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Assessment of Genetic Diversity in Walnut (*Juglans regia* L.) Genotypes from Southern and Southeastern Kazakhstan Using Microsatellite Markers

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Abstract

The walnut (*Juglans regia* L.) holds a significant position among Kazakhstan's nut crops, both in terms of cultivation and consumption. Although genetic resources of walnut are accessible within the country, they remain underexplored, yet they represent a valuable foundation for breeding initiatives and the development of locally adapted varieties. Currently, a comprehensive genetic profile of these resources is lacking, which hampers their effective utilization. The aim of this research was to evaluate the genetic diversity within a collection of walnut accessions collected from the southern and southeastern regions of Kazakhstan, including Almaty and Turkestan regions. To achieve this, eight SSR molecular markers were employed, providing insights into the genetic structure and relationships among the germplasm. All markers exhibited a high degree of polymorphism, with the WGA276 locus standing out as the most informative, displaying the greatest number of alleles and a high Shannon diversity index. The average expected heterozygosity (He) was 0.704 and was significantly higher than the observed heterozygosity (Ho = 0.547), which was confirmed by *t*-test (t = −6.426, $p < 0.05$). The findings indicated substantial genetic variation and intra-population polymorphism: on average, 5.875 alleles per locus were identified, and the Shannon index was 1.444. The population from Turkestan region (population 1, Sairam-Ugam) demonstrated the highest levels of diversity. Analyses of genetic structure, conducted through STRUCTURE, PCoA, and UPGMA methods, confirmed the existence of two genetically distinct groups exhibiting considerable diversity. Future research should focus on conserving the highly diverse populations in the Turkestan region to facilitate the development of stress-tolerant varieties. These findings highlight the importance of conserving and harnessing the genetic resources of Kazakhstan's *J. regia* populations for future breeding efforts.

Keywords: walnut; microsatellite markers; genetic diversity; polymorphism; population structure; breeding

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1. Introduction

Walnut (*Juglans regia* L.) is an economically valuable nut crop [1,2]. It contains bioactive compounds such as flavonoids and polyphenols, which exhibit antioxidant properties [2,3]. The elevated protein and lipid content of walnut kernels underpin their essential role in human nutrition [4].

The origin and dissemination of walnut in Asia are the result of intricate interactions between biogeographical processes and human activities. The spread of walnuts across the continent was significantly influenced by trade routes, notably the Silk Road and the Persian Royal Road, which served as "green corridors" enabling the movement of nuts despite geographical obstacles. These routes facilitated the widespread exchange of walnut germplasm throughout Asia, contributing to its broad distribution [5,6]. Currently, the primary range of walnut extends from the Balkan region eastward into Asia, encompassing countries such as China, Iran, Kazakhstan, Afghanistan, and Pakistan [7,8].

China is the world's largest walnut producer, accounting for approximately 50% of global output, followed by the United States, Iran, and other major producers [9–11]. From 2012 to 2021, global walnut production increased significantly—from 2.37 to 3.50 million tons [12]. According to the latest data from FAOSTAT, global walnut production has steadily increased over the past three years, reaching approximately 3.1 million tons in 2021, 3.2 million tons in 2022, and an estimated 3.3 million tons in 2023 [12]. This growth has been driven by rising demand, improved cultivation techniques, and the development of resilient varieties.

Investigating and evaluating the genetic diversity of Central Asian walnut germplasm is essential for regional breeding programs. In Kazakhstan, rising demand has spurred expanded cultivation and yield increases in suitable areas. Although Kazakhstan possesses a diverse gene pool of walnut resources, there is currently no established national breeding program dedicated to their improvement. Several international collaborations are underway to develop breeding technologies, involving joint projects with Russia, the United States, Bulgaria, the Czech Republic, and Poland, aimed at harnessing and conserving the region's genetic potential [13]. Kazakhstan's predominantly continental climate plays a crucial role in fostering a highly diverse genetic pool of walnuts, including genotypes adapted to withstand low temperatures and produce high-quality fruits. These genetic resources are of great importance for the development of targeted breeding programs focused on improving winter hardiness, enhancing resistance to pests and diseases, and increasing both yield and fruit quality. Leveraging this genetic diversity is vital for ensuring the sustainability and resilience of walnut cultivation in the region, ultimately supporting its economic and agricultural development [13].

A study was conducted on the genetic resources of walnut (*Juglans regia* L.) in Kazakhstan, aiming to identify and select promising genotypes for further use in breeding programs, as well as to assess the genetic diversity of this species [13,14]. As a result, the most promising genotypes were selected, which can be further utilized in breeding programs and for commercial cultivation. The region's favorable soil and climatic conditions present significant potential for the development of walnut industry in southern Kazakhstan. An evaluation of local walnut populations allowed for the selection of genotypes best suited to the specific environmental conditions of the southern and southeastern regions [15]. Previously, our research included mapping the distribution of walnut in this area, as well as the morphological characterization of walnut fruits [16]. Additional studies focused on the species' biological traits, its adaptation to local climate, and the impact of cold stress on its morphological, physiological, and phytochemical properties [17]. Valuable germplasm from natural populations was collected, preserved in vitro, and stored in a cryobank for long-term conservation and future utilization [18,19]. Moreover, efforts were made to assess the resistance of local varieties to pests and diseases, including bacterial blight caused by *Pantoea agglomerans*, leading to the identification of resistant genotypes [20]. Despite extensive research and the region's conducive natural conditions, the commercial cultivation of walnuts in Kazakhstan remains relatively underdeveloped.

