Application of Microsatellite and Inter Simple Sequence Markers for Certifying Trueness to Type of Grafted Ramets and Clustering of Persian Walnut Varieties

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Research Article

Keywords: genetic identity, microsatellite, inter simple sequence repeats, walnut, true-to-type

Posted Date: June 16th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-542091/v1

Abstract

The utility of seventeen Microsatellite (SSR) markers and fifteen inter simple sequence repeats (ISSR) markers for the identification of twenty eight ramets of 11 varieties of walnut (*Juglans regia*) was explored. Thirty nine individual genomes were screened using 61 and 38 scorable fragments from SSR and ISSR markers, respectively. The least polymorphic SSR locus was WGA004 (two alleles) and the most polymorphic (5 alleles) was WGA276. Polymorphism information content values ranged from 0.08 (WGA004) to 0.43 (WGA032) in SSR markers and from 0.11 (AGA (AC)7) to 0.49 (CAC(TGT)5) in ISSR markers, with an average of 0.29 and 0.19, respectively. In most cases, grafted varieties with identical names also had the same microsatellites profile. The principal coordinate analysis and clustering (UPGMA) based on the combined marker set emphasized two failures in grafting or off-types, ramets identified as Serr 4 (S4) and Vina 1 (V1). The presence of two off-type ramets in the walnut research orchard emphasizes the importance of using molecular certification for proving true-to-type of walnut orchards. Using 13 polymorphic SSRs, we tabulated a DNA fingerprint chart of 11 walnut varieties. Except for 'Chandler', each cultivar could be distinguished using a combination of only two SSR loci. The 13 SSRs markers evaluated in this study could be used in future to identify clones produced from the varieties.

Introduction

Persian walnut (*Juglans regia*) is widely cultivated for nut and wood production (McGranahan and Leslie 2012). Originally cultivated in Persia's plateau, it has been introduced all around the world (McGranahan 2007; Pollegioni et al. 2014). Besides morpho-agronomical characteristics, several biochemical (such as enzymes) and molecular markers have been established to identify walnut varieties and to estimate genetic diversity in walnut (Mahmoodi et al. 2013; Ali et al. 2016; Vischi et al. 2017). The identification of varieties based on phenotypic observations is slow, difficult, produces uncertain results because of environmentally induced variability, and it may even be impossible for juvenile or recently grafted plants (Pourkhaloee et al. 2017; Nadeem et al. 2018). Random amplified polymorphic DNA (RAPD) (Ahmed et al. 2012; Pop et al. 2013; Salieh et al. 2013), Restriction fragment length polymorphism (RFLP) (Fjellstrom et al. 1994), Inter-Simple Sequence Repeat (ISSR) (Jia et al. 2011; Grouh et al. 2011), simple sequence repeats or microsatellites (SSR) (Pop et al. 2013; Victory et al. 2006; Wang et al. 2008; Topcu et al. 2015; Vahdati et al. 2015), and amplified fragment length polymorphism (AFLP) (Kafkas et al. 2005, Bayazit et al. 2007) are the most common molecular markers which have been applied in walnut. DNA fingerprinting is commonly used to confirm the fidelity of varieties. Short tandem repeats DNA sequences known as microsatellites lie between conserved sequences and are scattered throughout the eukaryotic genomes (Bernard et al. 2018). Inter simple sequence repeat (ISSR) are also used to amplify DNA found between microsatellites, by using the PCR reaction with a repetitive sequence anchored by arbitrary or degenerate nucleotides. As a result, a large number of bands useful for fingerprinting purposes are obtained. ISSRs have been applied successfully in population genetic studies for a variety of organisms, including clonal plants (Wang et al. 2008).

SSR and ISSR are two of the main markers used in molecular studies because they are highly reproducible, show high level of polymorphism and are based on PCR procedure (Noor Shah et al. 2018). SSR and ISSR molecular markers are especially used in plant breeding for "DNA fingerprinting" important clones and for cultivar identification (Powell et al. 1996; Pollegioni et al. 2006; Dangl et al. 2005; Najafi et al. 2014). Microsatellite also appears to be the method of choice for fingerprinting varieties and germplasms (McGranahan 2007). SSRs and ISSRs permit rapid and accurate identification of errors such as misidentified trees before they are delivered to growers (Oraguzie et al. 2005). DNA fingerprinting by SSRs and ISSRs is a rapid method for quality control, confirmation of variety (true-to-type) and verification of identity (Matsumoto and Fukui 1996). DNA fingerprints also have great importance in certification programs and in protection of new releases from breeding programs (Sanchez et al. 2005; Han et al. 2016).

Our purpose was to use SSR and ISSR molecular markers to certify and prove the identities of clonal ramets in a walnut orchard by comparison with source plants. The identification of commercial Persian walnut varieties and/or proving their similarity using an SSR-based DNA and ISSR fingerprint and the publication of the alleles found in each cultivar at SSR and ISSR loci can be used to help verify true-to-type and to certify nursery stocks for commercial sale.

Materials And Methods

Plant material

In the present study, 11 varieties of Persian walnut (*Juglans regia*) growing at the mother orchard of Seed and Plant Improvement Research Institute in Kamalshahr, Karaj, Iran (35°40'N 51°19'E) were used as source of scion wood (Fig. 1). Twenty eight grafted ramets, propagated using the scion woods taken from the same 11 varieties described above, were grown at the walnut research orchard of Department of Horticulture, College of Aburaihan, University of Tehran, were used for SSR analysis (Table 1). Young leaves of mother plants and their clones were sampled from each tree in spring and stored at -80 °C until used for DNA extraction.

DNA extraction

DNA extraction from collected samples were performed using the method of Doyle and Doyle (Doyle and Doyle 1990) with some modifications as follows: about 100 mg of freeze-dried young leaves was ground in liquid nitrogen and leaves powder poured into a 1.5 mL tube; then 700 µL of preheated (65 °C) 2X CTAB buffer (2% CTAB, 2% PVP, 0.2% β-mercapto ethanol, 1.4 M NaCl (pH=8.0), 100 mM Tris-HCl (pH= 8.0), 20 mM Na_2EDTA (pH= 8.0) was added, the final mix was incubated at 65 °C for 40 min followed by mixing with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifugation at 13,000 g for 15 min. The resulting aqueous phase was taken and mixed twice with chloroform to remove contaminants. After centrifugation, the upper (aqueous) phase layer was taken and mixed with cold isopropanol for DNA precipitation. After centrifugation at 13,000 g for 10 min, nucleic acid precipitate was washed by 70 % ethanol for 0.5 hour, dried at room temperature and the resulting DNA pellet was dissolved in 50 µL TE buffer (pH=8). The DNA samples were stored at -20 °C. DNA was qualified in 2 % agarose gel in TAE buffer, stained with ethidium bromide (10 mg/ml) against phage DNA standard and visualized under UV light (Geldoc, UVP CO, USA). DNA quantity was determined using a spectrophotometer (Perkin Elmer Co.).

