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Determination of Genetic Diversity of Walnut (*Juglans regia* L.) Genotypes Grown in Khorasan Province Using RAPD, ISSR and SSR Markers

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ABSTRACT

Walnut (*Juglans regia* L.) is an important nut and timber tree species belonging to the family Juglandaceae. Iran ranks among the leading walnut-producing countries in the world, after the United States and China. Persian walnut is widely cultivated for its high-quality nuts and valuable wood. Due to its predominant seed propagation and outcrossing nature, walnut exhibits considerable genetic diversity, which can be exploited in breeding programs for crop improvement. In the present study, genetic variability, and relationships among 13 promising walnut genotypes were evaluated at the Khorasan Razavi Agricultural and Natural Resources Research Center using three molecular marker systems: RAPD, ISSR, and SSR. Among the 15 RAPD, 10 ISSR, and 15 SSR primers tested, 8 RAPD, 3 ISSR, and 6 SSR primers produced clear, reproducible, and scorable bands with a high level of polymorphism. RAPD, ISSR, and SSR primers generated a total of 93 amplification products, of which 76 were polymorphic. Genetic similarity based on RAPD, ISSR, and SSR markers ranged from 0.431 to 0.754, with a mean value of 0.592. A relatively moderate to high level of genetic diversity was observed between the Z30 and Ronde De Montignac genotypes. SSR analysis revealed 5 to 7 alleles per locus, all of which were polymorphic, while genetic similarity values ranged from 0.123 to 0.571 with an average of 0.347. Cluster analysis grouped all accessions into two major clusters with several subclusters, indicating considerable genetic variation among the studied genotypes. These findings provide useful information for walnut germplasm characterization, conservation, and future breeding programs.

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Introduction

Walnut (*Juglans regia* L.) belongs to the Juglandaceae family, and the genus Juglans comprises approximately 20 species with a chromosome number of 2n= 32 (Manning 1978; Stanford *et al*., 2000). Persian walnut is believed to have originated in the mountainous regions of Central Asia (Pollegioni *et al*., 2020). During the Wurm glaciations, walnut populations declined considerably in Southern Europe and Turkey but survived in refugial areas around the Black Sea and the Caspian Sea. Today, Persian walnut is widely cultivated for its high-quality edible nuts and as an important source of valuable timber (Hassani *et al*., 2020).

Accurate identification and characterization of walnut genotypes are essential for effective germplasm conservation, clarification of synonymy and homonymy among cultivars,

prevention of mislabelling, improvement of breeding strategies, and protection of intellectual property rights. Persian walnut germplasm exhibits substantial morphological and genetic variability, largely due to its outcrossing reproductive system and long history of seed propagation. This diversity can be evaluated using morphological, cytological, biochemical, and molecular markers. Traditionally, morphological traits related to tree, leaf, flower, nut, and kernel characteristics have been widely used to describe and classify walnut germplasm. However, the expression of many morphological traits is strongly influenced by environmental conditions and the long juvenile period of walnut, which often limits the reliability of phenotype-based identification (Guney *et al*., 2021).