Kazakhstan possesses extensive genetic resources related to the germplasm of walnut; however, their identification and detailed study have yet to be conducted. Kazakhstan has not yet established a national walnut breeding program, and systematic research on polymorphism analysis of SSR markers in local populations is still lacking. Investigating the genetic diversity within these resources is essential for effective management of the gene pool and for enhancing breeding efficiency. Molecular markers serve as a vital tool in this context, enabling the precise characterization of germplasm, the verification of cultivar origins, and the elucidation of genetic relationships among different accessions. Unlike morphological traits, molecular markers are unaffected by environmental conditions and can reveal cryptic genetic variation, thereby significantly broadening the scope for selective breeding and conservation of genetic diversity. Various types of molecular markers have been developed to facilitate genetic studies, including RFLP (Restriction Fragment Length Polymorphism) [21], RAPD (Random Amplified Polymorphic DNA) [22], AFLP (Amplified Fragment Length Polymorphism) [23,24], SSRs (Simple Sequence Repeats) [25], and SNP (Single Nucleotide Polymorphism) [26]. Among these, SSR markers are regarded as the most reliable due to their high polymorphism, with repeat motifs typically ranging from 1 to 6 base pairs [27–29]. Their widespread distribution across the genome and uniform coverage make SSRs a highly informative tool for various genetic analyses [30]. They are extensively employed in studies of biotic and abiotic stress responses, linkage map construction, analysis of genetic relationships among germplasm populations, and assessment of genetic diversity in *Juglans regia* [25,31–39]. Characterized by high polymorphism levels, co-dominant inheritance, and locus-specificity, SSR markers are invaluable for detailed genetic characterization. Investigating the genetic variability of walnut using molecular techniques is a crucial step toward establishing effective breeding strategies, understanding population structure, and elucidating genetic relationships among germplasm from different regions. Despite the availability of walnut genetic resources within Kazakhstan, these resources remain largely underexplored, limiting their potential for breeding and conservation efforts. They represent a valuable foundation for developing locally adapted varieties, yet a comprehensive genetic profile of these resources is currently lacking, which hampers their optimal utilization. The aim of this research is to evaluate the genetic diversity and population structure of *Juglans regia* accessions collected from the southern and southeastern regions of Kazakhstan, utilizing molecular markers to generate detailed insights into their genetic relationships and variability.

2. Materials and Methods

2.1. Plant Materials

The plant material for this study was collected from the primary walnut-growing regions of Kazakhstan, specifically from the Turkestan and Almaty regions, in 2024. A total of 75 samples were gathered through field collection across eight different sites in the southern and southeastern parts of the country, including four locations within Turkestan region and four within Almaty region. The sampling comprised 48 specimens from Turkestan and 27 from Almaty regions. Detailed information regarding the collected samples is provided in Table S2 while their sampling locations are illustrated in Figure 1.

The freshly collected leaves were placed in self-sealing bags and stored in a cooler with ice. After freezing in liquid nitrogen, the samples were kept in a freezer at a temperature of −80 °C to ensure preservation until further analysis.

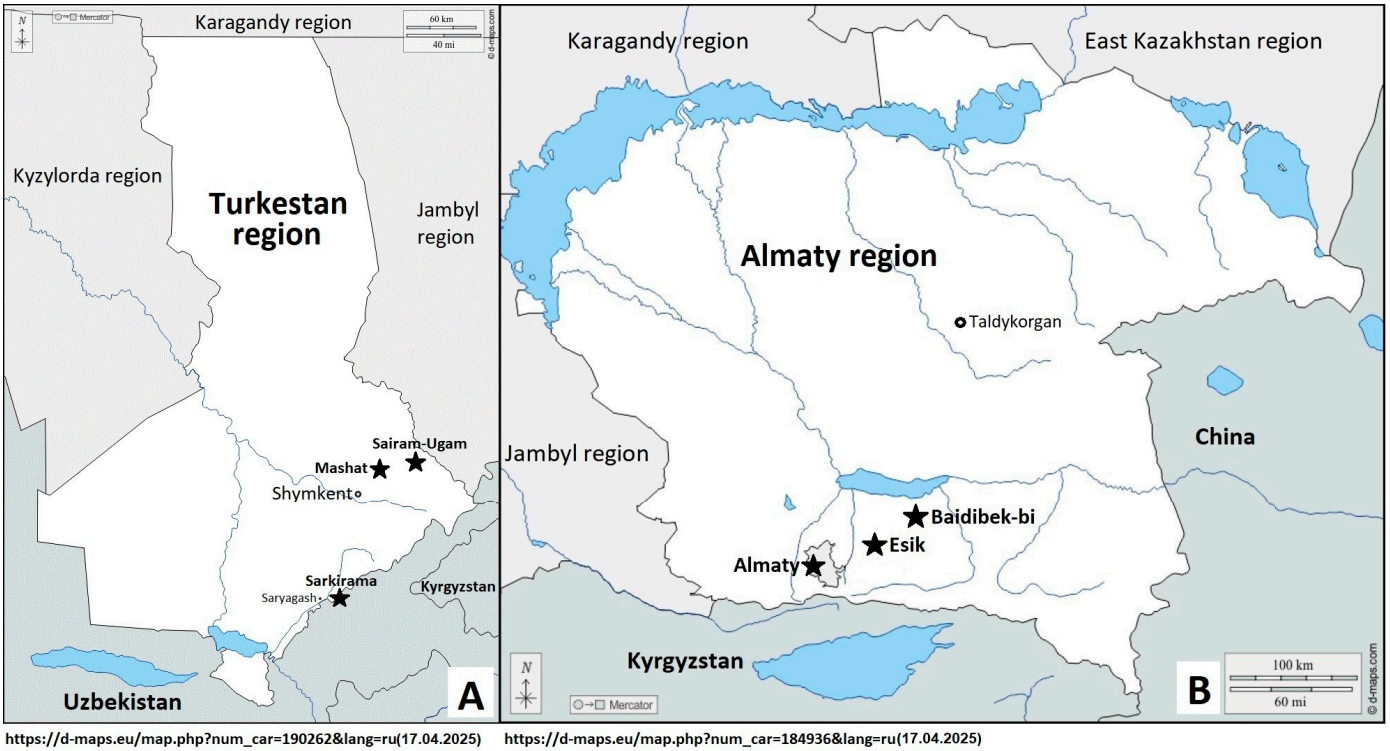

Figure 1. Distribution of walnut *(Juglans regia* L.) and collection sites on the map of the southern (**A**) and southeastern (**B**) regions of Kazakhstan.

