Article

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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: 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 laking, 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, 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 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. These findings highlight the importance of conserving and harnessing the genetic resources of Kazakhstan's *J. regia* populations for future breeding efforts.

Keywords: *Juglans regia* L.; microsatellite markers; genetic diversity; polymorphism; population structure; breeding

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1. Introduction

Juglans regia L. is a globally significant nut crop with considerable economic importance. Its kernels are distinguished by a rich profile of nutrients and phytochemicals that confer various health benefits [1-5]. These nuts serve as a valuable source of macronutrients, including high-quality proteins, healthy fatty acids, vitamins, and essential minerals such as omega-3 fatty acids [6-8]. Furthermore, they contain bioactive compounds such as flavonoids and polyphenols, which exhibit antioxidant properties [9,7]. The elevated protein and lipid content of walnut kernels underpin their essential role in human nutrition [**10**]. They are widely utilized in fresh consumption, confectionery manufacturing, and oil extraction, with fat content ranging from 65% to 70% [11, 12]. The superior nutritional profile of walnuts underscores their importance in dietary practices, leading to their recognition as a priority crop by the Food and Agriculture Organization (FAO) **[10].**

The origin and dissemination of *Juglans regia* 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 [13, 14]. Currently, the primary range of walnut extends from the Balkan region eastward into Asia, encompassing countries such as China, Iran, Kazakhstan, Afghanistan, and Pakistan [12, 15].

Currently, China stands as the world's largest producer of walnuts, contributing approximately 50.27% of the global output. Following China, the leading producers include the United States, Iran, Turkey, Ukraine, Chile, and several countries within the European Union [11, 16, 17]. Over the period from 2012 to 2021, global walnut production experienced a significant upward trend, increasing from approximately 2.37 million tons to over 3.50 million tons [18]. This growth can be attributed to increasing international demand, rising market prices, advancements in cultivation techniques, and the development and adoption of new high-yielding, pest- and disease-resistant varieties.

Investigating and evaluating the genetic diversity of walnut germplasm in Central Asia is crucial for advancing breeding initiatives tailored to this region. Recently, Kazakhstan has experienced a growing demand for walnuts, particularly in areas conducive to cultivation, which has led to increased yields and expanded production. 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 [19].

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 [19].

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 [19, 20]. As a result, the most promising genotypes were selected, which can be further utilized in breeding programs and for commercial cultivation. The region's favourable 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 south-eastern regions [21]. Previously, our research included mapping the distribution of *Juglans regia* in this area, as well as morphological characterization of walnut fruits [22]. 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 [23]. Valuable germplasm from natural populations was collected, preserved in vitro, and stored in a cryobank for long-term conservation and future utilization [24, 25]. 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 [26]. 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 *Juglans regia*; however, their identification and detailed study have yet to be conducted. 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 precise characterization of germplasm, verification of cultivar origins, and 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) [27], RAPD (Random Amplified Polymorphic DNA) [28], AFLP (Amplified Fragment Length Polymorphism) [29, 30], SSR (Simple Sequence Repeats) [31], and SNP (Single Nucleotide Polymorphism) [32]. 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 [33-35]. Their widespread distribution across the genome and uniform coverage make SSRs a highly informative tool for various genetic analyses [36]. 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 [31, 37-45]. 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.