Microsatellite primers and DNA amplification

Seventeen microsatellite primers (Table 2) were used to amplify microsatellites from the DNA of all 11 mother plants and their 28 clones (Table 1). Fourteen analyzed microsatellite loci (WGA1, WGA4, WGA9, WGA69, WGA89, WGA118, WGA202, WGA225, WGA276, WGA321, WGA331, WGA332, WGA349 and WGA376) developed by Dangl et al. (Dangl et al, 2005) and three microsatellite loci (WGA27, WGA32, WGA71) introduced for Persian walnut by Foroni et al. (Foroni et al. 2005; Foroni et al. 2006).

For each locus and sample, PCR reactions contained 60 ng genomic DNA, 2 mM MgCl2, 0.2 mM dNTP, 1X PCR buffer, 1 unit of Taq DNA polymerase (CinnaGen Co.), 50 ng μL^{1} of each primers (forward and reverse primers) in a final 20 µL volume. Microsatellites were amplified using a BioRad Gradient thermal cycler (Bio-Rad Laboratories, Inc., CA). PCR profiles were as follows: initial denaturation step at 94°C for 5 min, 35 amplification cycles of 45s at 94°C, 1 min at 52°C and 1 min at 72°C with a final extension of 10 min at 72°C. To prepare samples for loading, twenty microliters of denaturing dye solution (95% formamide, 0.5% bromophenol blue, and 0.5% xylene cyanol) was added to 15 µL of the remaining reaction product. The diluted samples were denatured by heating to 96°C for 3 min, and then immediately chilled on ice, 7 or 8 µL of the diluted mixture was loaded in every lane of warmed 6% polyacrylamide gels using a 49

well paper comb onto a BioRad sequencer, under denaturing conditions (7M urea, 1X TBE buffer (90 mM Tris - borate, pH 8.3, and 2 mM EDTA pH=8.0). DNA ladder (Fermentas) as allele size standard was run in every gel to determinate the PCR products size. Gels were stained with silver solution (Bassam and Gresshoff 2007) (Fig. 2).

ISSR amplification:

A total of fifteen 3'-anchored primers (MWG-Biotech) were tested for ISSR amplifications (Table 1). Eleven primers that showed clear and reproducible banding patterns were selected. Amplification reactions were carried out in 25 µl volumes containing 100 ng of template DNA, 1.1 unit of Taq-polymerase (Roche), 0.2 mM dNTPs (Roche), 1.5 pmol µl–1 of primer (MWG-Biotech), 10 mM Tris-HCl (pH 8.3) and 3 mM MgCl2. PCR reactions were performed under the following conditions: 2 min at 94°C for initial denaturation, 35 cycles of 30 s at 94°C (denaturation), 45 s at 44°C (annealing), and 60 s at 72°C (extension), followed by a final extension of 1 min at 72°C for final extension. PCR products were separated on 2.5% Agarose 0.5X TBE gels, ethidium-bromide stained and visualized on a UV light box. Photographs were taken of each gel using a Polaroid camera (DS-34). A 100 base-pair ladder was used to estimate the molecular size of bands. ISSR reactions were repeated at least 3 times and bands that were not consistent in all 3 reactions were discarded. Although a very high number of large amplicons were observed, the proximity between bands or their low intensity made scoring difficult. Highly reproducible bands were 300 to 1500 bp fragments. Candidate amplicons within the selected size-range were discarded if they were close in size to other fragments or unclear when they were presented in mother plants and their clones, simultaneously. ISSR bands were scored as absent (0) or present (1), assuming that each band position corresponded to 1 'locus' with 2 'alleles' (1, 0).

Data analysis

The SSR and ISSR alleles of each clone were scored as present (1) or absent (0). Nei's genetic distance was calculated among all samples on the basis of allele frequency data. Two similarity matrices were generated based on the proportion of shared amplification fragments separately for SSR and ISSR data (Nei and Li 1979). Number (Na) of alleles, effective number of alleles (Ne) per locus and observed heterozygosity (Ho) were calculated for SSR loci using Power Marker V3.2.5. The polymorphism information content of primers was calculated as:

$$PIC = 1 - \sum_{i}^{n} pi^2 - \sum_{i}^{n-1} \sum_{j=i+1}^{n} pi^2 pj^2 \text{ (Botstein et al. 1980)}$$

where p_i was the frequency of the i^{th} genotype of each SSR locus.

Cluster analysis was carried out using the Jaccard similarity test and an UPGMA clustering method (Durvasula and Rao, 2018) using the software Darwin V6.0.21. The data from the polymorphic markers were subjected to population structure analysis based on the admixture model-based clustering method in the software package STRUCTURE 2.3.4 (Falush et al., 2007). This model was run by varying the number of clusters (k) from 1 to 11 and 4 iteration for each K. A burn-in period of 10,000 and Markov Chain Monte Carlo (MCMC) replications of 50,000 after each burn-in was used. The Evanno method was used to estimate the ΔK which gives the best estimate for an optimum number of clusters (Evanno et al., 2005). The admixture model was repeated for the best K with a burn-in period of 50,000 and an MCMC of 100,000 at 1 iteration.

To determine the relationships among 39 samples, principal component analysis (PCA) was performed using DARwin V6.0.21 software.

Results And Discussion

SSR marker analysis

The identification of varieties within and between walnut orchards is important for better management of genetic resources in the collections. In this study, the amplification of seventeen SSR loci generated 61 total fragments (alleles) from 28 individual clonal plants of eleven Persian walnut varieties. The least polymorphic locus in our study was WGA004 (2 alleles per locus) and the most polymorphic (5 alleles) was at WGA276. The average number of alleles per locus was 3.5 (Table 2). The most common allele (91%) was at WGA004 (230 bp) (Table 2). The size range of amplicons was assayed between 162 (WGA069) and 277 bp (WGA331) (Table 2). Polymorphism at SSR loci in Persian walnut is population-dependent, but some loci have consistently been shown to be more or less polymorphic.

Dangl et al. (2005) studied the genetic diversity of 47 *J. regia* genotypes using 14 SSR primer pairs and identified 73 alleles with an average of 2.5 alleles per locus. Li et al. (2007) observed only three alleles at WGA001 and seven at WGA005 and WGA071. Foroni et al. (2006) analyzed 12 SSR loci and reported fewest alleles (N = 3) at WGA001 and WGA118, and the maximum number of alleles (N = 8) at WGA071. In the study of Bernard et al. (2018) among the 217 *J. regia* accessions, the number of alleles per locus ranges from 2 to 17 with an average of 8.92 and the number of effective alleles ranges from 1.18 to 4.61 with an average of 2.84.

Level of polymorphism strongly determines how useful an SSR locus will be; a small number of highly polymorphic microsatellite loci can often provide enough information to discriminate among genotypes. Foroni et al. (2005) used six SSR primer pairs to study the Italian walnut landraces in Sorento. Li et al. (2007) showed only five microsatellite markers were needed to obtain enough polymorphisms for the discrimination of 29 rootstocks of peach. In their study, the total number of observed alleles was 33, with an average of 5.5 alleles per locus. Vaiman et al. (1994) stated that if the PIC is higher than 0.5, then that locus has a high polymorphism level in the population examined; if it is between 0.25-0.5 the locus was considered moderately polymorphic, and if it is less than 0.25 its polymorphism is low. In this study PIC values ranged from 0.43 (WGA032) to 0.08 (WGA004) with a mean value of 0.29 (Table 3). Dangl et al. (2005) reported the highest PIC for WGA202 and WGA321 (0.74).