Molecular markers provide a more reliable alternative because they allow the detection of genetic variation directly at the DNA level and can be applied at any stage of plant development. Over the past decades, several DNA-based marker systems have been used to investigate genetic diversity in walnut, including isozymes (Wang *et al.*, 2008; Pollegioni *et al.*, 2020; Bujdoso *et al.*, 2021), restriction fragment length polymorphism (RFLP) (Fjellstrom *et al.*, 1994), randomly amplified polymorphic DNA (RAPD) (Ahmed *et al.*, 2012; Shah *et al.*, 2019; Sevindik *et al.*, 2023), inter-simple sequence repeats (ISSR) and simple sequence repeats (SSR) (Guney *et al.*, 2021; Abbasi Holasou *et al.*, 2023), amplified fragment length polymorphism (AFLP) (Kafkas *et al.*, 2005; Bayazit *et al.*, 2007; Xu *et al.*, 2012; Vafadar Shamasbi *et al.*, 2018), and single nucleotide polymorphisms (SNPs) (Ciarmiello *et al.*, 2011; Wang *et al.*, 2020). Among these, SSR markers are particularly valuable due to their high polymorphism, co-dominant inheritance, reproducibility, and efficient detection through PCR amplification (Bashir *et al.*, 2022). Microsatellite markers have been widely used to investigate genetic structure, population differentiation, and relationships among walnut cultivars worldwide (Shah *et al.*, 2018; Zhou *et al.*, 2021; Li *et al.*, 2023; Xiahou *et al.*, 2023; Kamdem *et al.*, 2024). Moreover, combining SSR markers with other marker systems such as RAPD and ISSR can provide a more comprehensive assessment of molecular diversity and genetic relationships within crop species (Ebrahimi *et al.*, 2016; Mahood *et al.*, 2020; Joshi *et al.*, 2020; Hussein *et al.*, 2023). In breeding programs, the identification of genetically diverse and superior genotypes from available germplasm is essential for selecting appropriate parental combinations. Therefore, integrating molecular marker analysis with conventional evaluation approaches can significantly improve the efficient utilization and management of walnut genetic resources.

In this study, thirteen walnut genotypes were selected from the available germplasm collection to represent the phenotypic and genetic diversity present within the population. These genotypes were chosen based on their morphological variation and their suitability for evaluating the effectiveness of different molecular marker systems. The main objective of this study was to assess the genetic diversity and relationships among *J. regia* genotypes cultivated in Khorasan Province, Iran, using three molecular marker systems: RAPD, ISSR, and SSR.

Materials and Methods

Plant material

Thirteen *J. regia* genotypes, including eight seedlings, two domestic cultivars, and three foreign cultivars, were used in this study (Table 1). These genotypes were specifically chosen to represent the range of morphological variation (e.g., nut size, shape, yield) and seedling versus cultivated backgrounds available in the Khorasan collection, allowing a comparative assessment of marker efficiency.

The seedling genotypes (381, 74, 226, 227, 443, 444, 339, and 340) originated from open pollination (*J. regia* × *J. regia*). Among these, genotypes 226 and 227 were derived from the K28 genotype, while 381, 74, 443, 444, 339, and 340 were progenies of the K21 genotype. Two newly developed Iranian cultivars, Z63 (cv. Jamal) and Z30 (cv. Damavand), were also included, both of which were recently released by Iranian horticultural researchers. The foreign cultivars used in this study were Hartley, Ronde de Montignac (RdM), and Chandler. All genotypes were obtained from the Mashhad Agricultural Research Institute, Iran.

Table 1. Walnut (*J. regia*) genotypes used in this study.

Genotypes	Origin	Descriptions	Parentages
381	Mashhad, Iran	Seedling	K21
Z63*	Karaj, Iran	cultivar	Introduced cultivar
74	Mashhad, Iran	Seedling	K21
227	Mashhad,Iran	Seedling	K28
444	Mashhad,Iran	Seedling	K21
443	Mashhad,Iran	Seedling	K21
Hartley	USA	cultivar	Foreign
340	Mashhad,Iran	Seedling	K21
339	Mashhad,Iran	Seedling	K21
Z30*	Karaj, Iran	cultivar	Introduced cultivar
Ronde de Montignac (RdM)	France	cultivar	Foreign
226	Mashhad,Iran	Seedling	K28
Chandler	USA	cultivar	Foreign

*=Z63 (Jamal) and Z30 (Damavand)

DNA extraction and PCR amplification

Young leaf samples were collected from individual trees, placed in an ice box, and transported to the laboratory. Genomic DNA was extracted on the same day using a modified cetyltrimethylammonium bromide (CTAB) method according to Dellaporta (Dellaporta *et al.*, 1983). DNA concentrations were measured spectrophotometrically at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). DNA quality was further assessed by electrophoresis on a 0.8% agarose gel.

