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Research Article

Phenological Differences, Genetic Diversity, and Population Structure of Genotypes Obtained from Seeds of Kaman-1 Walnut Cultivar

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Walnut (*Juglans regia* L.) is a diploid ($2n = 32$), deciduous, monoecious, and generally open-pollinated tree with nuts of high nutrient content. In this study, the phenological differences, genetic diversity, and population structure of Kaman-1 and its 79 progenies obtained by open pollination were characterized by ISSR primers and some important phenological traits. As a result of the phenological observations, it was determined that the progenies differ significantly from Kaman-1. Besides, using ISSR primers, walnut genotypes were found to have genetic similarities ranging from 0.52 to 0.99. UPGMA cluster analysis showed that accessions from 2 different groups were classified, and population structure analysis confirmed this finding. Based on the results, a significant variation both phenologically and genetically was found within the walnut accessions. Also, this study confirmed that the progenies obtained from the Kaman-1 walnut cultivar have a quite wide variation and that ISSR primers and phenological traits are an important tool in determining genetic diversity.

1. Introduction

The genus *Juglans* consists of about 22 different species and all species produce nuts. However, *Juglans regia* L., known as Persian or English walnut, is the only species widely grown for nut production. Walnuts are native to the mountain valleys of Central Asia. Firstly, they were introduced into Europe by the Greeks and then were introduced into North America by colonists. Today, the genotypes of Persian walnut are grown in North and South America, Europe, and Asia, and this fruit species is the most widely grown nut in the World [1]. World walnut production was about 3.700.000 tons and the harvested area was about 1.200.000 ha in 2018. Turkey, which has a wide genetic diversity in walnuts, is ranked fourth in the world after the United States, China, and Iran in both production area and quantity [2].

Juglans regia L. is a monoecious species bearing staminate and pistillate flowers separately on the same tree. Flowers are wind-pollinated. Walnuts are generally cross-compatible and dichogamous, but a small number of genotypes are homogamous [3]. Dichogamy can lead to poor pollination and nut set in walnut orchards, and commercial plantings sometimes include one or more pollenizer genotypes to supplement pollen availability from the main cultivar [4]. Male inflorescences (catkins) each consist of 100 to 160 flowers and can produce around 2 million pollen grains. Female inflorescences of most walnut species have one to three individual flowers [5].

Walnut consumption has increased in recent years, due partly to scientific studies on their health benefits, including reduced risk of cardiovascular and Alzheimer's disease [6, 7]. Walnut kernels include proteins, fats, dietary fibers, plant sterols, phytochemicals, and microelements. Most of the fat contents of walnut are unsaturated essential fatty acids that are beneficial to human health [8, 9]. For this reason, breeding studies on walnuts are gaining more and more importance in the world.

Walnut cultivation and breeding programs require more time and labor compared to other plants due to their long juvenile period. Also, the fact that the walnut is heterozygous makes it difficult to produce acceptable new cultivars by seed propagation. However, this situation provides an important genetic diversity opportunity for plant breeding. In recent years, the application of new molecular and genetic techniques has revolutionized walnut breeding and shortened breeding time. DNA markers have played an important role in understanding the genetic diversity of different germplasm. DNA markers, as well as morphological markers, have been used for many years in determining genetic differences. Genetic diversity is the basis of an organism's ability to adapt to a changing environment through natural selection. Populations with little genetic variation are more vulnerable to the arrival of new pests or diseases, pollution, changes in climate, habitat destruction, and other events [10]. High variability increases the ability to withstand these adversities. It also increases plant breeder's ability to produce new cultivars.

Morphological markers have been used effectively for many years to detect differences between walnut genotypes. In walnut breeding programs, one of the most studied phenological parameters is leafing and defoliation period, because these traits are very important to avoid crop losses from late spring and early autumn frosts. Traditionally, morphological descriptors, for example, UPOV [11] and Descriptors for Walnut [12], have been used for description and identification of walnut genotypes [13]. When there is an excessive similarity in the morphological trait investigated, the morphological distinction becomes difficult. Moreover, this method is affected by environmental conditions. DNA markers are being increasingly used for precise genetic characterization, ascertaining origin, and elucidating the dispersal route, owning to their reproducibility, reliability, and independence from environmental conditions [14]. RFLP markers were initially used to determine genetic diversity in walnut genotypes [15]. Subsequently, RAPDs [16, 17], ISSRs [18, 19], AFLPs [20, 21], and SSRs [22–26] markers were effectively used to determine genetic diversity for the walnut tree. The use of intersimple sequence repeat (ISSR) analysis overcomes many of the technical limitations of RFLP and RAPD analyses and has higher reproducibility than RAPDs. ISSR markers involve the PCR amplification of DNA using single primers composed of microsatellite sequences [27].

Walnut is a heterozygous fruit species due to its tendency to dichogamy. For this reason, walnut genotypes obtained by open pollination show significant genetic diversity, but the degree of genetic diversity is not known. In particular, the studies that determine the degree of both phenological and genetic variation in walnut progenies have remained limited. Therefore, in this study, genetic and phenological variations that occurred by open pollination progenies of a superior walnut cultivar were investigated to find out how Kaman-1 progenies differ from this cultivar. As a result, here we report on studies of key phenological traits and the distribution of ISSR markers in several open-pollinated progenies collected in Kaman-1.

2. Materials and Methods

2.1. Plant Material and DNA Extraction. In this study, we used a total of 80 walnut genotypes: 79 progenies obtained by open-pollinated seeds of Kaman-1 and this walnut cultivar. Seeds were planted in 2017, in pots in the greenhouse at Kahramanmaras Sutçu Imam University. In spring 2019, 3 to 5 young, disease-free leaves were collected from each genotype for DNA extraction. Genomic DNA was extracted from samples using the CTAB method of Doyle and Doyle [28] with minor modifications by Bardak [29].