Observed heterozygosity in our study populations was moderate and nearly identical (H_O = 0.56), a value somewhat lower than in similar studies. The per-locus observed heterozygosity ranged from 0.18 in WGA004 to 0.90 in WGA032 (Table 3). Foroni et al. (2005) found that in their population the average observed heterozygosity was 0.68, and the locus with the highest observed heterozygosity was WGA071 (0.81). In the study of Ebrahimi et al. (2011), the average value of observed heterozygosity across loci was 0.72. The per locus observed heterozygosity (Ho) ranged from 0.10 to 0.68 with a mean value of 0.47 in the study of Bernard et al. (2018).

The sizes of SSR alleles at a locus can vary across populations and can vary among genotypes even for clonal varieties (Nybom et al. 2014). Allele sizes we observed for some commercial varieties at some loci matched the sizes mentioned in studies by Dangl et al. (2005) and Foroni et al. (2005, 2006). New alleles found in this research were allele d at WGA001 found in Iranian varieties, Z63 and K72, allele d in WGA071 found in the Iranian variety Z30, and allele e at WGA276 found in the Iranian variety Z53 and ‘Pedro’ (Table 2).

In this study, WGA001, WGA009, WGA069 and WGA202 were the most informative markers, while WGA004, WGA027, WGA032 and WGA376 did not have any polymorphism useful for discriminating among these samples. Using 13 polymorphic SSRs, we tabulated a DNA fingerprint chart of 11 maternal varieties (Table 5). Allele sizes for each of the five replicates of each clone were largely consistent (Table 2), and some allelic combinations at a locus were unique to the varieties we tested (Table 5). The bold, underlined entries were unique allele combinations that can be used to identify clones (Table 5). Except for ‘Chandler’, each cultivar could be distinguished using a combination of only two SSR loci. These 13 SSR markers were powerful enough to produce a unique DNA fingerprint for each of the 11

maternal varieties, so they can be used to certify clonal identity with respect to varieties in this study. According to the UPOV guidelines (UPOV International Union for the Protection of New Varieties of Plants 2010) for DNA fingerprinting using molecular markers for the protection of new plant varieties, only markers with distinct PCR bands, high reproducibility, and reasonable polymorphism can be selected to use for this purpose. SSR primer sets for cultivar identification have been identified for many economically important plants, such as *Camellia* (Chen et al. 2016), olive (Pan et al. 2008), *Brassica* (Wei et al. 2012) and walnut (Ebrahimi et al. 2011; Mahmoodi et al. 2013).

ISSR marker analysis

ISSR markers are helpful in fields of genetic diversity, phylogenetic studies; gene tagging, genome mapping and evolutionary biology in a wide range of plant species. They have been effectively used in many tree species, such as walnut (*J. regia* L.) (Mahmoodi et al. 2012; Shah et al. 2019).

ISSR analysis of 15 markers produced 38 scorable fragments, of which 33 were polymorphic (Table 6). The number of alleles observed at each ISSR locus varied from 1 (AGA (AC) 7) to 10 (CAC (TGT) 5) with an average of 3.78 alleles per locus. The size ranges of the bands were between 100 to 1000 bp. The most prolific ISSR primer was CAC (TGT) 5 amplifying 10 polymorphic bands, while the least productive markers amplifying single monomorphic loci were AGA (AC) 7. ISSR markers have the potential to discriminate walnut varieties from each other because they generate a large number of products per primer. PIC values range from 0.11 (AGA (AC) 7) to 0.49 (CAC(TGT)5) with an average of 0.19.

Nei's genetic distance based on ISSR markers ranged from 0.15 to 0.74 with an average of 0.58 among all the walnut varieties in our study, which was lower than the findings of Mahmoodi et al. (2012) who used ISSR analysis for the characterization and categorizing of walnut genotypes of Jammu and Kashmir. Effective number of alleles (Ne) and Nei's gene diversity were 1.48 and 0.29, respectively (Shah et al. 2019). Ghanbari et al. (2019) evaluated thirty one genotypes of walnut using 10 ISSR markers and identified a total of 26 polymorphic alleles, with an average of seven alleles per locus (Ghanbari et al. 2019). In this study, the lowest pair-wise dissimilarity (15%) was observed between 'Vina' and 'Franquette' while as highest pair-wise dissimilarity (74%) was observed between 'Serr' and Z30, which reinforced results of the SSR markers. The Mantel test showing no significant coefficient of correlation between SSR and ISSR distance matrices ($r = 0.34$, $P = 0.05$).

Cluster analysis based on combination of SSR and ISSR markers

To better visualize the relationships among the varieties, the data from both the primer types (SSR and ISSR) was pooled and a dendrogram was developed from this data. The dendrogram generated from the UPGMA cluster analysis showed considerable variation among all 11 varieties (Fig. 3). The optimum number of sub-populations K, which best explain the population structure of the accession was estimated to be 3 (K=3) using the Evanno method (Evanno et al., 2005). The population structure from the structural analysis is shown in Fig. 4, which depicts the three sub-populations composed of the 39 accession studied.

Cluster analysis revealed that within the first group, three sub clusters were seen. In the first sub cluster, Z63 and 'Lara' samples were closely grouped together. Z53 and Z30 and their ramets were grouped in the second and the third sub clusters of first group, respectively. K72, 'Hartley', 'Serr' and their ramets were separated in the second group, with the exception of Serr.4, which was genetically distinct but similar to other 'Serr' ramets. The third group included ramets of 'Franquette' 'Chandler', 'Vina' and 'Pedro'. Vina1 was separated from other 'Vina' samples in this group (as an off-type); it was a distinct clade and was in fact genetically distinct from all other clones. Except for two samples that appeared to be off-types, a unique genotype was obtained for the ramets of each clone, and each ramet of each clone had the same genotype. These results confirm the ramet's true-to-type except for the Vina.1 and Serr.4 variants, so Jaccard's

coefficient between the ramets and the stock block clones was 1. The Jaccard's coefficient between Vina. 1 and Vina was 0.28 and between Serr. 4 and Serr was 0.56 (Table 4). Principal coordinate analyses (Fig 5) confirmed the results of the cluster analysis and revealed three well-separated clusters. All ramets of the same source genotypes were placed with their source clones, with the exception of Vina1 and Serr4. The reasons for misclassification may have included mislabeling of samples, failed grafts, and collecting the wrong scion wood from the mother orchard. This kind of mistake usually happens when grafters do not use right labeling protocols and/or do not use different painting colors to characterize each cultivar.

Conclusion

Our results demonstrate the use of microsatellites and Inter-simple Sequence Repeat markers for identification and verification of Persian walnut (*Juglans regia*) varieties and the testing of trueness to type of grafted ramets. Applied research of this type is essential to the nursery trade, which needs simple, low-cost methods for certifying stock and the prevention of errors in propagation. Our results also show the value of using microsatellites to verify genotype identification in breeding programs where propagation errors can result in misallocation of effort, poor data, and even failure to show progress.

Declarations

Disclaimer:

Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Dept. of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

Data archiving statement

The authors confirm that the data supporting the findings of this study are available within the article.