DNA amplification was performed using RAPD, ISSR, and SSR markers. The specific primers and their sequences are presented in Table 2. All PCR reactions were carried out in a Primus thermal cycler in a final volume of 25 µL containing 10x PCR buffer, sterile MQ water, and 0.2 mM dNTPs, with specific variations in DNA template, primer concentrations, $MgCl_2$, *Taq* DNA polymerase, and cycling profiles optimized for each marker type.

For RAPD analysis (8 primers, CinnaGen, Tehran, Iran), the reaction mixture contained 25ng DNA, 2 µM primer, 1.5 mM $MgCl_2$, and 1U *Taq* polymerase. The thermal profile consisted of an initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 38°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min, then brought down to 4 ºC.

For ISSR analysis (10 primers screened, 3 analyzed), the mixture included 25 ng template DNA, 0.4 µM primer, 1.5 mM $MgCl_2$, 0.2 mM, and 1U *Taq* polymerase. Cycling conditions were an initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 45 s, annealing at 38–51°C for 1 min, and extension at 72°C for 2 min, ending with a final 6 min extension at 72°C.

For SSR amplification, the reaction comprised 10 ng DNA, 0.2 µM of each primer, 1.9 mM $MgCl_2$, and 0.25U *Taq* polymerase. A touchdown PCR program was utilized: an initial denaturation at 94°C for 5 min; 15 cycles of 94°C for 45 s, 58°C (decreasing by 0.2°C per cycle) for 45 s, and 72°C for 45 s; followed by 20 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 10 min.

Following amplification, products of the dominant markers (RAPD and ISSR) were separated on 1.2% agarose gels, respectively, in 0.5x TBE buffer for 2 h at 65V. The different agarose concentrations were utilized to optimally resolve the specific fragment size ranges generated by each technique. Upon completion, the gels were stained with ethidium bromide (0.05%) and visualized on a UV transilluminator. For the co-dominant SSR markers, PCR amplification was first confirmed by running 10 µL of the product on a 2% agarose gel. To achieve the high resolution required for allele sizing, the products were subsequently resolved on 6% non-denaturing polyacrylamide gels and visualized using silver staining. All raw gel images are available from the corresponding author upon reasonable request.

Data analysis

Distinct scoring methodologies were applied depending on the marker system. Band/allele

scoring was performed directly from clear and reproducible gels to ensure consistent fragment calling. The amplified fragments for the dominant markers (RAPD and ISSR) were scored as binary data (1 for presence, 0 for absence). The genetic associations for these markers were evaluated by calculating Jaccard's similarity coefficient for pairwise comparisons based on the proportion of shared bands. The similarity matrix was subjected to Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis, and a dendrogram was generated using NTSYS-PC V. 2.1 software.

Table 2. RAPD, ISSR and SSR primer survey for determination of 13 walnut (*J. regia*) genotypes.

Primer name	Marker type	Sequence (5′→3′)	Total amplification fragments	Polymorphic fragments
R1	RAPD	GTGACGTAGG	7	5
R2	RAPD	GTCCACACGG	10	8
R3	RAPD	TGCCGAGCTG	7	7
R4	RAPD	GGGACGTTGG	6	3
R5	RAPD	CATGACCGCC	3	1
R6	RAPD	GGGTAACGCC	3	1
R7	RAPD	TCGGCGATAG	4	4
R8	RAPD	AGGTGACCGT	3	2
Total RAPD			43	31
P1	ISSR	CTC-TCT-CTC-TCT-CTC-TA	4	2
P2	ISSR	CAC-ACA-CAC-ACA-CAC-AG	5	2
P3	ISSR	TGT-GTG-TGT-GTG-TGT-GA	4	2
Total ISSR			13	6
WGA069	SSR	AGA-TGC-ACA-GAC-CAA-CCC-TC	7	7
WGA004	SSR	TAA-GCC-AAC-ATG-GTA-TGC-CA	7	7
WGA001	SSR	CGC-GCA-CAT-ACG-TAA-ATC-AC	5	5
WGA009	SSR	CCA-TTG-CTC-TGT-GAT-TGG-G	6	6
WGA118	SSR	TGT-GCT-CTG-ATC-TGC-CTC-C	5	5
WGA202	SSR	CCC-ATC-TAC-CGT-TGC-ACT-TT	7	7
Total SSR			37	37