2.2. Morphological Characterization. Phenological observations of genotypes were taken for 2 consecutive years, 2018 and 2019. To make an accurate phenological comparison between Kaman-1 walnut cultivar and its progenies, samples from a 1-year-old grafted Kaman-1 sapling were used. The phenological description was carried out by using the Descriptors for Walnut [12] and Sütyemez [30]. Definitions of phenological traits are presented in Table 1.

2.3. ISSR Analysis. Extracted genomic DNA was PCR-amplified using 12 ISSR primer pairs (Table 2). PCR reactions were performed in a 20 μl volume. The reaction mixture contained 2 μL 10x PCR buffer, 5 mM dNTP (Vivantis), 1 μL ISSR primer, 1.5 μL $MgCl_2$, 1 μL Taq DNA polymerase, 12 μL dH_2O, and 1 μL genomic DNA. The PCR-amplification program consisted of one cycle at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final cycle at 72°C for 10 min. Amplified PCR products were separated by gel electrophoresis using 3% agarose gel. Then, the genomic DNA was stained with a dyeing solution containing ethidium bromide (1 lt pure water and 300 μl ethidium bromide) for 15 minutes. The stained DNA bands were visualized under UV light. Fragment lengths were scored in the range of 200–1000 bp.

2.4. Data Analyses. The products of ISSR were scored manually as present (1) or absent (0) and data recorded. Polymorphic information content (PIC) values provide an estimate of the discriminatory power of a marker by taking into account not only the number of alleles at a locus but also the relative frequencies of those alleles in the population under study [31]. According to the scoring results we obtained, PIC values of the primers were calculated by Laborda et al. [32] using Excel software. The frequency of alleles per locus was calculated using the following formula:

$$\mathrm{PIC} = 1 - \sum Pij2j = 1, \quad (1)$$

Table 1: Definitions used in the determination of phenological traits.

No	Traits	Description
1	Time of leaf budburst	When over 50% of terminal buds have enlarged and the bud scales have split exposing the green leaves inside
2	Leafing time	The date when 50% of terminal buds have enlarged and the bud scales have split exposing the green leaves
3	Time of leaf yellowing	The date when more than 50% of the leaves on the tree turn yellow
4	Time of defoliation	The date when all the leaves on the tree fell

Table 2: Sequences of ISSR primer pairs used in the genetic diversity of 80 walnut genotypes.

No.	Primer	Sequence
1	ISSR1	CACACACACACAA
2	ISSR3	CACACACACACAGG
3	ISSR4	CACACACACACAGC
4	ISSR5	CACACACACACAG
5	ISSR6	CACACACACACACAGT
6	ISSR7	ACACACACACACACACCG
7	ISSR8	ACACACACACACACACACC
8	ISSR9	ACACACACACACACACTG
9	ISSR11	GAGAGAGAGAGAGAGATC
10	ISSR12	GAGAGAGAGAGAGAGAGAC
11	ISSR13	AGAGAGAGAGAGAGAGC
12	ISSR15	ATATATATATATATAT

Table 3: Units, *n*, maximum, minimum, mean, and standard deviation of phonological traits in the walnut genotypes.

Traits	Units	N	Min	Max	Mean ± SD*	CV (%)*
Date of budburst	Julian date	80	69	117	89.96 ± 9.06	10.07
Leafing date	Julian date	80	81	125	98,19 ± 8.54	8.70
Leaf yellowing	Julian date	80	304	328	311,76 ± 5.76	1.85
Defoliation	Julian date	80	321	353	336,31 ± 6.23	1.85

*SD: Standard deviation; (CV%): coefficient of variance.

where *Pij* is the frequency of the *j*th allele for primer *i*. The level of genetic distance between pairs of genotypes was estimated using pairwise comparison [33].

The genetic distance was used for cluster analysis with the Popgen software, version 3.2. The dendrogram of walnut genotypes according to the "Unweighted Pair Group of Arithmetic Means (UPGMA)" method, was drawn using the NTSYSpc v. 2.02 program [34]. The dendrogram was constructed on the basis of Dice's similarity coefficient [35]. The cluster analysis in the STRUCTURE 2.3.4 package software was also applied to infer population structure in walnut genotypes. Five runs of STRUCTURE were done by setting the number of clusters (*K*) from 1 to 10. Each run consisted of a burn-in period of 10.000 steps followed by 100.000 Monte Carlo Markov Chain (MCMC) replicates [36]. The results of the analysis were recorded in the zip file and this file was uploaded to the Structure Harvester web page (http://taylor0.biology.ucla.edu/structureharvester/) and the ideal ΔK value was determined.

Phenotypic data for quantitative morphological traits were recorded as days from January 1 for statistical analysis. The data were analyzed statistically with descriptive statistics, cluster analyses, principal component analyses (PCA), and correlation by using the JMP13 Statistical Package Program for morphological diversity based on phenological traits. Phenological pairwise distances of the walnut genotypes were clustered using Ward's method [37]. The morphological and genetic differences obtained in the study were compared with each other.

3. Results and Discussion

3.1. Phenotypic Diversity. Significant variation was detected between progenies and Kaman-1. The date of budburst of genotypes varied between the 69th day and the 117th day of the year, and the foliation periods varied between the 81st day and the 125th day. Also, leaf yellowing and defoliation periods of progenies were determined to vary between the 304th day and the 353rd day of the year. Average budburst, leafing, leaf yellowing, and defoliation dates in the accessions were 56, 96, 302, and 354 Julian days, respectively. Genotype 80 had the earliest dates of budburst and leafing, whereas Genotype 3 had the latest defoliation date. The highest coefficient of variability (10.07) was observed in date of budburst, while the lowest was leaf yellowing and defoliation with 1.85 (Table 3).