The plant material was collected from trees growing in the vicinity of settlements and in mountainous areas. The populations consisted of mature walnut trees, all of which originated from open-pollinated seedlings derived from orchards or home gardens. From each population, between 15 and 40 individual trees were sampled, depending on availability (total N = 102 individuals), to ensure representative coverage [40]. Tree density and accessibility varied across different sites. The sampled walnut trees were grouped into six geographic populations: three in Turkestan region (Sairam-Ugam, Mashat, and Sarkyrama) and three in Almaty region (Almaty city, Baidibek Bi, and Esik) (Table S2 and Figure 1).

Figure 2 displays photographs of selected walnut samples collected during field expeditions in the Turkestan and Almaty regions.

2.2. DNA Extraction and Molecular Analysis Using SSR Markers

In 2024, five young leaves were collected from each of 75 walnut trees during the active growth phase. Thus, the total number of trees included in the study is 75. Samples were immediately preserved in silica gel to prevent polyphenol oxidation and homogenized in liquid nitrogen using an optimized CTAB protocol with modern modifications [41]. DNA quality evaluation and quantification was performed by using a SmartSpecTMPlus Spectrophotometer (Bio-RAD Laboratories, Inc., Hercules, CA, USA). DNA concentration was standardized to 30 ng/µL for PCR using Tris-EDTA buffer (pH 8.0). The protocol was specifically adapted for tannin-rich tissues, as confirmed by successful SSR marker amplification in downstream experiments. To minimize fragmentation, lysis time was reduced to 30 min, and temperature was maintained at 65 °C throughout the process. DNA was quantified spectrophotometrically (260 and 280 nm) and visualized on 1.0% agarose gels stained with ethidium bromide. Eight SSR molecular markers (WGA001, WGA027, WGA042, WGA118, WGA009, WGA202, WGA276, and WGA376) were chosen for the genotypic characterization of walnut cultivars based on a comprehensive review of existing

scientific studies [42,43]. The selection criteria included their uniform distribution across the walnut genome and the criterion of polymorphism information content (PIC) > 0.5 in previous studies. These markers were specifically selected to ensure robust genotyping results. Detailed information regarding each primer, including sequence, annealing temperature, and expected amplicon size, is presented in Table 1.

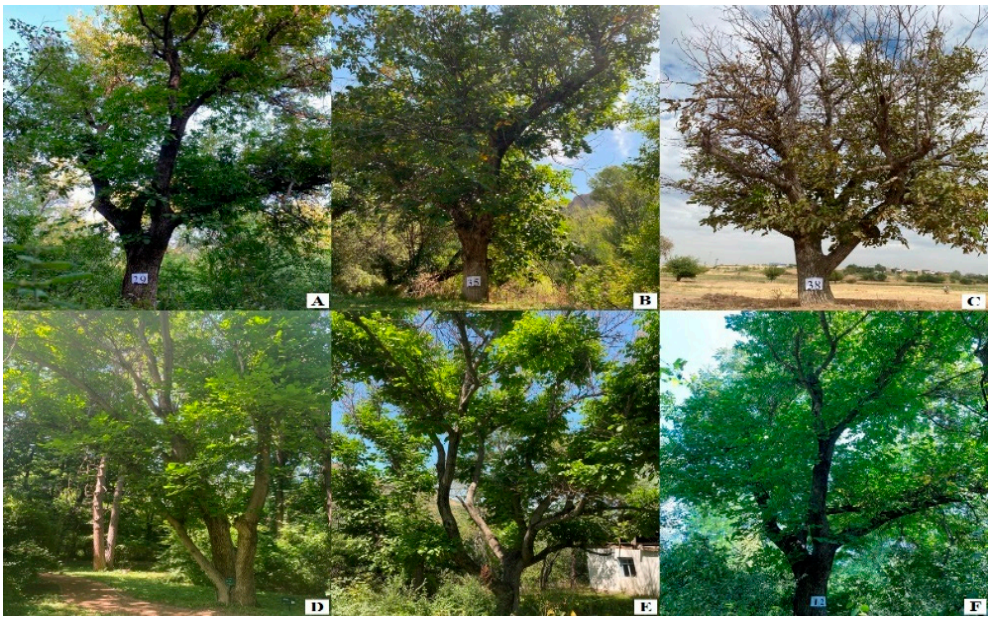

Figure 2. Walnut (*Juglans regia* L.) trees from various populations: (**A**) Sairam-Ugam State National Nature Park, (**B**) Mashat v., (**C**) Sarkyrama v. in Turkestan Region, (**D**) Almaty city, (**E**) Baidibek-bi, and (**F**) Esik in Almaty Region.

Table 1. Details and characteristics of selected SSR markers used for analysis of walnut genetic diversity.

Primer	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Annealing Temperature (°C)	Reference
WGA001	ATTGGAAGGGAAGGGAAATG	CGCGCACATACGTAAATCAC	56	Dangl et al., 2005 [42]
WGA027	AACCCTACAACGCCTTGATG	TGCTCAGGCTCCACTTCC	57	Woeste et al., 2002 [43]
WGA042	GTGGGTTCGACCGTGAAC	AACTTTGCACCACATCCACA	55	Shah et al., 2020 [35]
WGA118	TGTGCTCTGATCTGCCTCC	GGGTGGGTGAAAAGTAGCAA	60	Dangl et al., 2005 [42]
WGA009	CATCAAAGCAAGCAATGGG	CCATTGCTCTGTGATTGGG	56	Dangl et al., 2005 [42]
WGA202	CCCATCTACCGTTGCACTTT	GCTGGTGGTTCTATCATGGG	62	Dangl et al., 2005 [42]
WGA276	CTCACTTTCTCGGCTCTTCC	GGTCTTATGTGGGCAGTCGT	60	Dangl et al., 2005 [42]
WGA376	GCCCTCAAAGTGATGAACGT	TCATCCATATTTACCCCTTTCG	56	Dangl et al., 2005 [42]

The PCR reaction mixture (25 µL) consisted of 2.5 µL of genomic DNA (30 ng), 1 µL of each primer (1 pM/µL) (Sigma-Aldrich, St. Louis, MO, USA), 2.5 µL of a dNTP mixture (2.5 mM; dATP, dCTP, dGTP, and dTTP in aqueous solution) (ZAO Sileks, Moscow, Russia), 2.5 µL of $MgCl_2$ (25 mM), 0.2 µL of Taq polymerase (5 U/µL) (ZAO Sileks, Sayansk, Russia), 2.5 µL of 10× PCR buffer, and 12.8 µL of ddH_2O. Amplification products were separated using TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8), with ethidium bromide added for visualization. A 100 bp DNA ladder (Fermentas, Vilnius, Lithuania) was used as a molecular size marker to estimate fragment lengths. PCR was performed on a Bio-Rad

T100™ Thermal Cycler (Bio-RAD, Hercules, CA, USA) with the following conditions: an initial denaturation step at 94 °C for 5 min; 35 cycles consisting of denaturation at 94 °C for 30 s and extension at 72 °C for 30 s; and a final extension step at 72 °C for 5 min.