Conversely, SSR markers were scored by recording the specific allele fragment sizes (bp) for each genotype. This size-based scoring allowed for the proper calculation of co-dominant variability parameters. The following parameters were estimated from the microsatellite data: effective number of alleles per locus ($N_e = 1/\Sigma p_i^2$, where p_i is the frequency of the *i*-th allele); observed heterozygosity (H_0, calculated as the number of heterozygous genotypes divided by the total number of genotypes); and the polymorphism information content (PIC).

Results and Discussion

In the present study, three molecular marker systems, RAPD, ISSR, and SSR, were employed to assess the genetic diversity and relationships among walnut genotypes. The integration of dominant (RAPD and ISSR) and codominant (SSR) markers provided a comprehensive evaluation of the genetic structure, as multi-marker approaches are recognized for offering complementary and reliable insights into germplasm variability.

RAPED and ISSR genetic diversity

During the RAPD screening stage, 15 custom-designed 10-mer primers were evaluated across all genotypes. Among these, 8 primers produced clear and reproducible polymorphic amplification patterns (Table 2). A total of 43 bands were generated, of which 30 were polymorphic, corresponding to a polymorphism rate of 69.7%. The number of bands per primer ranged from 3 to 10, averaging 5.37. Genetic similarity coefficients among the 13 walnut genotypes varied from 0.526 to 0.903. The highest similarity value (0.903) was observed between genotypes 340 and 339, indicating a close genetic relationship between these two genotypes. In contrast, the lowest similarity coefficient (0.526) was detected between genotypes 226 and 381, suggesting considerable genetic divergence. The UPGMA dendrogram constructed from RAPD data grouped the genotypes into two main clusters (Fig. 1). Cluster I included Hartley, 340, 339, Z30, RdM, 226, and Chandler, whereas Cluster II comprised 381, Z63, 74, 227, 444, and 443. This clustering pattern indicates the presence of

substantial genetic variability within the studied germplasm.

ISSR markers demonstrated higher reproducibility than RAPD, consistent with previous reports attributing this to longer primer length and higher annealing temperature (Goulao *et al.*, 2001). In this study, three ISSR primers generated a total of 13 amplification bands, with an average of 4.3 bands per primer. Among these, eight bands were polymorphic, resulting in a polymorphism rate of 61.53% (Table 2). Genetic similarity coefficients derived from ISSR data ranged from 0.500 to 0.920. The highest similarity value (0.920) was observed between genotypes 444 and 443, indicating strong genetic relatedness. Conversely, the lowest similarity coefficient (0.500) was found between genotypes 74 and Chandler, suggesting significant genetic differentiation. The ISSR-based UPGMA dendrogram also separated the genotypes into two major clusters (Fig. 2). Cluster I consisted of Hartley, 381, 340, Z63, and 74, whereas Cluster II included 339, Z30, 226, 227, 444, 443, Chandler, and RdM.