Determination of phenological characteristics of walnut genotypes such as leafing and defoliation date is very important in terms of breeding new genotypes adapted to growing regions with late spring and early autumn frosts. To date, much research has been carried out on walnut to determine these traits [38–43]. Walnut genotypes differed due to the influence of both genetic and ecological factors in the traits studied.

Results of the phenotypic cluster analysis conducted in this study showed that genotypes could be separated into 2 major groups and 5 subgroups. The heat map showing the relationships between genotypes and phenological traits is presented in Figure 1. Kaman-1 was in the first subgroup with 16 progenies. Progenies, which are phenologically late especially in terms of leaf yellowing and defoliation periods, were included in the fifth subgroup. Results of the cluster analysis also partially confirmed the results of Principal Component Analyses (PCA) performed on the walnut genotypes (Table 4 and Figure 2.). This dendrogram, obtained using phenological data and Ward's method, revealed the phenological variation between genotypes. Arzani et al. [44] characterized 58 different walnut genotypes in terms of important phenological and pomological traits and effectively used the trait dendrogram to distinguish genotypes with superior properties.

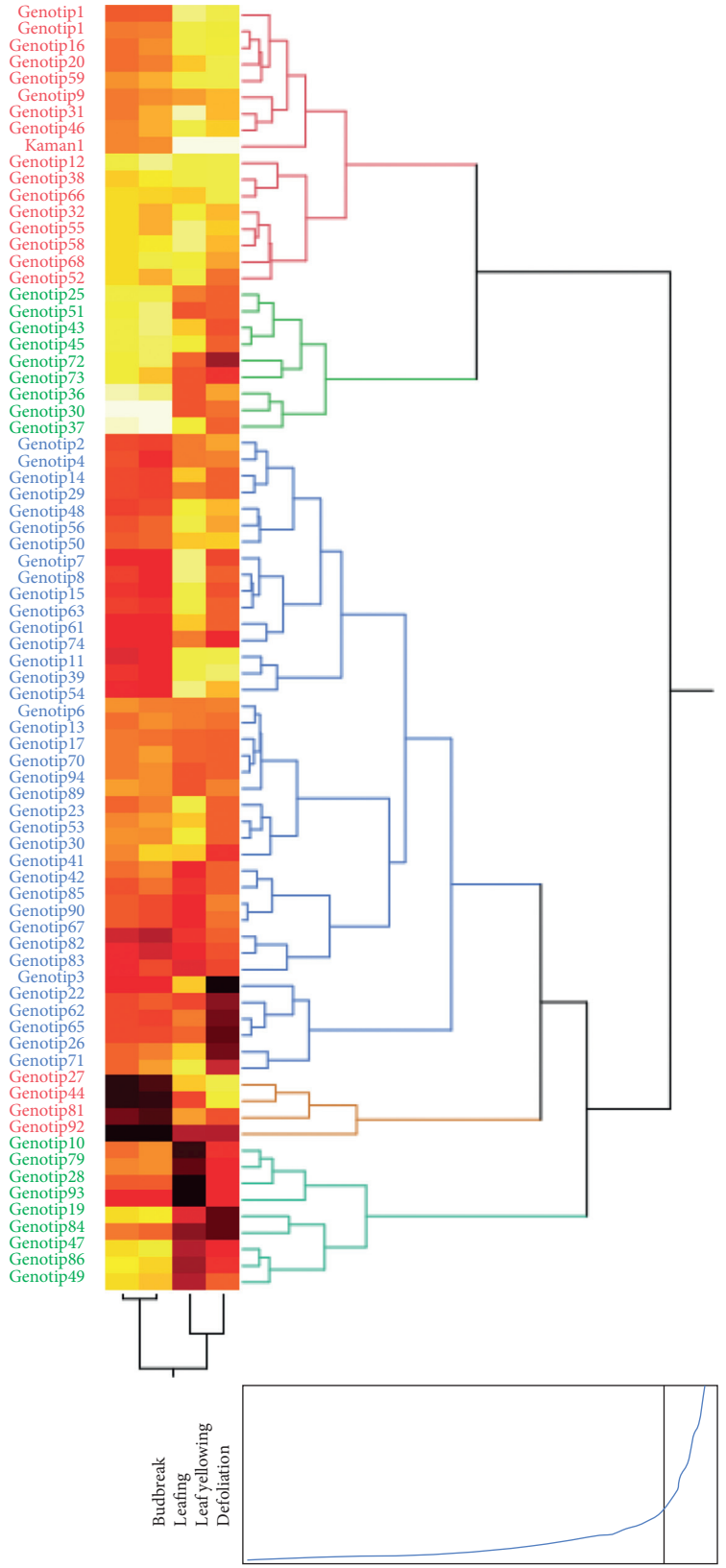

Figure 1: Phenotypic clustering of walnut genotypes based on Ward's phenological pairwise distance and phenological heat map.

Table 4: Eigenvectors of principal components (PC) of morphological traits in the walnut population.