Funding information

Funding for this research was provided by the University of Tehran.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgment

The authors appreciate Iran National Science Foundation (INSF), University of Tehran and Center of Excellence for Walnut Improvement and the Technology for their supports. We also acknowledge Mohammad Mehdi Arab for reviewing the paper and giving some valuable comments.

References

Ahmed N, Mir JI, Reyazul R, Ahmad Rather N, Rashid R, Wani H, Shafı W, Mir H, Sheikh MA (2012) SSR and RAPD analysis of genetic diversity in walnut (*Juglans regia* L.) genotypes from Jammu and Kashmir, India. Physiol Mol Biol Pla 18(2): 149–160

Ali AM, Zubair SJ, Abbas AM, Jubrael JM (2016) Genetic diversity among Walnuts (*Juglans regia*) population in Kurdistan Region–Iraq using AFLP-PCR. ZANCO J Pure Appl Sci 28:50–55

Bassam B, Gresshoff P (2007) Silver staining DNA in polyacrylamide gels. Nat Protoc 2: 2649–2654

Bayazit S, Kazan K, Gu¨lbitti SC, Evik V, Ayanog˘lu H, Ergu¨l A (2007) AFLP analysis of genetic diversity in low chill requiring walnut(*Juglans regia* L.) genotypes from Hatay, Turkey. Sci Hortic 111:394–398

Bernard A, Barreneche T, Lheureux F, Dirlewanger E (2018) Analysis of genetic diversity and structure in a worldwide walnut (*Juglans regia* L.) germplasm using SSR markers. PLoS ONE 11: 1-19

Botstein D, White RL, Skolnick M, Davis RW (1980) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 32:314–331

Chen YN, Dai XG, Hou J, Guan HW, Wang YX, Li Y, Yin TM (2016) DNA fingerprinting of oil camellia cultivars with SSR markers. Tree Genet Genomes 12: 1-8

Dangl GS, Woeste MK, Aradhya A, Koehmstedt CH, Simon D, Potter CA, McGranahan G (2005) Characterization of 14 Microsatellite markers for genetic analysis and cultivar identification of walnut. J Am Soc Hortic Sci 130(3): 348-354

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12: 13–15

Durvasula, R., Rao, D.V.S., 2018. Extremophiles: Nature's Amazing Adapters. CRC Press, Boca Raton

Ebrahimi AR, Fatahi Z, Zamani A (2011) Analysis of genetic diversity among some Persian walnut genotypes (*Juglans regia* L.) using morphological traits and SSRs markers. Sci Hortic 130: 146–151

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals

using the software STRUCTURE: a simulation study. Mol Ecol 14: 2611–2620

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. Mol Ecol Notes 7:574–578

Fjellstrom RG (1994) Genetic diversity of walnuts (*Juglans* L.) species determined by restriction fragment length polymorphisms. PhD Diss., Univ. California, Davis.

Foroni I, Woeste K, Monti LM, Rao R (2006) Identification of 'Sorrento' walnut using simple sequence repeats (SSRs). Genet Resour Crop Evol 54:1081-1094

Foroni I, Rao R, Woeste K, Gallitelli M (2005) Characterization of *Juglans regia L*. with SSR markers and evaluation of genetic relationships among cultivars and the Sorrento landrace. J Hortic Sci Biotech 80(1): 49-53

Ghanbari A, Faraji M, Behnamian M, Estaji A, Pyrayesh A, Fahim S (2019) Genetic Diversity Evaluation of Some Walnut (*Juglans regia* L.) Genotypes in Meshkin-Shahr by ISSR Marker. J of Nuts 10(1): 1-8.

Grouh MSH, Vahdati K, Lotfi M, Hassani D, Pirvali Biranvand N (2011) Production of haploids in Persian walnut through parthenogenesis induced by gamma-irradiated pollen. J Amer Soc Hortic Sci 136(3): 198–204.

Han H, Woeste KE, Hu Y, Dang M, Zhang T, Gao T, Zhou H (2016) Genetic diversity and population structure of common walnut (*Juglans regia*) in China based on EST-SSRs and the nuclear gene phenylalanine ammonia-lyase (PAL). Tree Genet Genomes 12:111

Jia XD, Wang T, Zhai M, et al. (2011) Genetic diversity and identification of chinese- grown pecan using ISSR and SSR markers. Molecules 16(12):10078–10079

Kafkas S, Ozkan H, Sutyemez M (2005) DNA polymorphism and assessment of genetic relationships in walnut genotypes based on AFLP and SAMPL markers. J Am Soc Hortic Sci 130: 585-590

Li X, Reighard G, Swire-Clark GA, Vance Baird W (2007) Peach rootstock identification by DNA-fingerprinting with microsatellite (SSR) markers. J Am Pomologic Soc 61(4):162-166

Mahmoodi R, Rahmani F, Rezaee R (2013) Genetic diversity among *Juglans regia* L. genotypes assessed by morphological traits and microsatellite markers. Span J Agric 11(2):431–437

Mahmoodi, R., Rahmani, F., Paktarmani, R. (2012): Genetic diversity of Persian walnut from Iran as revealed by intersimple sequence repeat (ISSR) markers. J Am Pomol Soc. 66:101-106

Matsumoto S, Fukui H (1996) Identification of rose cultivars and clonal plants by random amplified polymorphic DNA. Hort Sci 67: 49-54

McGranahan G, Leslie C (2012) Walnut. In: Badenes ML, Byrne DH (eds) Fruit Breeding. Springer, Boston, pp 827–846

McGranahan G (2007) Molecular genetics of walnuts – a breeder's perspective. Proc. IS on Biotechnol. Temp. Fruit Crops and Trop. Species. Acta Hortic 738

Nadeem MA, Muhammad AN, Muhammad Q, Yıldız D, Gonul C, Mehtap Y, et al. (2018) DNA molecular markers in plant breeding:current status and recent advancements in genomic selection andgenome editing. Biotechnol Biotec Eq 32:261–285

Najafi F, Mardi M, Fakheri B, Pirseyedi M, Mehdinejad N, Farsi M (2014) Isolation and characterization of novel microsatellite markers in walnut (*Juglans regia* L.). Am J Plant Sci 5(3):409–415

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. PNAS 76: 5269–5273

Noor Shah U, Mir JI, Ahmed N, Fazili KM (2018) Assessment of germplasm diversity and genetic relationships among walnut (*Juglans regia* L.) genotypes through microsatellite markers. J Saudi Soc Agric Sci 17: 339-350

Nybom H, Weising K, Rotter B (2014) DNA fingerprinting in botany: past, present, future. Investig. Genet. 5 (1): 1–35

Oraguzie N, Yamamoto T, Soejima J, De Silva N (2005) DNA fingerprinting of apple (*Malus spp.*) rootstocks using Simple Sequence Repeats. Plant Breed 124(2):197-202

Pollegioni P, Woeste KE, Chiocchini F, Olimpieri I, Tortolano V, Clark J, Hemery GE, Mapelli S, Maria EM (2014) Landscape genetics of Persian walnut (*Juglans regia* L.) across its Asian range. Tree Genetic Genome 10: 1027–1043

Pollegioni P, Major A, Bartoli S, Ducci F, Proietti R, Malvolti ME, Avanzato D (2006) Application of Microsatellite and Dominant Molecular Markers for the Discrimination of Species and Interspecific Hybrids in Genus Juglans. Acta Hortic 598-609

Pop IF, Cristina Vicol A, Botu M, Andrei Raica P, Vahdati K, Pamfil D (2013) Relationships of walnut cultivars in a germplasm collection: Comparative analysis of phenotypic and molecular data. Sci Hortic 153:124–135.