Amplification products were analyzed using a 2% agarose gel, with TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8) employed for separation. Ethidium bromide was added to visualize the DNA fragments. A 100 bp DNA ladder (Fermentas, Vilnius, Lithuania) served as a molecular size reference to estimate fragment lengths. The Gel Documentation System (Gel Doc XR+, Bio-RAD, Hercules, CA, USA) was utilized to capture and analyze the results. Each sample was subjected to three independent tests to ensure reliability and reproducibility of the data.

2.3. Data Analysis

For the analysis of SSR data, GenAlEx 6.5 software was utilized [44,45]. Genetic diversity within the studied walnut populations was evaluated using several key parameters: the total number of observed alleles (NA), observed heterozygosity (Ho), expected heterozygosity (He), effective number of alleles (Ne), Shannon's information index (I). Further genetic analysis included an Analysis of Molecular Variance (AMOVA). The resulting genetic distance matrix was used for Principal Coordinate Analysis (PCoA) and to construct a UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram using PAST 4.03 software [46]. This approach enabled the identification of genetic clusters within the population and provided insights into the extent of genetic admixture among them.

To improve the clustering of walnut genotypes, we applied the model-based Bayesian clustering algorithm implemented in STRUCTURE v.2.3.4 [47]. The results generated by STRUCTURE were further analyzed using STRUCTURE HARVESTER v.0.6.1 to identify the optimal number of genetic clusters (K) [48].

3. Results

3.1. Analysis of the Genetic Diversity of Walnut (Juglans regia)

Using eight pairs of SSR markers, the genetic diversity and population structure of 75 walnut genotypes were assessed, along with an analysis of polymorphism at SSR loci. DNA profiles specific to the studied walnut samples were generated. The results of the genetic diversity analysis are summarized in Table 2. A total of 47 alleles were identified across all markers, with an average of 5.875 alleles per locus. The number of alleles per locus (Na) ranged from 2, observed at loci WGA027 and WGA042, to 10 at locus WGA276.

Table 2. Polymorphism levels of SSR markers and genetic diversity parameters.

Locus	Na	Ne	I	Ho	He
WGA001	6.000	3.882	1.507	0.612	0.742
WGA027	2.000	1.930	0.675	0.238	0.482
WGA042	2.000	1.943	0.679	0.341	0.485
WGA118	3.000	2.665	1.039	0.593	0.625
WGA009	6.000	4.592	1.650	0.625	0.782
WGA202	9.000	5.619	1.916	0.571	0.822
WGA276	10.000	6.664	2.095	0.717	0.850
WGA376	9.000	6.458	1.995	0.676	0.845
Mean	5.875	4.219	1.444	0.547	0.704

Note: Na—number of detected alleles; Ne—effective number of alleles; Ho—observed heterozygosity; He—expected heterozygosity; I—Shannon information index.

The effective number of alleles (Ne) ranged from 1.930 to 6.664, with an average of 4.219 (Table 2). Expected heterozygosity (He) varied between 0.482 and 0.850, averaging 0.704. Observed heterozygosity (Ho), in turn, ranged from 0.238 to 0.717, with an overall

mean of 0.547. The highest observed heterozygosity and expected heterozygosity was detected at locus WGA276. The Shannon diversity index ranged from 0.675 to 2.095, with an average value of 1.444. The marker WGA027, exhibiting the lowest polymorphism, also showed the smallest Shannon index (I), whereas the most polymorphic marker, WGA276, demonstrated the highest diversity index.

The selected walnut samples from six locations, representing two geographic regions (Turkestan and Almaty regions), were compared based on several genetic parameters. These included the number of alleles (Na), the effective number of alleles (Ne), the Shannon diversity index (I), observed heterozygosity (Ho), and expected heterozygosity (He). The analysis of genetic diversity across the six populations is summarized in Table 3.

Table 3. Comparison of six walnut populations from different regions of Kazakhstan based on genetic parameters.

Pop	Location	Na	Ne	I	Ho	He
pop1	S-U	4.625	3.658	1.307	0.697	0.683
pop2	MA	2.375	1.851	0.670	0.367	0.388
pop3	SA	2.500	1.877	0.642	0.290	0.363
pop4	AL	3.500	2.612	1.000	0.437	0.555
pop5	BA	1.125	1.075	0.157	0.063	0.109
pop6	ES	3.875	3.420	1.187	0.634	0.639

Note: pop1–pop3—walnut populations from the Turkestan region (Sairam-Ugam, S-U; Mashat, MA; Sarkyrama, SA); pop4–pop6—populations from the Almaty region (Almaty, AL; Baidibek-bi, BA and Esik, ES).

The study results indicated that the average number of alleles per locus ranged from 1.125 to 4.625. The effective number of alleles (Ne) varied between 1.075 and 3.658. Heterozygosity parameters showed that expected heterozygosity (He) ranged from 0.109 to 0.683, while observed heterozygosity (Ho) fluctuated from 0.063 to 0.697. The Shannon diversity index spanned from 0.157 to 1.307. The lowest levels of genetic diversity were observed in population 5 (pop5) from Baidibek Bi in Almaty region, as evidenced by their low values of Na, Ne, and Shannon index. The Baidibek Bi population may have allele loss due to isolation of small populations", enhancing the correlation of the results. Conversely, the highest genetic diversity was recorded in population 1 (pop1) from Sairam-Ugam in Turkestan region.