Traits	PC1	PC2	PC3	PC4
Date of budburst	0,70	−0,12	0,03	0,71
Leafing date	0,70	−0,10	−0,03	−0,71
Leaf yellowing	0,11	0,70	−0,71	0,04
Defoliation	0,11	0,70	0,71	−0,01
% of variance	49,50	36,32	13,38	0,80
Cumulative variance	49,50	85,82	99,20	100,00

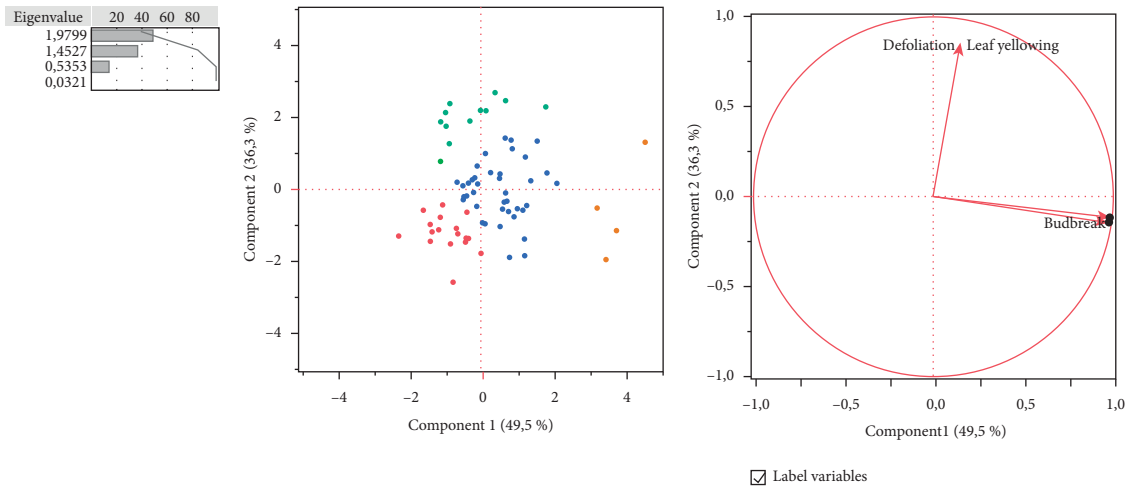

Figure 2: Principal component analyses biplot of walnut population.

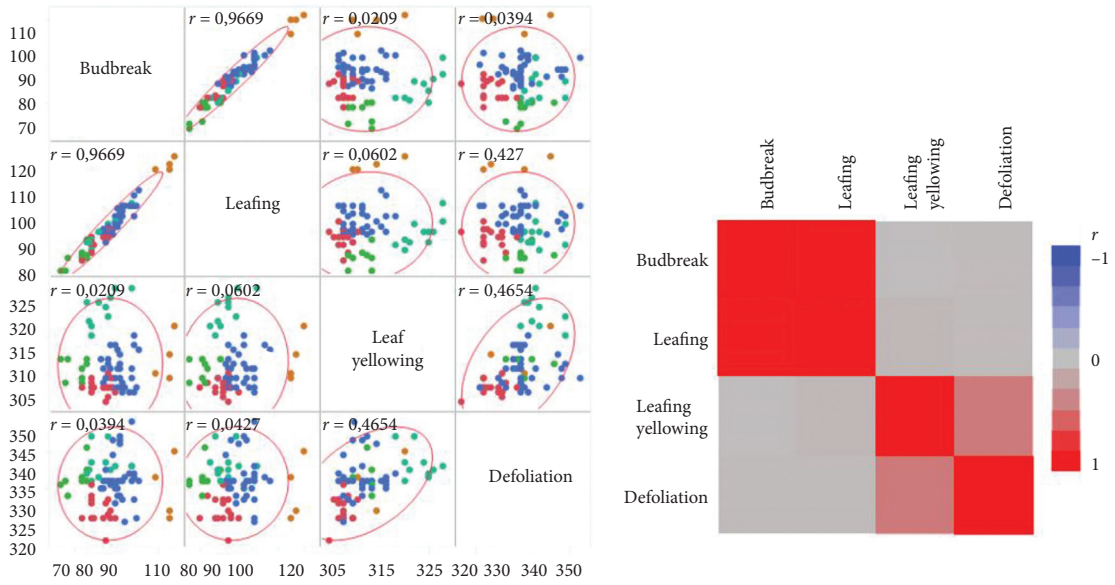

Figure 3: Scatterplot matrix and heatmap of correlations of phenological traits.

Correlation of coefficient between different traits of accessions revealed significant positive correlations among 4 phenological traits. The strongest positive correlation ($r = 0,97$) in the examined phenological traits was determined between dates of budburst and leafing. Also, a significant positive correlation ($r = 0,47$) was determined between leaf yellowing and defoliation dates. Amiri et al. [45] found a positive correlation between leafing date and defoliation dates ($r = 0,30$). Besides, significant correlations between leafing date and some horticultural traits were determined on walnut by other researchers [46–48]. Correlations between the studied phenological traits and individuals are presented in Figure 1 and the scatterplot matrix and heatmap of correlations determined between phenological traits are presented in Figure 3.