Powell W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S, Rafalski A (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Mol Breed 2:225–238

Pourkhaloee A, Khosh-Khui M, Arens P, Salehi H, Razi H, Niazi A, Afsharifar A, van Tuyl J (2017) Genetic diversity and population structure of Iranian tulips revealed by EST-SSR and NBS-LRR markers. Int J of Hortic Sci Technol 4(2):167-182

Rohlf FJ (1998) NTSYS-pc, numerical taxonomy and multivariate analysis system, version 2.01. Exeter Publishing. Ltd. Setauket. New York

Shah NU, Mir JI, Ahmed N, Fazili KM (2019) Genetic Diversity Analysis of Walnut (*Juglans regia* L.) from Kashmir Valley Using RAPD and ISSR Markers. Agrotech 8:1-12

Shahriari M, Omrani A, Falahati-Anbaran M, Ghareyazei B, Nankali A (2008) Identification of Irannian olive cultivars byusing RAPD and Micrisatellite markers. Acta Hortic 791: 109–115

Salieh HMF, Tahir RAN, Faraj JM (2013) Assessment of genetic relationship among some Iraqi walnut genotypes (*Juglans regia* L.) in Sulaimani region using RAPD and SSR molecular markers. J Agric Sci 9(3):351–362

Sanchez-Perez R, Ruiz D, Dicenta F, Egea J, Martınez-Gomez P (2005) Application of simple sequence repeat (SSR) markers in apricot breeding: molecular characterization, protection, and genetic relationships. Hort Sci 103: 305–315

Topcu H, Coban, N, Woeste K, Sütyemez M, Kafkas S (2015) Developing new microsatellite markers in walnut (*Juglans regia* L.) from *Juglans nigra* genomic GA enriched library. Ekin J Crop Breed Genet 12:93-99

UPOV (2010) Guidelines for DNA profiling: molecular marker selection and database construction (BBMT guidelines). UPOV, Geneva, Switzerland

Vahdati K, Pourtaklu SM, Karimi Rouhollah, Barzehkar Rouhollah, Amiri R, Mozaffari M, Woeste K (2015) Genetic diversity and gene flow of some Persian walnut populations in southeast of Iran revealed by SSR markers. Plant Syst Evol 301:691–699

Vahdati K, Arab MM, Sarikhani S, Sadat-Hosseini M, Leslie CA, Brown PJ (2019) Advances in Persian walnut breeding strategies, Chapter 13. In: JM Al-Khayri, SM Jain and DV Johnson (eds.) Advances in plant breeding strategies, Vol 4. Nut 139 and Beverage Crops. Springer,Cham,Switzerland, In press.

Vaiman D, Mercier G (1994) A set of 99 microsatellite, characterization, synteny mapping and polymorphism, mamm. Genome 5: 288–297

Vischi, M, Chiabà C, Raranciuc S, Poggetti L, Messina R, Ermacora P, Cipriani G, Paffetti D, Vettori C, Testolin R (2017) Genetic Diversity of Walnut (*Juglans Regia* L.) in the Eastern Italian Alps. Forests 8(3):81-95

Victory ER, Glubitz JC, Rhods OE, Woest KE (2006) Genetic homogenieity in *Juglans nigra* at nuclear microsatellites. Am J Bot 93:118–126

Wang H, Pei D (2008) Genetic diversity and structure of walnut populations in Central and Southwestern China revealed by microsatellite markers. J Am Soc Hortic Sci 133, 197–203

Wei L, Zhao C, Li J (2012) Screening of core simple sequence repeat primer pairs and establishment of a multiplex polymerase chain reaction system for Brassica genomes A and C. Plant Breed 131:457–459

Tables

Table 1. Persian walnut varieties and codes used for fingerprinting using SSR and ISSR markers.

Variety[1]	Code	Ramet name in walnut research orchard, College of Aburaihan[2]	Code of Ramet
Z53	1	Z53.1	2
		Z53.2	3
		Z53.3	4
Z63	5	Z63.1	6
		Z63. 2	7
		Z63. 3	8
		Z63. 4	9
‘Lara’	10	Lara. 1	11
		Lara. 2	12
		Lara. 3	13
		Lara. 4	14
‘Vina’	15	Vina. 1	16
		Vina. 2	17
		Vina. 3	18
‘Franquette’	19	Franquette. 1	20
		Franquette. 2	21
		Franquette. 3	22
‘Serr’	23	Serr. 1	24
		Serr. 2	25
		Serr. 3	26
		Serr. 4	27
‘Pedro’	28	Pedro. 1	29
‘Hartley’	30	Hartley. 1	31
		Hartley. 2	32
Z30	33	Z30. 1	34
K72	35	K72. 1	36
		K72. 2	37
‘Chandler’	38	Chandler. 1	39

[1]: The stock block of the studied varieties was located at Seed and Plant Improvement Research Institute in Karaj, Iran.

[2]: Walnut research orchard located at Department of Horticulture, College of Aburaihan, University of Tehran, Pakdasht, Iran.

Table 2. The loci names, allele name, size and frequency used for SSR fingerprinting of walnut*.

Locus	Allele name and size (bp)	Frequency	Locus	Allele name and size (bp)	Frequency
WGA001 ATTGGAAGGGAAGGGAAATG CGCGCACACTACGTAAATCAC	a, 186	0.1	WGA202	a, 259	0.43
	b, 190	0.25	CCCATCTACCGTTGCACTTT	b, 263	0.14
	c, 192	0.47	GCTGGTGGTTCTATCATGGG	c, 266	0.29
	d, 198	0.16		d, 273	0.12
WGA004 TGTTGCATTGACCCACTTGT TAAGCCCAACATGGTATGCCA	a, 230	0.91	WGA225	a, 189	0.35
	b, 236	0.08	AATCCCTCTCCTGGGCAG TGTTCCACTGACCACTTCCA	b, 196	0.37
WGA009 CATCAAAGCAAGCAATGGG CCATTGCTCTGTGATTGGG	a, 231	0.2		c, 202	0.26
	b, 240	0.28	WGA276	a, 174	0.21
	c, 244	0.25	CTCACTTTCTCGGCTCTTCC	b, 180	0.03
	d, 246	0.25	GGTCTTATGTGGGCAGTCGT	c, 190	0.29
WGA027 AACCCTACAACGCCTTGATG TGCTCAGGCTCCACTTCC	a, 203	0.12		d, 192	0.30
	b, 206	0.74		e, 211	0.14
	c, 208	0.12	WGA321	a, 222	0.24
WGA032 CTCGGTAAGCCACACCAATT ACGGGCAGTGTATGCATGTA	a, 166	0.1	TCCAATCGAAACTCCAAAGG	b, 237	0.06
	b, 170	0.15	GTCCAAAGACGATGATGATGGA	c, 241	0.53
	c, 182	0.21		d, 245	0.15
	d, 196	0.52	WGA331	a, 273	0.38
WGA069 TTAGTTAGCAAACCCACCCG AGATGCACAGACCAACCCTC	a, 162	0.47	TCCCCCTGAAATCTTCTCCT	b, 275	0.44
	b, 178	0.05	CGGTGGTGTAAGGCAAATG	c, 277	0.16
	c, 180	0.25	WGA332	a, 214	0.67