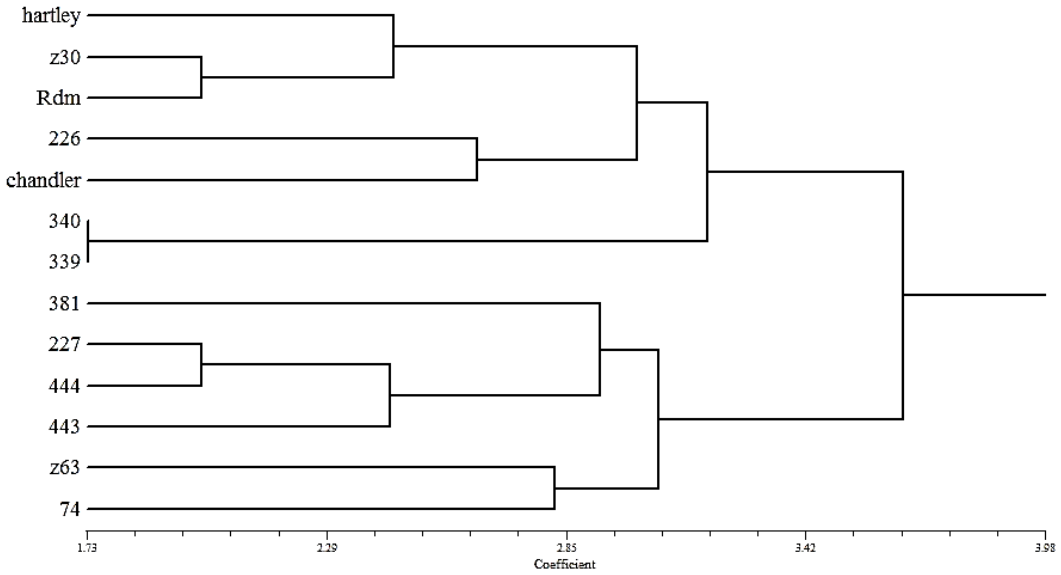

Fig. 1. Dendrogram showing relationship between 13 different walnut (*J. regia*) genotypes/cultivars based on RAPD analysis using UPGMA.

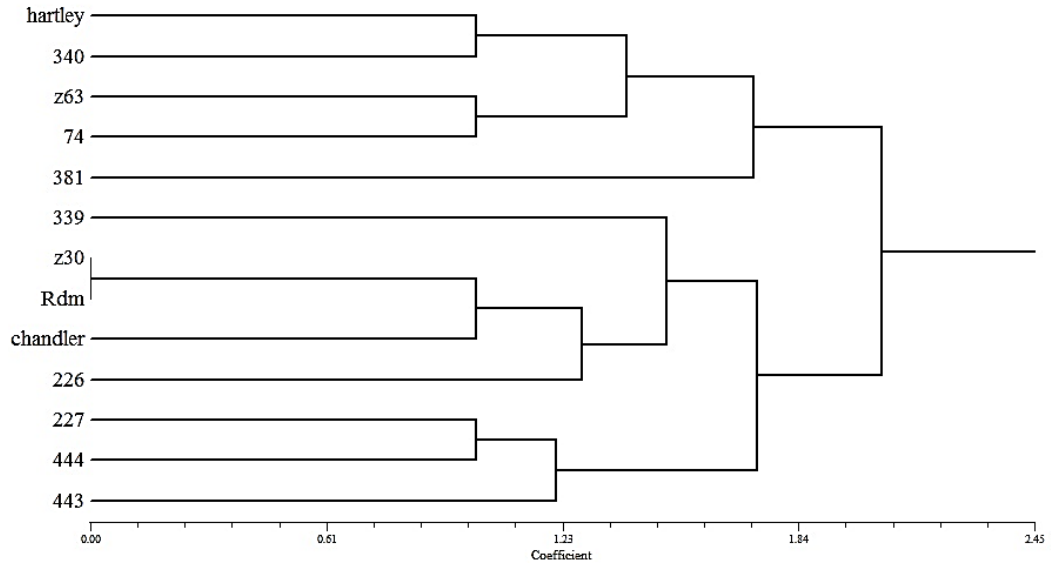

Fig. 2. Dendrogram showing relationship between 13 different walnut (*J. regia*) genotypes/cultivars based on ISSR analysis using UPGMA.

The polymorphism levels observed for RAPD (69.7%) and ISSR (61.53%) indicate a considerable degree of genetic variability among the studied walnut genotypes. However, these values are slightly lower than those reported in some previous studies. For instance, Sevindik *et al.* reported polymorphism rates of 74.54% for RAPD and 70.58% for ISSR in Turkish walnut populations. Similarly, Dogan (Dogan *et al.* 2014) reported polymorphism levels of 71.1% and 69.1% for RAPD and ISSR markers, respectively, in an analysis of 59 walnut genotypes. Such differences may be attributed to variations in the genetic background of the studied populations, differences in geographic origin, and the specific primers used in each study. Nevertheless, the reproducibility observed in ISSR markers confirms their reliability for assessing genetic diversity in walnut germplasm.