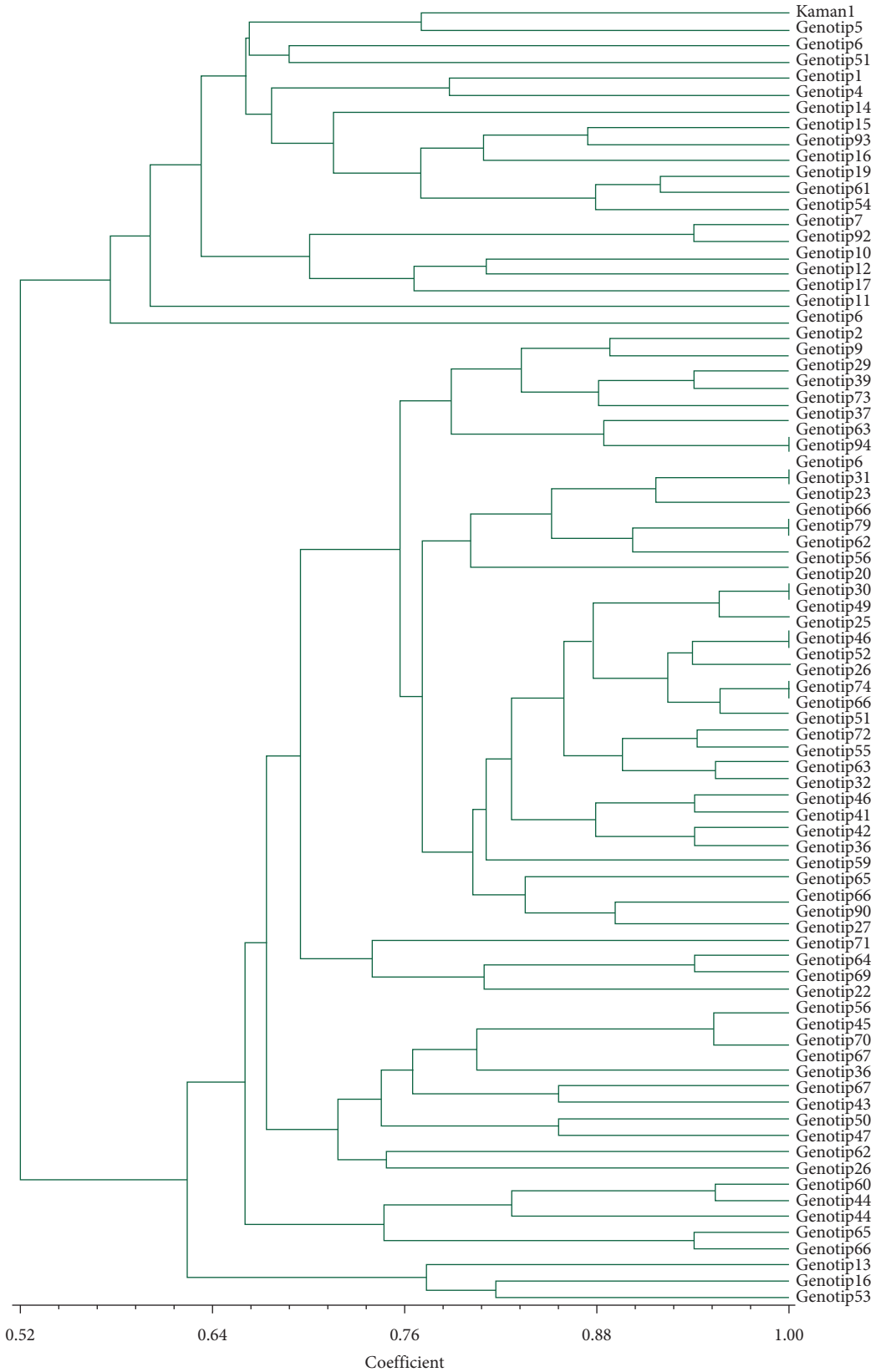

Figure 4: UPGMA dendrogram of walnut genotypes.

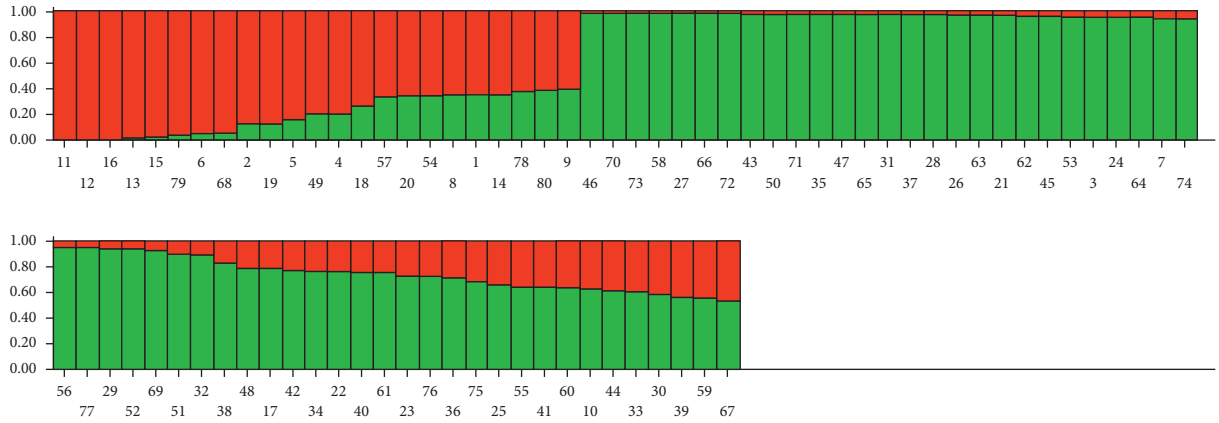

Figure 5: Population structure of 80 walnut genotypes ($\Delta K = 2$).

3.2. Polymorphism Analysis. 12 ISSR primer pairs were used to characterize genetic diversity among the walnut genotypes. Bands were obtained from 6 of these primer pairs and PIC values of allele numbers are presented in Table 4. A total of 44 bands were distinctly amplified within the 80 walnut genotypes. Of all the amplified bands, 44 amplified bands (84.53%) were polymorphic. Among the walnut population, a total of 38 alleles were detected. The number of alleles revealed by the ISSR analysis ranged from 3 to 9 alleles per locus with a mean value of 6.33 alleles per locus. Besides, polymorphism information content values ranged from 0.81 to 0.99 with a mean PIC value of 0.91.

Several studies have been conducted on walnut (*Juglans regia* L.) which determine genetic diversity using the ISSRs [18, 49–51]. However, the number of studies that determine the genetic diversity in seedlings is quite limited. Li et al. [52] used ISSR to determine the genetic diversity of some walnut seedlings. The results showed that 101 loci were detected by 9 ISSR primers screened out from 36 primers and 89 loci were polymorphic, accounting for 88.12%. Although the primers used were different, similar results were obtained with the rate of polymorphism obtained in our study.