	d, 182	0.21	ACGTCGTTCTGCACTCCTCT	b, 220	0.19
WGA071 ACCCGAGAGATTTCTGGGAT GGACCCAGCTCCTCTTCTCT	a, 200	0.48	GCCACAGGAACGAGTGCT	c, 225	0.12
	b, 208	0.21	WGA349	a, 265	0.08
	c, 210	0.26	GTGGCGAAAGTTTATTTTTTGC	b, 270	0.56
	d, 214	0.02	ACAAATGCACAGCAGCAAAC	c, 274	0.34
WGA089 ACCCATCTTTCACGTGTGTG TGCCTAATTAGCAATTTCCA	a, 212	0.06	WGA376	a, 242	0.06
	b, 216	0.65	GCCCTCAAAGTGATGAACGT	b, 246	0.29
	c, 218	0.25	TCATCCATATTTACCCCTTTCG	c, 254	0.64
	d, 222	0.02			
WGA118 TGTGCTCTGATCTGCCTCC GGGTGGGTGAAAAGTAGCAA	a, 184	0.11			
	b, 192	0.20			
	c, 197	0.14			
	d, 199	0.53			

Table 3. Observed number of alleles (Na), effective number of alleles (Ne), observed heterozygosity (Ho) and polymorphism information content (PIC) for 17 SSR loci in 11 *J. regia* varieties and clones.

	Na	Ne	Ho	PIC
WGA001	4	3.03	0.29	0.16
WGA004	2	1.19	0.18	0.08
WGA009	4	3.95	0.72	0.39
WGA027	3	1.70	0.52	0.26
WGA032	4	2.79	0.90	0.43
WGA069	4	2.93	0.54	0.22
WGA071	4	2.79	0.80	0.42
WGA089	4	2.00	0.31	0.16
WGA118	4	2.73	0.62	0.33
WGA202	4	3.19	0.67	0.37
WGA225	3	2.94	0.75	0.42
WGA276	5	3.99	0.67	0.40
WGA321	4	2.65	0.49	0.26
WGA331	3	2.65	0.49	0.26
WGA332	3	1.94	0.42	0.22
WGA349	3	2.24	0.44	0.22
WGA376	3	1.99	0.72	0.40
Mean	3.59	2.63	0.56	0.29

Table 4: Jaccard’s coefficient based on SSR markers between clone individuals and the stock block clones of the studied walnut varieties.

The stock block clones	The walnut research orchard clones	Jaccard coefficient	Loci with same alleles	Loci with different alleles
Z53	Z53. 1	1	17	0
	Z53. 2	1	17	0
	Z53. 3	1	17	0
Z63	Z63. 1	1	17	0
	Z63. 2	1	17	0
	Z63. 3	1	17	0
	Z63. 4	1	17	0
‘Lara’	Lara. 1	1	17	0
	Lara. 2	1	17	0
	Lara. 3	1	17	0
	Lara. 4	1	17	0
‘Vina’	Vina. 1	0.28	3	14
	Vina. 2	1	17	0
	Vina. 3	1	17	0
‘Franquette’	Franquette. 1	1	17	0
	Franquette. 2	1	17	0
	Franquette. 3	1	17	0
‘Serr’	Serr. 1	1	17	0
	Serr. 2	1	17	0
	Serr. 3	1	17	0
	Serr. 4	0.56	4	13
‘Pedro’	Pedro. 1	1	17	0
‘Hartley’	Hartley. 1	1	17	0
	Hartley. 2	1	17	0
Z30	Z30. 1	1	17	0
K72	K72. 1	1	17	0
	K72. 2	1	17	0
‘Chandler’	Chandler. 1	1	17	0

Table 5. DNA fingerprints of each walnut cultivar using walnut SSR markers.

	Z53	Z63	Lara	Vina	Franquette	Serr	Pedro	Hartley	Z30	K72	Chandler
WGA001	cc	dd	bb	Bb	Bc	Ac	cc	Cc	cc	cd	cc
WGA009	ac	ac	dd	Bb	Bd	Bc	Ab	Bd	cc	ab	ad
WGA069	cc	ac	ad	Aa	Ab	Cd	Aa	Dd	aa	ac	aa
WGA071	bc	ab	ac	Aa	Ac	Aa	Ac	Ab	bd	bc	ac
WGA089	bb	bb	bc	Ab	Bb	Cc	Ab	Bb	bb	cc	bb
WGA118	ad	ad	dd	Cc	Cd	Bd	Bd	Dd	bd	bb	bd
WGA202	cc	ac	aa	Ad	Bb	Ac	Bc	Ac	ad	ad	ad
WGA225	ab	ab	aa	Ab	Ab	Bc	Bc	Bc	ab	cc	bc
WGA276	de	aa	cd	Cc	Cc	Ad	Ce	Bd	de	ad	cd
WGA321	ac	ad	cc	Cc	Cc	Ab	Ac	Cc	dd	cc	ad
WGA331	bc	bb	ab	Bb	Aa	Aa	Cc	Ab	bc	bc	aa
WGA332	aa	aa	bc	Ac	Aa	Ab	Ab	Aa	aa	aa	bc
WGA349	bc	bb	bb	Bc	Cc	Bc	Bb	Bb	aa	ac	bc

The bold, underlined entries are the specific genotypes that can be used to identify clones.

Table 6. Primer sequence, total number of fragments, number of polymorphic fragments and polymorphism information content (PIC) of 15 ISSR primers used across 39 genotypes of walnut.

	Primer sequence	Total number of fragments	Number of polymorphic fragments	PIC
1	(TC)8AG	2	1	0.25
2	(CA)8GT	3	3	0.26
3	(GA)8G	0	0	0
4	(GT)8C	2	2	0.16
5	(AC)8TG	8	8	0.44
6	(AGTG)4	3	2	0.28
7	(GATA)5	2	1	0.22
8	(TCT)6	0	0	0
9	(GT)8TC	2	1	0.16
10	(GA)8TC	0	0	0
11	(AC)8CT	0	0	0
12	(TGT)5CAC	3	2	0.28
13	CAC(TGT)5	10	10	0.49
14	ACA(CT)7	2	2	0.20
15	AGA(AC)7	1	1	0.11
	Mean	2.53	2.20	0.19