SSR allelic diversity

Microsatellite (SSR) analysis was conducted using 15 primer pairs, of which six successfully amplified polymorphic loci across the studied genotypes (Table 2). A total of 37 alleles were detected, with the number of alleles per locus ranging from 5 to 7 and an average of 6.1 alleles per locus. The amplified fragments ranged in size from 144 to 250 bp, and all loci exhibited 100% polymorphism. The UPGMA dendrogram generated from SSR data separated the genotypes into two clusters (Fig. 3). Cluster I was further divided into two subclusters: the first included Hartley, Rdm, 340, 339, and Z30, whereas the second comprised genotypes 226, 227, 443, 444, 381, 74, and Chandler. Cluster II contained only genotype Z63, suggesting that this genotype is genetically more distinct compared with the others.

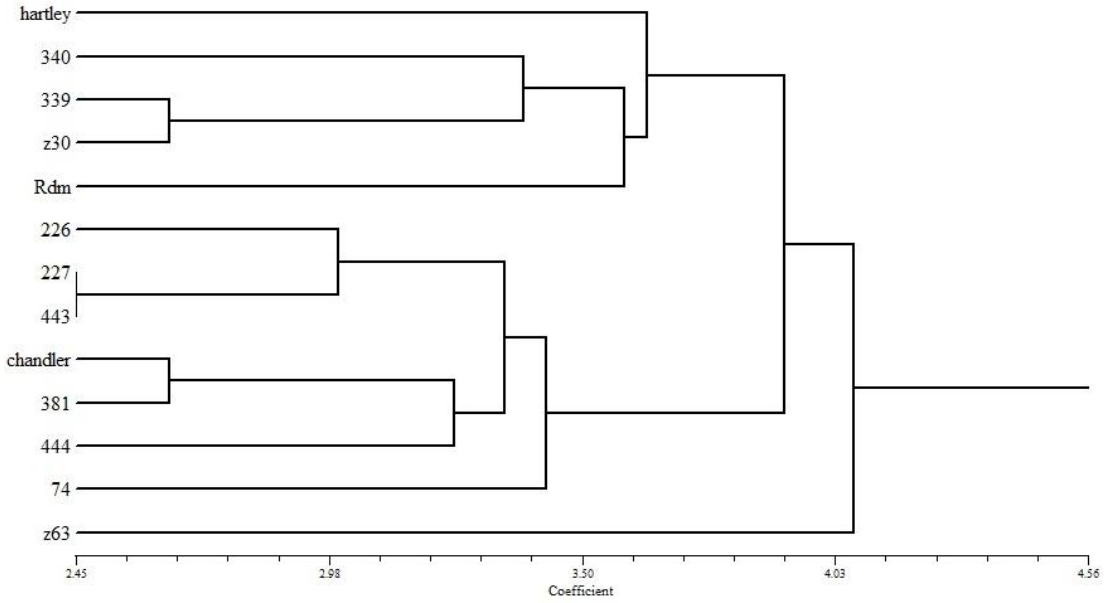

Fig. 3. Dendrogram showing relationship between 13 different walnut (*J. regia*) genotypes/cultivars based on SSR analysis using UPGMA.

Genetic diversity parameters calculated for the six microsatellite loci are presented in Table 3. SSR loci with a higher number of alleles generally exhibited higher heterozygosity values, indicating their strong discriminatory potential.

Discriminatory power of SSR markers

The PIC values calculated for the SSR loci ranged from 0.6847 to 0.8020, with an overall mean value of 0.7468. The highest PIC value was recorded for locus WGA202 (0.8020), indicating its high discriminatory power for distinguishing walnut genotypes. In contrast, locus WGA009 showed the lowest PIC value (0.6847), although it still demonstrated substantial informativeness. SSRs, due to their hypervariability, high reproducibility, Mendelian inheritance, and co-dominant expression, are ideal for parentage analysis and fine-scale genotyping (Streiff *et al*., 1999; Bashir *et al*., 2022).