3.3. Genetic Relationships and Population Structure. The ISSRs data were used to generate a dendrogram of 80 walnut genotypes, shown in Figure 4. Genotypes were found to be genetically similar to 0.52–0.99. Li et al. [52] reported that the genetic similarity rate ranged from 0.67 to 0.79 in a study conducted to determine the genetic diversity of 61 walnut genotypes obtained from 4 seedling populations. In another study conducted by Sharifi et al. [53], ISSR markers were used to determine the genetic diversity of 82 walnut genotypes. As a result of this study, Nei's genetic diversity values ranged from 0.13 to 0.24. The differences in the findings obtained are due to the differences in the populations used.

The genetic similarity coefficients of the walnut genotypes were lowest between Genotype 18, Genotype 27, Genotype 22, and Genotype 36, while it was highest was between Genotype 3, Genotype 42, Genotype 10, Genotype 48, Genotype 30, and Genotype 93. In the dendrogram, 2 main groups were revealed. The first group included Kaman-1 and its 19 progenies and the remaining 60 genotypes were in the other group. Genotype 5, Genotype 1, and Genotype 12 were very close to each other, both phenologically and genetically.

In this study, structural genetic analysis was also conducted using STRUCTURE 2.3.4 and Structure Harvester. As a result of the analysis, the highest Delta k value was in $\Delta K = 2$. For this reason, we determined that our walnut accessions are divided into 2 main groups using Delta $K = 2$ value and were similar to the results obtained by UPGMA analysis (Figure 5). According to these findings, 28.75% (23 genotypes) of the accessions were found in Cluster I, and the remaining genotypes (57 genotypes) were in Cluster II. In this study, it is worth noting that the walnut genotypes were always morphologically and genetically divided into 2 groups.

4. Conclusion

Turkey has an important genetic diversity in walnuts, and Kaman-1 is an important local cultivar in Turkey. In this study, we demonstrated genetic and morphological differences between progenies of Kaman-1 and this cultivar. We found that, when compared to Kaman-1, the progenies leafed and defoliated earlier or later than Kaman-1, while some had values very close to this cultivar. In the dendrogram, we created using Ward's method with phenological parameters, and we obtained 2 major groups. Besides, we used 12 ISSR markers to determine genetic diversity and obtained 44 polymorphic bands. As a result of the genetic analysis, both the UPGMA dendrogram and the structure genotypes were divided into 2 main clusters. Within the scope of the study, it was quite remarkable that the clusters obtained with both morphological and genetic parameters showed similarity. As a result, it was determined that genotypes obtained from seeds of Kaman-1 have a significant genetic diversity. Besides, the use of both genetic and morphological parameters in characterizing a population provides a clearer distinction and provides an important resource for future breeding work.

Data Availability

The main data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

[1] G. McGranahan and C. Leslie, "Walnut," in *Fruit Breeding. Handbook of Plant Breeding*, M. Badenes and D. Byrne, Eds., Vol. 8, Springer, Boston, MA, USA, 2012.

[2] Faostat, *Food and Agricultural Organization of the United Nations*, Rome, Italy, 2020, https://faostat.fao.org.

[3] W. H. Krueger, "Pollination of English walnuts: practices and problems," *HortTechnology*, vol. 10, no. 1, pp. 127–130, 2000.

[4] M. Sutyemez, "Comparison of AFLP polymorphism in progeny derived from dichogamous and homogamous walnut genotypes," *Pakistan Journal of Biological Sciences*, vol. 9, no. 12, pp. 2303–2307, 2006.

[5] A. Bernard, F. Lheureux, and E. Dirlewanger, "Walnut: past and future of genetic improvement," *Tree Genetics and Genomes*, vol. 14, no. 1, p. 1, 2018.

[6] I. E. Orhan, I. P. Suntar, and E. K. Akkol, "In vitroneuroprotective effects of the leaf and fruit extracts ofJuglans regiaL. (walnut) through enzymes linked to Alzheimer's disease and antioxidant activity," *International Journal of Food Sciences and Nutrition*, vol. 62, no. 8, pp. 781–786, 2011.

[7] C. Sánchez-González, C. J. Ciudad, V. Noé, and M. Izquierdo-Pulido, "Health benefits of walnut polyphenols: an exploration beyond their lipid profile," *Critical Reviews in Food Science and Nutrition*, vol. 57, no. 16, pp. 3373–3383, 2017.

[8] S. Taneva, S. Momchilova, I. Marekov, E. Blagoeva, and M. Nikolova, "Free and esterified sterols in walnuts and hazelnuts in three stages during kernel development," *Comptes rendus de l'Académie bulgare des Sciences*, vol. 66, no. 12, pp. 1681–1688, 2013.

[9] E. Kafkas, A. Burgut, H. Ozcan et al., "Fatty acid, total phenol and tocopherol profiles of some walnut cultivars: a comparative study," *Food and Nutrition Sciences*, vol. 08, no. 12, pp. 1074–1084, 2017.

[10] L. Xiang, X. L. Li, X. S. Wang et al., "Genetic diversity and population structure of Distylium chinense revealed by ISSR and SRAP analysis in the three gorges reservoir region of the yangtze river, China," *Global Ecology and Conservation*, vol. 21, Article ID e00805, 2020.

[11] UPOV, *International Union for the Protection of New Varieties of Plants. Guidelines for the Conduct of Tests for Distinctness, Uniformity and Stability. Walnut (Juglans regia)*, pp. 1–37, UPOV, Geneva, Switzerland, 2015.