3.2. Population Structure Analysis

For the subsequent genetic analysis, the population structure of 75 walnut genotypes was analyzed (Figures 3 and 4). The STRUCTURE 2.3.4 software evaluated the likelihood of classifying individuals into different numbers of clusters. The optimal number of groups was identified based on the ΔK statistic.

The analysis utilized a model incorporating admixture and correlated allele frequencies, with the ΔK method relying on the rate of change of the second-order likelihood function [48]. A clear peak was detected at K = 2, confirming the most probable number of genetic clusters, as shown in Figure 3.

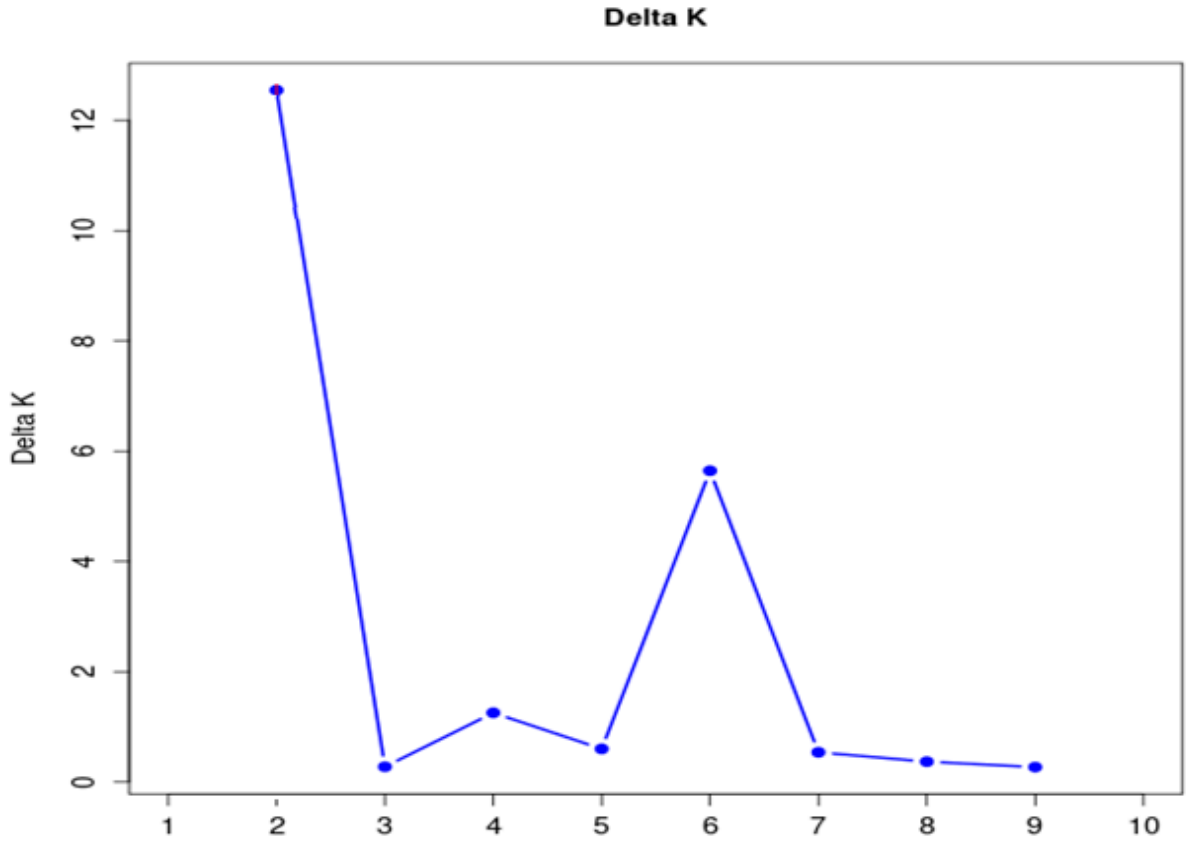

Figure 3. Population structure of 75 walnut genotypes based on 8 SSR markers, as determined by STRUCTURE analysis, assuming K = 2.

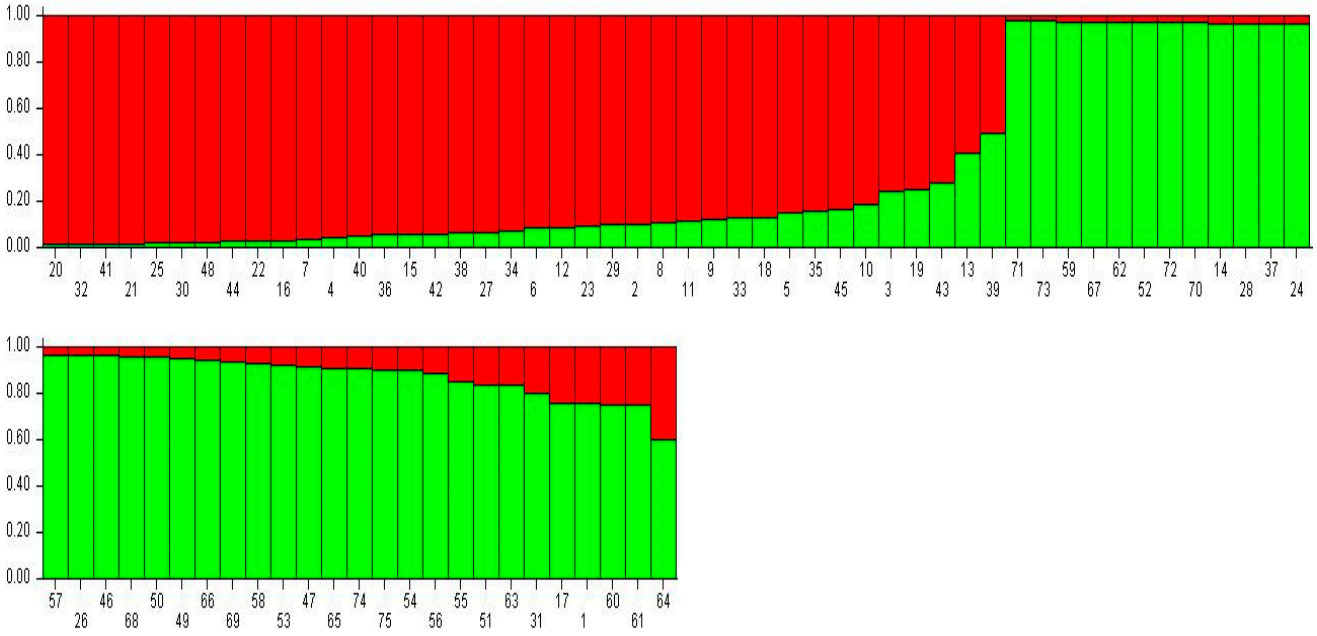

Figure 4. Genetic structure of 75 walnut genotypes analyzed using 8 SSR markers. Each color (red, green) represents a different genetic cluster among the samples.