Table 3. Genetic diversity parameters estimated for six microsatellite primers in 13 walnut (*J. regia*) genotypes.

Primer	Major allele frquency	No. of obs.	Allele No	GeneDiversity	Heterozygosity	PIC
WGA 069	0.2500	10.0000	7.0000	0.8050	1.0000	0.7769
WGA004	0.2692	13.0000	7.0000	0.7988	1.0000	0.7703
WGA 001	0.3333	12.0000	5.0000	0.7326	0.3333	0.6847
WGA009	0.2727	11.0000	6.0000	0.7934	0.9091	0.7620
WGA118	0.4231	13.0000	5.0000	0.7249	0.7692	0.6848
WGA202	0.2308	13.0000	7.0000	0.8254	1.0000	0.8020
Mean	0.2965	12.0000	6.1667	0.7800	0.8353	0.7468

As observed in other species, their high polymorphism levels positively influence identity and exclusion probabilities (He *et al.*, 2002; Dwiningsih *et al.*, 2020; Achard *et al*., 2020; Enyew *et al*., 2022). Comparable results have been documented by Ahmed *et al*. in 82 walnut genotypes from the North Western Himalayan region of Jammu and Kashmir, India, using 13 SSR and 20 RAPD primers. A high level of genetic diversity was observed, with alleles per locus ranging from 1 to 5 for SSR primers and 2 to 6 for RAPD primers. The proportion of polymorphic loci was 100%, with similarity ranging from 12% to 79%, averaging 49% (Ahmed *et al*., 2012). This high level of polymorphism probably reflects the outcrossing nature of walnut, since similar results have been obtained using a combination of SSR and RAPD markers in other crops (Pandey *et al*., 2021; Akshitha *et al*., 2022). Among the three marker systems evaluated in this study, SSR markers proved to be the most informative and reliable for assessing genetic diversity. The high number of detected alleles and the 100% polymorphism rate highlight the strong potential of SSR markers to reveal genetic variation. These findings are consistent with previous studies, including Dogan (Dogan *et al.* 2014), who reported a polymorphism rate of 99.1% and high allelic diversity for SSR markers in walnut. The high level of polymorphism detected by SSR markers is mainly attributed to their codominant inheritance and their ability to detect multiple alleles at a single locus, particularly in highly heterozygous and outcrossing species such as *J. regia*.

Relationships Among Walnut Genotypes

The combined molecular data provided a clear resolution of genetic relationships among the studied walnut genotypes. Genetic similarity coefficients derived from SSR analysis ranged from 0.431 to 0.754, indicating moderate to high levels of genetic diversity within the germplasm. The UPGMA dendrogram based on SSR data separated the genotypes into two major clusters, each further subdivided into distinct subclusters (Fig. 4). The first main cluster contained two subclusters: the first including Hartley, RdM, 339, 340, and Z30, while the second included genotypes 226 and Chandler. The second main cluster also consisted of two subclusters, comprising genotypes 443, 444, 227, and 381 in one group and genotypes 74 and Z63 in the other. Notably, Hartley and Chandler were grouped within the same main cluster and exhibited a similarity coefficient of 0.583, indicating a moderate level of genetic relatedness between these widely cultivated cultivars.

Several Mashhad genotypes, including Hartley, Z30, RdM, 340, 339, 226, and Chandler, tended to cluster together, suggesting relatively close genetic relationships among these genotypes. However, some Mashhad genotypes such as 339, 340, and 226 showed a certain degree of genetic differentiation from other genotypes, highlighting the presence of valuable intra-population diversity.