[12] IPGRI, *Descriptors for walnut (Juglans spp.)*, pp. 1–54, International Plant Genetic Resources Institute, Rome, Italy, 1994.

[13] M. Ghasemi, K. Arzani, and D. Hassani, "Evaluation and identification of walnut (*Juglans regia* L.) genotypes in Markazi province of Iran," *Crop Breeding Journal*, vol. 2, no. 2, pp. 119–124, 2012.

[14] H. Zaher, B. Boulouha, M. Baaziz, L. Sikaoui, F. Gaboun, and S. M. Udupa, "Morphological and genetic diversity in olive (*Olea europaea* subsp. europaea L.) clones and varieties," *Plant Omics Journal*, vol. 4, no. 7, pp. 370–376, 2011.

[15] R. G. Fjellstrom and D. E. Parfitt, "Walnut (*Juglans* spp.) genetic diversity determined by restriction fragment length polymorphisms," *Genome*, vol. 37, no. 4, pp. 690–700, 1994.

[16] F. P. Nicese, J. I. Hormaza, and G. H. McGranahan, "Molecular characterization and genetic relatedness among walnut (*Juglans regia* L.) genotypes based on RAPD markers," *Euphytica*, vol. 101, no. 2, pp. 199–206, 1998.

[17] U. Erturk and Z. Dalkilic, "Determination of genetic relationship among some walnut (*Juglans regia* L.) genotypes and their early-bearing progenies using RAPD markers," *Romanian Biotechnol Lett.*vol. 16, pp. 5944–5952, 2011.

[18] D. Potter, F. Gao, G. Aiello, C. Leslie, and G. McGranahan, "Intersimple sequence repeat markers for fingerprinting and determining genetic relationships of walnut (*Juglans regia*) cultivars," *Journal of the American Society for Horticultural Science*, vol. 127, no. 1, pp. 75–81, 2002.

[19] A. Ji, Y. Wang, G. Wu, W. Wu, H. Yang, and Q. Wang, "Genetic diversity and population structure of North China mountain walnut revealed by ISSR," *American Journal of Plant Sciences*, vol. 05, no. 21, pp. 3194–3202, 2014.

[20] S. Kafkas, H. Ozkan, and M. Sütyemez, "DNA polymorphism and assessment of genetic relationships in walnut genotypes based on AFLP and SAMPL markers," *Journal of the American Society for Horticultural Science*, vol. 130, no. 4, pp. 585–590, 2005.

[21] S. Bayazit, K. Kazan, S. Gülbitti, V. Çevik, H. Ayanoğlu, and A. Ergül, "AFLP analysis of genetic diversity in low chill requiring walnut (Juglans regia L.) genotypes from Hatay, Turkey," *Scientia Horticulturae*, vol. 111, no. 4, pp. 394–398, 2007.

[22] I. Foroni, K. Woeste, L. M. Monti, and R. Rao, "Identification of "Sorrento" walnut using simple sequence repeats (SSRs)," *Genetic Resources and Crop Evolution*, vol. 54, no. 5, pp. 1081–1094, 2007.

[23] S. Mohsenipoor, K. Vahdati, R. Amiri, and M. R. Mozaffari, "Study of the genetic structure and gene flow in Persian walnut (*Juglans regia* L.) using SSR markers," *Acta Horticulturae*, vol. 861, no. 861, pp. 133–142, 2010.

[24] Y.-H. Hu, P. Zhao, Q. Zhang et al., "De novo assembly and characterization of transcriptome using Illumina sequencing and development of twenty five microsatellite markers for an endemic tree *Juglans hopeiensis* Hu in China," *Biochemical Systematics and Ecology*, vol. 63, pp. 201–211, 2015.

[25] M. Vischi, C. Chiabà, S. Raranciuc et al., "Genetic diversity of walnut (*Juglans regia* L.) in the Eastern Italian Alps," *Forests*, vol. 8, no. 3, pp. 81–94, 2017.

[26] K. Vahdati, S. Mohseni Pourtaklu, R. Karimi et al., "Genetic diversity and gene flow of some Persian walnut populations in southeast of Iran revealed by SSR markers," *Plant Systematics and Evolution*, vol. 301, no. 2, pp. 691–699, 2015.

[27] J. C. Huang and M. Sun, "Genetic diversity and relationships of sweetpotato and its wild relatives in Ipomoea series Batatas (*Convolvulaceae*) as revealed by inter-simple sequence repeat (ISSR) and restriction analysis of chloroplast DNA," *Theoretical and Applied Genetics*, vol. 100, no. 7, pp. 1050–1060, 2000.

[28] J. J. Doyle and J. L. Doyle, "A Rapid DNA Isolation procedure for small quantities of fresh leaf tissue," *Phytochemical Bulletin*, vol. 19, pp. 11–15, 1987.

[29] A. Bardak, "Pamukta ilişkilendirme haritalaması yöntemiyle markör geliştirme," *Bursa Tarım Kongresi*, vol. 13-15, pp. 1–10, 2017.

[30] M. Sütyemez, ""Kahramanmaraş bölgesinde ceviz (*Juglans regia* L.) seleksiyonu ve seçilmiş bazı tiplerin döllenme biyolojileri üzerine araştırmalar," Çukurova Üniversitesi Fen Bilimleri Enstitüsü," *Doktora tezi*, vol. 416, 1998.

[31] A. Bardak and Y. Bölek, "Genetic diversity of diploid and tetraploid cottons determined by SSR and ISSR markers," *Turkish Journal of Field Crops*, vol. 17, no. 2, pp. 139–144, 2012.