Based on the amplification of 8 SSR markers across 75 walnut samples, a genetic distance was calculated. The genotypes were grouped into two clusters. The first cluster mainly included individuals numbered 20, 32, 41, 21, 25, 30, 48, 44, 22, 16, 7, 4, 40, 36, 15, 42, 38, 27, 34, 6, 12, 23, 29, 2, 8, 11, 9, 33, 18, 5, 35, 45, 10, 3, 19, 43, 13, and 39 from

Turkestan region (populations pop1 (S-U), pop2 (MA), and pop3 (SA). The average genetic distance (expected heterozygosity) within this cluster was 0.486 (Figure 4). The second cluster comprised genotypes numbered 71, 73, 59, 67, 62, 52, 72, 70, 14, 28, 37, 24, 57, 26, 46, 68, 50, 49, 66, 69, 58, 53, 47, 65, 74, 75, 54, 56, 55, 51, 63, 31, 17, 1, 60, 61, and 64, collected from Almaty region and Turkestan region (populations: pop1 (S-U), pop2 (MA), and pop3 (SA), pop4 (AL), pop5 (BA), and pop6 (ES)). The average genetic distance (expected heterozygosity) within this cluster was 0.747 (Figure 4). To confirm the significance of differences between the groups, additional statistical analysis was performed using analysis of variance (ANOVA). ANOVA revealed statistically significant differences between the groups (F = 2.872, p = 0.00018) in Table S3. Thus, our results confirm that the differences in genetic distances between the analyzed clusters are statistically significant.

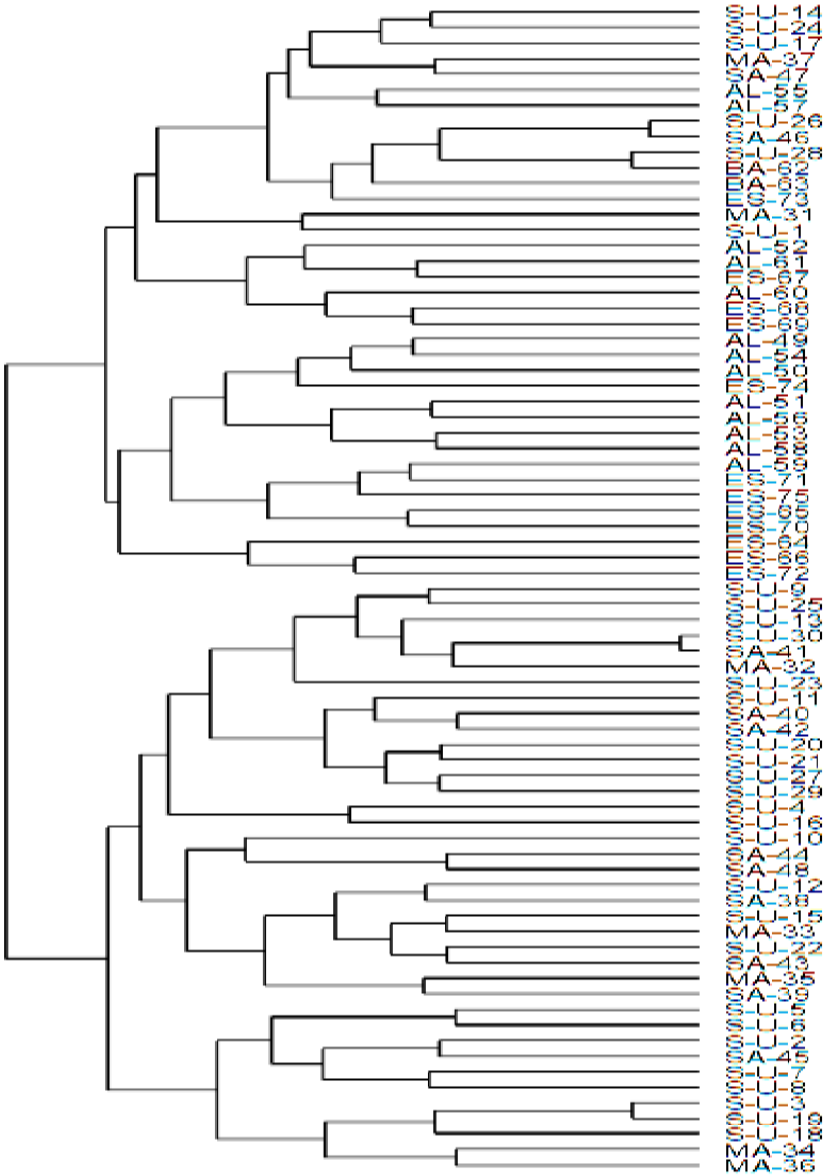

Figure 5. Clustering analysis for 75 walnut individuals collected from the 6 locations of Kazakhstan using the UPGMA method.