The clustering pattern observed in this study may reflect both the evolutionary history and geographic origin of the studied germplasm. Similar relationships between genetic structure and geographic distribution have been reported in previous large-scale walnut studies. Bernard *et al.* (2018), using PCoA and STRUCTURE analyses on 253 walnut accessions, demonstrated a clear separation of global walnut populations into Eastern Europe/Asia and Western Europe/Americas. Similarly, Dogan *et al*. (2014) observed clustering patterns largely associated with geographic origin among Turkish and international walnut cultivars.

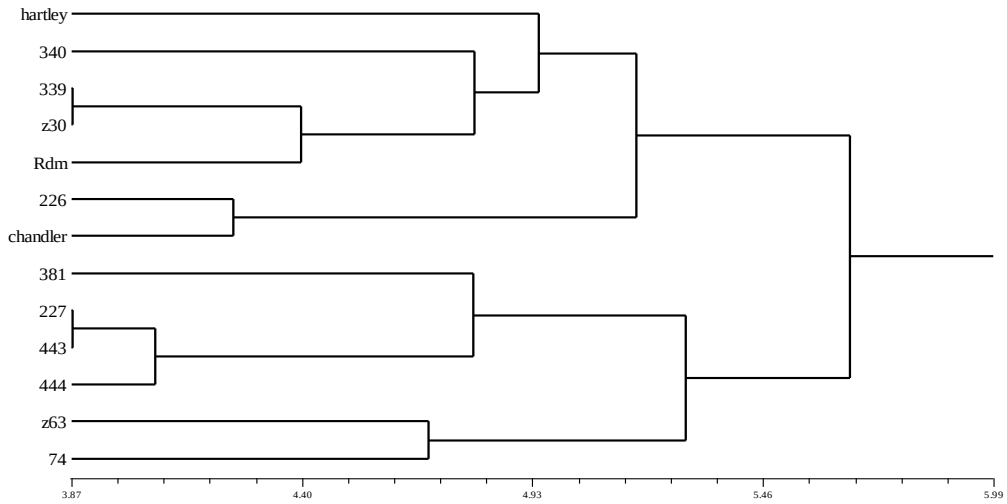

Fig. 4. Dendrogram showing relationship between 13 different walnut (*J. regia*) genotypes/cultivars based on combined RAPD, ISSR, and SSR analysis using UPGMA.

The results of this study confirm that different molecular marker systems vary in their efficiency for revealing genetic diversity, depending on factors such as reproducibility, cost, speed, and information content. RAPD markers provide rapid and cost-effective screening of genetic diversity, while ISSR markers offer improved reproducibility. In contrast, SSR markers provide the highest level of informativeness due to their codominant inheritance and high allelic diversity. Although UPGMA clustering revealed genetic relationships, we acknowledge that additional analyses such as AMOVA, PCoA, and STRUCTURE would provide deeper insights into population differentiation. These are planned for future studies with larger sample sizes

Overall, SSR and ISSR assays were more reliable than RAPD markers for walnut germplasm characterization, with SSR providing the highest resolution for distinguishing closely related genotypes. The considerable genetic diversity observed among the studied walnut genotypes is consistent with the biological characteristics of *J. regia*, particularly its outcrossing reproductive system. These findings emphasize the importance of comprehensive molecular characterization for effective germplasm conservation and for the selection of genetically diverse parental genotypes in future walnut breeding programs.

Conclusion

This study demonstrated that the combined use of RAPD, ISSR, and SSR markers provides a comprehensive and reliable assessment of genetic diversity in walnut (*J. regia L.*). All three marker systems revealed considerable polymorphism, with SSRs exhibiting the highest discriminatory power due to their co-dominant inheritance and high allelic richness. The clustering patterns showed clear genetic differentiation among the studied genotypes, highlighting the presence of valuable diversity within the germplasm. Genotypes such as Z63 and 381 displayed distinct genetic profiles and may represent promising candidates for future breeding, conservation, and core collection development. Overall, the findings emphasize the importance of integrating multiple molecular markers for effective germplasm characterization and for guiding strategic walnut breeding programs.

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Conflict of interests

The authors declare no conflict of interest.

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