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

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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 Sütyemaz [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 MgCl₂, 1 μL Taq DNA polymerase, 12 μL dH₂O, 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 μL 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:

\[
PIC = 1 - \sum Pij2j = 1, \tag{1}
\]
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 over 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  | CACACACACACAAAA                 |
| 2  | ISSR3  | CACACACACAGGG                   |
| 3  | ISSR4  | CACACACACAGC                    |
| 4  | ISSR5  | CACACACACACAG                   |
| 5  | ISSR6  | CACACACACACAGT                  |
| 6  | ISSR7  | ACACACACACACACCG                |
| 7  | ISSR8  | ACACACACACACACACCC              |
| 8  | ISSR9  | ACACACACACACACACTG              |
| 9  | ISSR11 | GAGAGAGAGAGAGAGAGATC            |
| 10 | ISSR12 | GAGAGAGAGAGAGAGAGAGAC           |
| 11 | ISSR13 | GAGAGAGAGAGAGAGAGAGC            |
| 12 | ISSR15 | ATATATATATATATATATATATATATATATATATAT |

where $P_{ij}$ 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 $\Delta K$ 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 354th 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.
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.

### 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.](image)

![Figure 3: Scatterplot matrix and heatmap of correlations of phenological traits.](image)
Figure 4: UPGMA dendrogram of walnut genotypes.
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 in 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. 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.

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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