3.3. Cluster Analysis Using UPGMA and Principal Coordinate Analysis (PCoA)

Genetic distances among walnut groups based on origin were assessed using cluster analysis with the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and Principal Coordinate Analysis (PCoA). The list of studied samples, along with their

population designations and geographical locations, is provided in Table 3 in the main text and Table S1 in the Supplementary Materials. The dendrogram of 75 walnut genotypes, generated through cluster analysis using the UPGMA method, revealed that the studied material formed subgroups of individuals with distinct differences, as illustrated in Figure 5. The analysis divided the samples into two main branches, further subdivided into six sub-branches. The first branch comprised 37 genotypes of walnut (49.3%), including populations from Almaty and Turkestan region (S-U pop1, MA pop2, SA pop3, AL pop4, BA pop5, and ES pop6). The second branch included 38 genotypes from Turkestan region (50.7%), with 24 samples from Sairam-Ugam and 14 from Mashat and Sarkyrama. The results indicated that genotypes from the same geographic origin did not consistently cluster together (Figure 5). For example, the first sub-branch of the first main branch contained not only genotypes from Almaty but also ten samples (S-U-14, S-U-24, S-U-17, MA-37, SA-47, S-U-26, SA-46, S-U-28, MA-31, S-U-1) originating from Turkestan region.

The results of the Principal Coordinate Analysis (PCoA) confirmed the findings of the UPGMA cluster analysis. Based on PCoA, two distinct groups of samples were identified (Figure 6). The PCoA plot of 75 walnut genotypes illustrated a clear overlap between the two clusters, which reflects the low genetic distances among the studied individuals. Each point in the graph of Figure 6 represents a walnut sample collected in a different geographic region and location: Turkestan region (Sairam-Ugam, S-U; Mashat, MA; Sarkyrama, SA); Almaty region (Almaty, AL; Baidibek-bi, BA and Esik, ES). The first group comprised genotypes from Almaty region and Turkestan region (pop1-pop6 S-U; MA; SA; AL; BA; ES). The second group included samples from Turkestan region (pop1-pop3, SU; MA; SA).

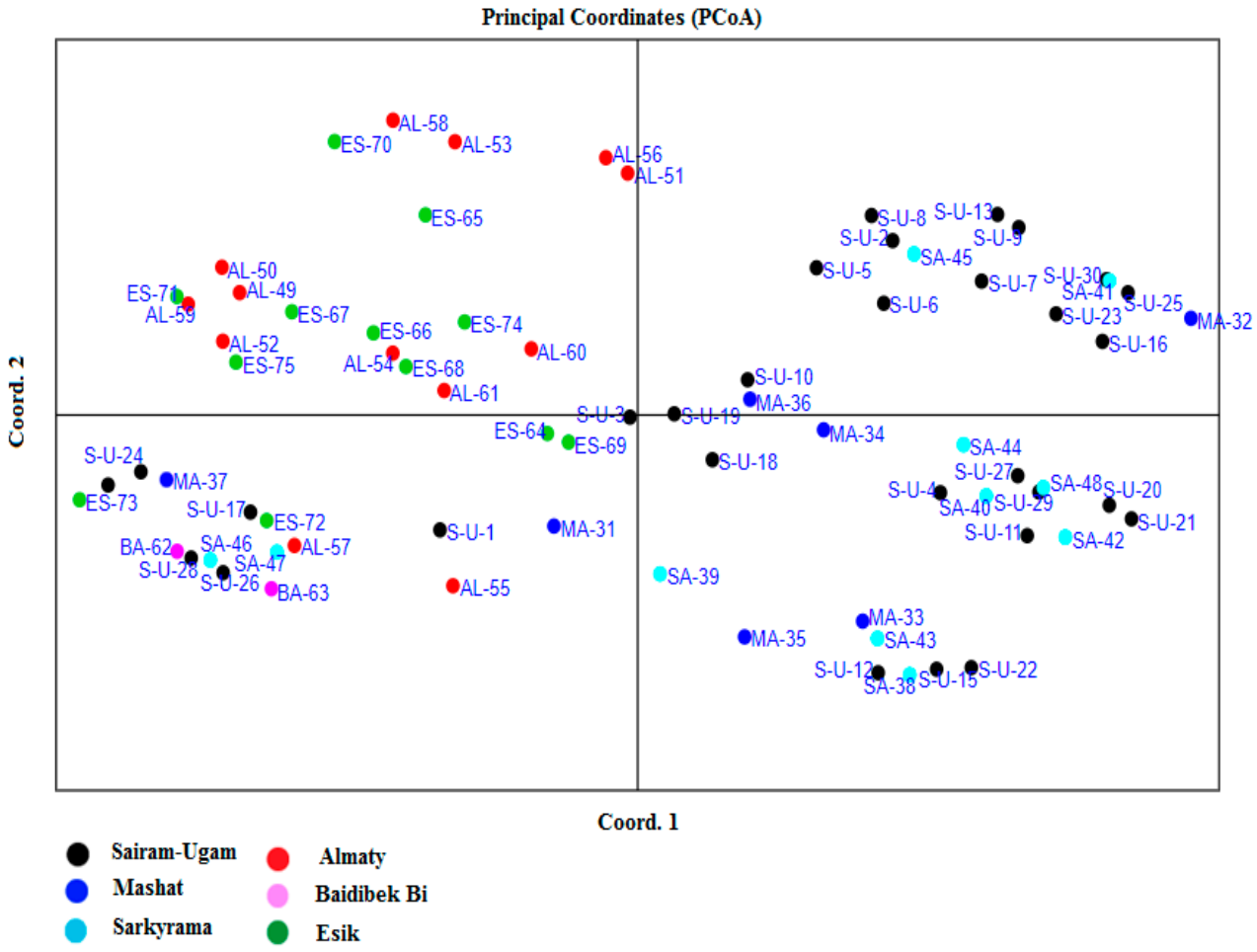

Figure 6. Principal Coordinate Analysis (PCoA) of 75 walnut genotypes.

The Principal Coordinate Analysis (PCoA) plot of 75 walnut genotypes illustrated a clear overlap between the two clusters, which reflects the low genetic distances among the studied individuals. Therefore, the analysis of the 75 walnut samples demonstrated considerable genetic variation among genotypes from different regions, highlighting a broad genetic diversity present within the studied collection.