[32] P. R. Laborda, K. M. Oliveira, A. A. F. Garcia, M. E. A. G. Z. Paterniani, and A. P. De Souza, "Tropical maize germplasm: what can we say about its genetic diversity in the light of molecular markers?" *Theoretical and Applied Genetics*, vol. 111, no. 7, pp. 1288–1299, 2005.

[33] M. Nei, "Genetic distance between populations," *The American Naturalist*, vol. 106, no. 949, pp. 283–292, 1972.

[34] F. J. Rohlf, *NTSYSpc Numerical Taxonomy and Multivariate Analysis System Version 2.0 User Guide*, pp. 1–43, Applied Biostatistics Inc., Port Jefferson, NY, USA, 1998.

[35] L. R. Dice, "Measures of the amount of ecologic association between species," *Ecology*, vol. 26, no. 3, pp. 297–302, 1945.

[36] M. J. Hubisz, D. Falush, M. Stephens, and J. K. Pritchard, "Inferring weak population structure with the assistance of sample group information," *Molecular Ecology Resources*, vol. 9, no. 5, pp. 1322–1332, 2009.

[37] M. R. Anderberg, *Cluster Analysis for Applications*, pp. 2–18, Academic Press, New York, NY, USA, 1973.

[38] Y. Akca and S. Ozongun, "Selection of late leafing, late flowering, laterally fruitful walnut(Juglans regia)types in Turkey," *New Zealand Journal of Crop and Horticultural Science*, vol. 32, no. 4, pp. 337–342, 2004.

[39] A. Ebrahimi, R. Fatahi, and Z. Zamani, "Analysis of genetic diversity among some Persian walnut genotypes (*Juglans regia* L.) using morphological traits and SSRs markers," *Scientia Horticulturae*, vol. 130, no. 1, pp. 146–151, 2011.

[40] Ş. B. Bükücü and M. Sütyemez M, "The determination of the chilling requirements of some walnut (*Juglans regia* L.) cultivars and types," *Turkish Journal of Agricultural and Natural Sciences*, vol. 3, no. 4, pp. 305–310, 2016.

[41] A. Hassankhah, K. Vahdati, M. Rahemi, D. Hassani, and S. Sarikhani Khorami, "Persian walnut phenology: effect of chilling and heat requirements on budbreak and flowering date," *International Journal of Horticultural Science and Technology*, vol. 4, no. 2, pp. 259–271, 2017.

[42] S. Kefayati, A. S. Ikhsan, M. Sutyemez et al., "First simple sequence repeat-based genetic linkage map reveals a major QTL for leafing time in walnut (*Juglans regia* L.)," *Tree Genetics and Genomes*, vol. 15, no. 1, pp. 1–13, 2019.

[43] A. A. Aslamarz, K. Vahdati, M. Rahemi, and D. Hassani, "Evaluation of chilling-heat requirements of some Persian walnut cultivars," *VI International Walnut Symposium*, vol. 861, pp. 317–320, 2009.

[44] K. Arzani, H. Mansouri-Ardakan, A. Vezvaei, and M. R. Roozban, "Morphological variation among Persian walnut (*Juglans regia*) genotypes from central Iran," *New Zealand Journal of Crop and Horticultural Science*, vol. 36, no. 3, pp. 159–168, 2008.

[45] R. Amiri, K. Vahdati, S. Mohsenipoor, M. R. Mozaffari, and C. Leslie, "Correlations between some horticultural traits in walnut," *HortScience*, vol. 45, no. 11, pp. 1690–1694, 2010.

[46] A. Ebrahimi, A. Khadivi-Khub, Z. Nosrati, and R. Karimi, "Identification of superior walnut (*Juglans regia*) genotypes with late leafing and high kernel quality in Iran," *Scientia Horticulturae*, vol. 193, pp. 195–201, 2015.

[47] A. Khadivi-Khub, A. Ebrahimi, F. Sheibani, and A. Esmaeili, "Phenological and pomological characterization of Persian walnut to select promising trees," *Euphytica*, vol. 205, no. 2, pp. 557–567, 2015.

[48] B. Abedi and T. Parvaneh, "Study of correlations between horticultural traits and variables affecting kernel percentage of walnut (*Juglans regia* L.)," *Journal of Nuts*, vol. 7, no. 1, pp. 35–44, 2016.

[49] P. Pollegioni, S. Bartoli, F. Cannata, and M. E. Malvolti, "Genetic differentiation of four Italian walnut (*Juglans regia* L.) varieties by inter simple sequence repeat (ISSR)," *Journal of Genetics and Breeding*, vol. 57, no. 3, pp. 231–240, 2003.

[50] M. V. Christopoulos, D. Rouskas, E. Tsantili, and P. J. Bebeli, "Germplasm diversity and genetic relationships among walnut (*Juglans regia* L.) cultivars and Greek local selections revealed by Inter-Simple Sequence Repeat (ISSR) markers," *Scientia Horticulturae*, vol. 125, no. 4, pp. 584–592, 2010.

[51] R. Mahmoodi, F. Rahmani, and R. Paktarmani, "Genetic diversity of Persian walnut from Iran as revealed by inter-simple sequence repeat (ISSR) markers," *Journal of the American Pomological Society*, vol. 66, no. 2, pp. 101–106, 2012.

[52] G. T. Li, C. X. Ai, L. S. Zhang, H. R. Wei, and Q. Z. Liu, "ISSR analysis of genetic diversity among seedling walnut (*Juglans* spp.) populations," *Journal of Plant Genetic Resources*, vol. 12, no. 4, pp. 640–645, 2011.

[53] S. Sharifi, R. Amiri Fahliani, and A. Masoumi Asl, "Walnut genetic diversity investigation using phenological and morphological characteristics and ISSR markers," *Journal of Agricultural Science and Technology*, vol. 23, no. 1, pp. 187–200, 2021.