Figures

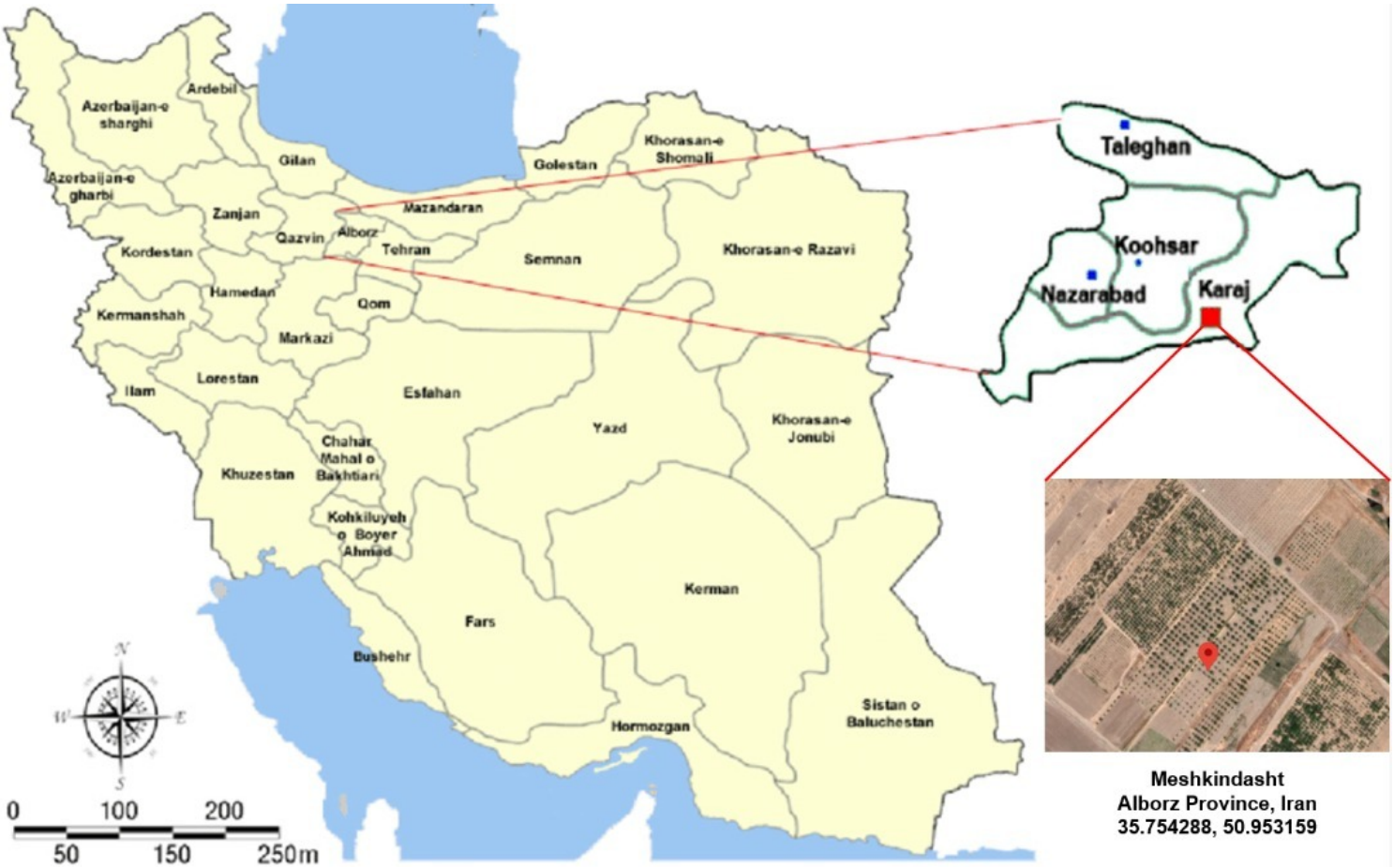

Figure 1

Geolocation of the walnut orchard of Seed and Plant Improvement Research Institute in Kamalshahr, Karaj, and the source of scion wood and of leaf samples for this study. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

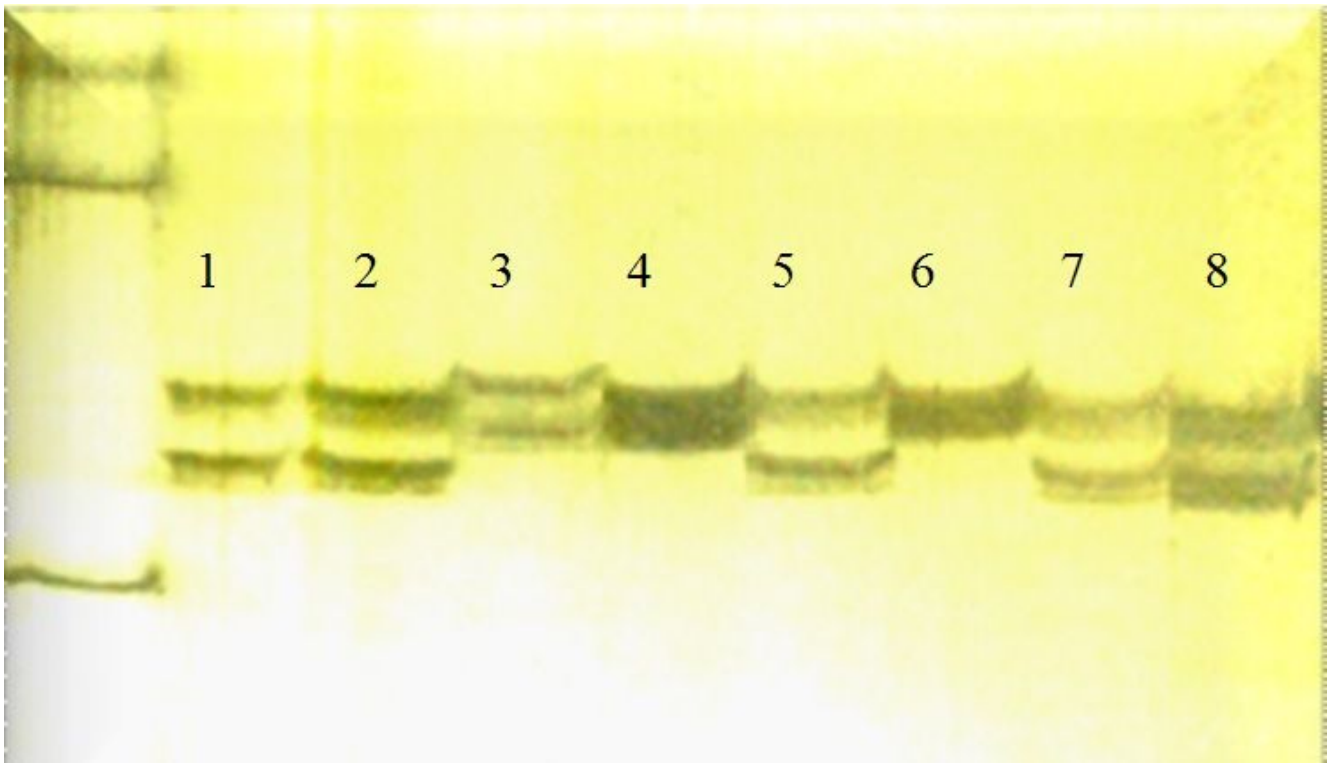

Figure 2

SSR gel profile of 8 walnut varieties using primer WGA321. The first column is 100 bp molecular size ladder. (1) Z63.1, (2) Chandler.1, (3) Serr.1, (4) Z53.1, (5) Z63.2, (6) Pedro.1, (7) Z63.3, (8) Z30.1.