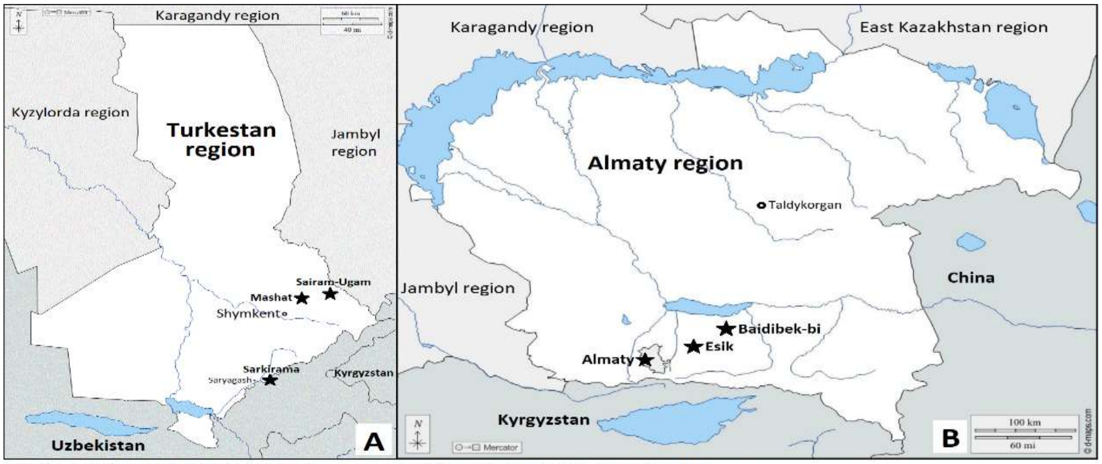
The aim of this research is to evaluate the genetic diversity and population structure of Juglans regia accessions collected from the southern and southeastern regions of Kazakhstan, utilizing molecular markers to generate detailed insights into their genetic relationships and variability.

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 1, 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.



https://d-maps.eu/map.php?num_car=190262&lang=ru https://d-maps.eu/map.php?num_car=184936&lang=ru

Figure 1. Distribution of *Juglans regia* 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 *Juglans regia* 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 [46]. 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 1 and Figure 1).

Table 1. Collection sites: key geographic information for each sampling location in the southern and southeastern regions of Kazakhstan.

No.	Geographic Region, Location	Population/Local Walnut Sample	Latitude	Longitude	Altitude (m)
1	Turkestan region, S-U	S-U-01	N 42 39 995	E 070 15 080	820
2	Turkestan region, S-U	S-U-02	N 42 39 995	E 070 15 095	817
3	Turkestan region, S-U	S-U-03	N 42 39 992	E 070 15 098	815
4	Turkestan region, S-U	S-U-04	N 42 39 991	E 070 15 119	811
5	Turkestan region, S-U	S-U-05	N 42 39 988	E 070 15 119	808
6	Turkestan region, S-U	S-U-06	N 42 39 982	E 070 15 117	804
7	Turkestan region, S-U	S-U-07	N 42 39 981	E 070 15 113	811
8	Turkestan region, S-U	S-U-08	N 42 39 980	E 070 15 115	806
9	Turkestan region, S-U	S-U-09	N 42 39 975	E 070 15 113	804
10	Turkestan region, S-U	S-U-10	N 42 39 986	E 070 15 130	810
11	Turkestan region, S-U	S-U-11	N 42 39 995	E 070 15 139	814
12	Turkestan region, S-U	S-U-12	N 42 40 001	E 070 15 135	811
13	Turkestan region, S-U	S-U-13	N 42 40 010	E 070 15 136	805
14	Turkestan region, S-U	S-U-14	N 42 399 95	E 070 15 112	799
15	Turkestan region, S-U	S-U-15	N 42 39 955	E 070 15 112	804
16	Turkestan region, S-U	S-U-16	N 42 39 985	E 070 15 153	810
17	Turkestan region, S-U	S-U-17	N 42 39 994	E 070 15 156	808
18	Turkestan region, S-U	S-U-18	N 42 39 999	E 070 15 161	811
19	Turkestan region, S-U	S-U-19	N 42 40 020	E 070 15 154	817
20	Turkestan region, S-U	S-U-20	N 42 40 021	E 070 15 157	816
21	Turkestan region, S-U	S-U-21	N 42 40 017	E 070 15 157	816
22	Turkestan region, S-U	S-U-22	N 42 40 039	E 070 15 135	819