4. Discussion

Walnut (*Juglans regia*) populations exhibit a high level of genetic diversity. Identifying valuable traits and understanding their genetic variability are key priorities for breeding programs. Molecular markers serve as powerful tools for analyzing genome structure and genetic variation. Among these, SSR (microsatellite) markers are most frequently used in walnut diversity studies. Previous studies have confirmed the effectiveness of SSR markers for analyzing the population structure of J. regia in Central Asia. Li et al. [49] developed 22 polymorphic SSR markers, which exhibited high PIC values and low population differentiation. Ye et al. [50] identified three genetic clusters among 1.000 individuals from 46 populations, noting the influence of natural and anthropogenic factors on genetic admixture. Shahi Shavvon et al. [51] detected moderate genetic variability and gene flow across 27 populations in the Iranian Plateau. Eser et al. [52] identified 551 polymorphic SSR loci, con-firming their applicability for germplasm analysis. As a result of this research, a comparative analysis of microsatellite polymorphism was conducted among local walnut populations from different geographic regions of Kazakhstan. Our findings confirm that *Juglans regia* populations in Kazakhstan exhibit a high level of genetic diversity, consistent with data reported in international studies. The average number of alleles per locus (Na = 5.87) and the effective number of alleles (Ne = 4.21) align closely with results from Ebrahimi et al. (2011), who analyzed 31 samples and identified an average of 5.10 alleles per locus [53]. Similar values have been observed in Turkish studies, such Orhan et al. (2020), where the mean number of alleles reached 6.43, indicating a richer gene pool within Turkish populations [54]. The Kazakh population is blocked by mountains (such as the Tianshan Mountains), and the gene flow is lower than that in the plain areas of Turkey. These differences are likely driven by historical factors, including long-term breeding activities and migration of genetic material across regions. Such variability underscores the importance of conserving diverse genetic resources to enhance breeding strategies and adapt to environmental challenges. In recent studies, Kairova et al. (2025) evaluated the genetic diversity and population structure of wild and cultivated Juglans regia in Kazakhstan. Their results revealed that wild walnut populations in Kazakhstan form distinct genetic clusters, reflecting historical isolation and adaptation, while cultivars exhibit a more dispersed genetic profile, indicative of admixture and artificial selection. They showed that inbreeding coefficients (F) were highest in wild Kazakh walnuts (0.17), indicating genetic bottlenecks and habitat fragmentation, whereas local genotypes showed lower inbreeding levels (0.08), suggesting a more diverse genetic background [14].

Regarding heterozygosity metrics, our results reveal a significant discrepancy between the expected heterozygosity (He = 0.70) and the observed heterozygosity (Ho = 0.54). Similar patterns of heterozygote deficiency have been reported in studies by Bernard et al. (2018), Plugatar et al. (2023), and Aradhya et al. (2010), where a low level of heterozygosity was consistently observed. As proposed by Manthos et al. (2023), such disparities may indicate the influence of inbreeding, limited gene flow, or intensive selection pressures [55–58]. The observed heterozygosity deficit in Kazakhstani walnut (*Juglans regia*) populations may be explained not only by inbreeding and selection pressure but also by adaptation to the region's specific continental climate conditions, characterized by extreme seasonal temperature fluctuations and pronounced aridity, which generate intense selective

pressures. In the context of our study, these findings may reflect a historical isolation of populations, emphasizing the need for further investigation into their demographic history and gene flow dynamics. Understanding these factors is crucial for developing strategies to maintain genetic diversity and improve breeding programs.

The Shannon information index (I), reaching 2.095 at locus WGA276, indicates a high level of genetic variability within the studied populations. Similar values have been reported in the study by Manthos et al. (2023) and Suprun et al. (2025), confirming the broad applicability and high informativeness of the selected markers [58,59]. However, the level of polymorphism at the WGA276 locus in our studies was higher than in the study by Shamlu et al. (2018), the average number of alleles per locus was about 7.9 [60]. The genetic background differences of walnuts in Central Asia may stem from the history of introduction along the Silk Road. This suggests a more diverse gene pool in the Kazakh populations, which is a critical factor to consider in conservation strategies and breeding programs aimed at maintaining and utilizing genetic diversity effectively.

Regarding population structure, the identification of two clusters that do not correlate with geographic location aligns with findings from previous studies, such as Bernard et al. (2020) and Plugatar et al. (2023), which also reported high levels of within-population differentiation and a lack of clear geographic structuring [56,61]. This pattern may be attributed to historical migration events and interregional exchanges of genetic material, highlighting the importance of incorporating analyses of migration dynamics and historical demographic processes in future research. Such insights are essential for understanding the mechanisms shaping genetic diversity and for developing effective conservation and breeding strategies.

Analysis of population structure using STRUCTURE, UPGMA, and PCoA methods identified two major genetic clusters that do not align with the geographic origins of the samples. This suggests a high level of genetic differentiation within the studied populations. The presence of these unique genetic forms offers valuable opportunities for their integration into breeding programs aimed at improving winter hardiness, pest and disease resistance, and overall agronomic traits. Harnessing this genetic diversity can facilitate the development of superior cultivars adapted to local conditions and contribute to the broadening of the genetic base of cultivated walnuts.

5. Conclusions

This study confirmed the high genetic diversity of Kazakh *Juglans regia* populations, highlighting their potential for use in breeding programs. All markers exhibited a high degree of polymorphism, with the WGA276 locus standing out as the most informative, displaying the greatest number of alleles and a high Shannon diversity index. The average expected heterozygosity (He) was 0.704, markedly higher than the observed heterozygosity (Ho), which was 0.547. The findings indicated substantial genetic variation and intra-population polymorphism: on average, 5.875 alleles per locus were identified, and the Shannon index was 1.444. The pronounced polymorphism of SSR markers, particularly at locus WGA276, underscores their effectiveness in assessing intra-population variation. Population structure analysis revealed two main genetic clusters primarily linked to geographic origin, although significant internal differentiation suggests long-term isolation and local adaptation. The population from the Turkestan region possesses the most diverse gene pool and represents a valuable resource for developing resilient and high-yielding cultivars. These findings emphasize the importance of conserving and strategically utilizing genetic diversity to enhance the adaptive capacity of the species, forming a foundation for future conservation and gene pool expansion initiatives.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae11070810/s1, Table S1: Descriptive Statistics and Paired t-Test Results; Table S2: Collection sites: key geographic information for each sampling location in the southern and southeastern regions of Kazakhstan; Table S3: ANOVA analysis of genetic diversity in *Juglans regia*.

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