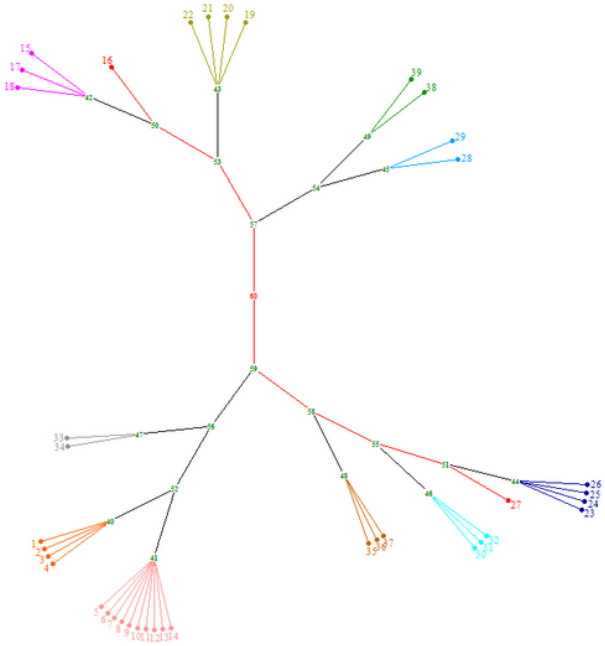

Figure 3

Dendrogram of 39 walnut clones based on UPGMA analysis using Jacard's similarity coefficient. Serr. 4p (number 27) and Vina. 1p (number 16) were two failures in grafting or off-types, ramets. (1) Z53, (2) Z53.1, (3) Z53.2, (4) Z53.3, (5) Z63, (6) Z63.1, (7) Z63. 2, (8) Z63. 3, (9) Z63. 4, (10) Lara, (11) Lara. 1, (12) Lara. 2, (13) Lara. 3, (14) Lara. 4, (15) Vina,

(16) Vina. 1, (17) Vina. 2, (18) Vina. 3, (19) ‘Franquette’, (20) Franquette. 1, (21) Franquette. 2, (22) Franquette. 3, (23)‘Serr’, (24) Serr. 1, (25) Serr. 2, (26) Serr. 3, (27) Serr. 4, (28) ‘Pedro’ (29) Pedro. 1, (30)‘Hartley’, (31) Hartley. 1, (32) Hartley. 2, (33) Z30, (34) Z30. 1, (35) K72, (36) K72. 1, (37) K72. 2, (38)‘Chandler’, (39) Chandler. 1.

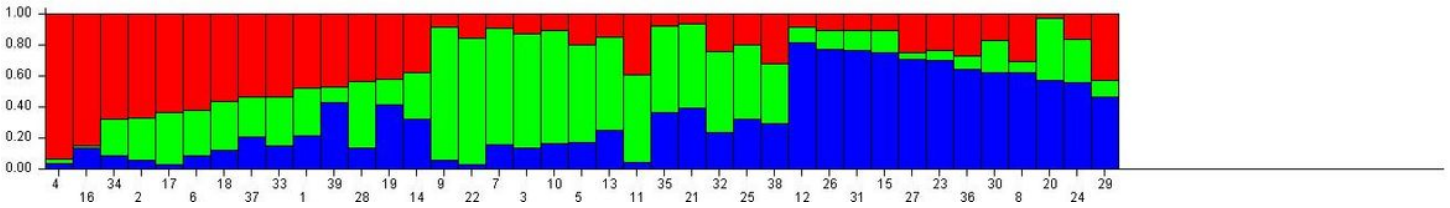

Figure 4

Estimated population structure of 39 walnut varieties as revealed by polymorphic SSR and ISSR markers for (K=3). (1) Z53, (2) Z53.1, (3) Z53.2, (4) Z53.3, (5) Z63, (6) Z63.1, (7) Z63. 2, (8) Z63. 3, (9) Z63. 4, (10) Lara, (11) Lara. 1, (12) Lara. 2, (13) Lara. 3, (14) Lara. 4, (15) Vina, (16) Vina. 1, (17) Vina. 2, (18) Vina. 3, (19) ‘Franquette’, (20) Franquette. 1, (21) Franquette. 2, (22) Franquette. 3, (23)‘Serr’, (24) Serr. 1, (25) Serr. 2, (26) Serr. 3, (27) Serr. 4, (28) ‘Pedro’ (29) Pedro. 1, (30)‘Hartley’, (31) Hartley. 1, (32) Hartley. 2, (33) Z30, (34) Z30. 1, (35) K72, (36) K72. 1, (37) K72. 2, (38)‘Chandler’, (39) Chandler. 1.

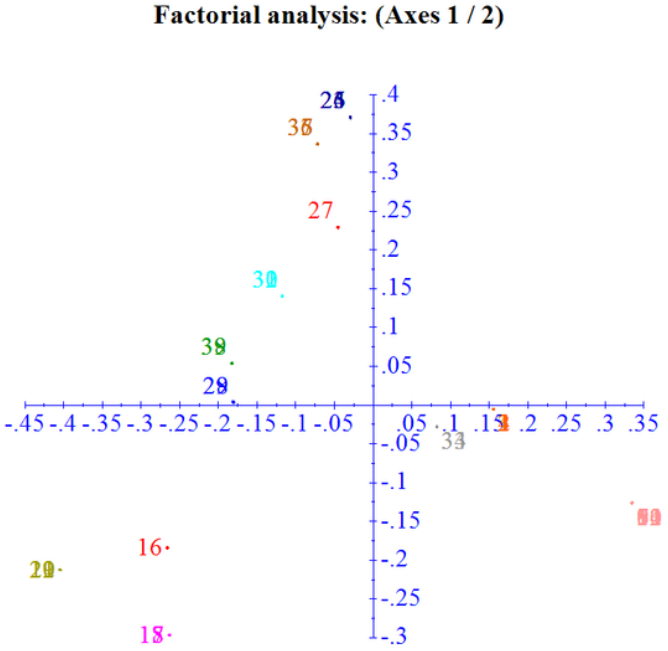

Figure 5

Factor analysis scatter plot of 39 varieties of walnut based on SSR and ISSR markers using DARWin software. Vina. 1 (number 16) and Serr. 4 (number 27) are two failures in grafting or off-types, ramets. (1) Z53, (2) Z53.1, (3) Z53.2, (4) Z53.3, (5) Z63, (6) Z63.1, (7) Z63. 2, (8) Z63. 3, (9) Z63. 4, (10) Lara, (11) Lara. 1, (12) Lara. 2, (13) Lara. 3, (14) Lara. 4, (15) Vina, (16) Vina. 1, (17) Vina. 2, (18) Vina. 3, (19) ‘Franquette’, (20) Franquette. 1, (21) Franquette. 2, (22) Franquette. 3, (23)‘Serr’, (24) Serr. 1, (25) Serr. 2, (26) Serr. 3, (27) Serr. 4, (28) ‘Pedro’ (29) Pedro. 1, (30)‘Hartley’, (31) Hartley. 1, (32) Hartley. 2, (33) Z30, (34) Z30. 1, (35) K72, (36) K72. 1, (37) K72. 2, (38)‘Chandler’, (39) Chandler. 1.