، RAPD، SSR، منطقة هورامان.

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