23	Turkestan region, S-U	S-U-23	N 42 40 041	E 070 15 140	820
24	Turkestan region, S-U	S-U-24	N 42 40 051	E 070 15 069	831
25	Turkestan region, S-U	S-U-25	N 42 40 049	E 070 15 067	829
26	Turkestan region, S-U	S-U-26	N 42 40 041	E 070 15 073	828
27	Turkestan region, S-U	S-U-27	N 42 39 994	E 070 15 082	821
28	Turkestan region, S-U	S-U-28	N 42 39 987	E 070 15 088	816
29	Turkestan region, S-U	S-U-29	N 42 39 986	E 070 15 097	815
30	Turkestan region, S-U	S-U-30	N 42 39 984	E 070 15 068	845
31	Turkestan region, MA	MA -01	N 42 25 665	E 070 03 024	669
32	Turkestan region, MA	MA -02	N 42 25 670	E 070 03 025	665
33	Turkestan region, MA	MA-03	N 42 25 672	E 070 03 030	664
34	Turkestan region, MA	MA-04	N 42 25 675	E 070 03 028	664
35	Turkestan region, MA	MA-05	N 42 25 672	E 070 02 669	660
36	Turkestan region, MA	MA-06	N 42 25 671	E 070 02 682	662
37	Turkestan region, MA	MA-07	N 42 25 670	E 070 02 642	664
38	Turkestan region, SA	SA-01	N 41 27 773	E 069 25 611	579
39	Turkestan region, SA	SA-02	N 41 27 794	E 069 25 634	577
40	Turkestan region, SA	SA-03	N 41 27 796	E 069 25 641	580
41	Turkestan region, SA	SA-04	N 41 27 802	E 069 25 649	578
42	Turkestan region, SA	SA-05	N 41 27 813	E 069 25 671	578
43	Turkestan region, SA	SA-06	N 41 27 860	E 069 25 729	578
44	Turkestan region, SA	SA-07	N 41 27 874	E 069 25 744	576
45	Turkestan region, SA	SA-08	N 41 27 958	E 069 25 637	575
46	Turkestan region, SA	SA-09	N 41 27 955	E 069 25 636	576
47	Turkestan region, SA	SA-10	N 41 27 692	E 069 25 686	577
48	Turkestan region, SA	SA-11	N 41 27 693	E 069 25 684	574
49	Almaty region, AL	AL-01	N 43 179 141	E 076 867 784	898
50	Almaty region, AL	AL-02	N 43 197 52	E 076 84 600	895
51	Almaty region, AL	AL-03	N 43 19 733	E 076 84 664	896
52	Almaty region, AL	AL-04	N 43 19 756	E 076 84 601	895
53	Almaty region, AL	AL-05	N 43 197 55	E 076 84 602	894
54	Almaty region, AL	AL-06	N 43 20 214	E 076 84 941	896
55	Almaty region, AL	AL-07	N 43 13 322	E 076 55 063	880
56	Almaty region, AL	AL-08	N 43 13 331	E 076 55 262	883
57	Almaty region, AL	AL-09	N 43 13 013	E 076 54 955	896
58	Almaty region, AL	AL-10	N 43 13 011	E 076 54 954	895
59	Almaty region, AL	AL-11	N 43 13 010	E 076 54 950	892
60	Almaty region, AL	AL-12	N 43 13 012	E 076 54 951	898
61	Almaty region, AL	AL-13	N 43 13 013	E 076 54 956	891
62	Almaty region, BA	BA-01	N 43 51 353	E 077 69 19	720
63	Almaty region, BA	BA-02	N 43 51 329	E 077 69 359	725
64	Almaty region, ES	ES-01	N 43 18 085	E 077 29 997	1286
65	Almaty region, ES	ES-02	N 43 18 086	E 077 29 993	1284
66	Almaty region, ES	ES-03	N 43 18 081	E 077 30 000	1287
67	Almaty region, ES	ES-04	N 43 341 342	E 077 471 442	1285
68	Almaty region, ES	ES-05	N 43 341 11	E 077 481 579	1286
69	Almaty region, ES	ES-06	N 43 341 054	E 077 480 984	1284
70	Almaty region, ES	ES-07	N 43 341 010	E 077 480 994	1286

71	Almaty region, ES	ES-08	N 43 18 164	E 077 29 976	1283
72	Almaty region, ES	ES-09	N 43 18 162	E 077 29 973	1282
73	Almaty region, ES	ES-10	N 43 18 112	E 077 30 004	1291
74	Almaty region, ES	ES-11	N 43 18 107	E 077 29 990	1286
75	Almaty region, ES	ES-12	N 43 18 104	E 077 29 991	1284

Note: Geographic region, location are: Turkestan region (Sairam-Ugam, S-U; Mashat, MA; Sarkyrama, SA); Almaty region (Almaty, AL; Baidibek-bi, BA and Esik, ES).

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

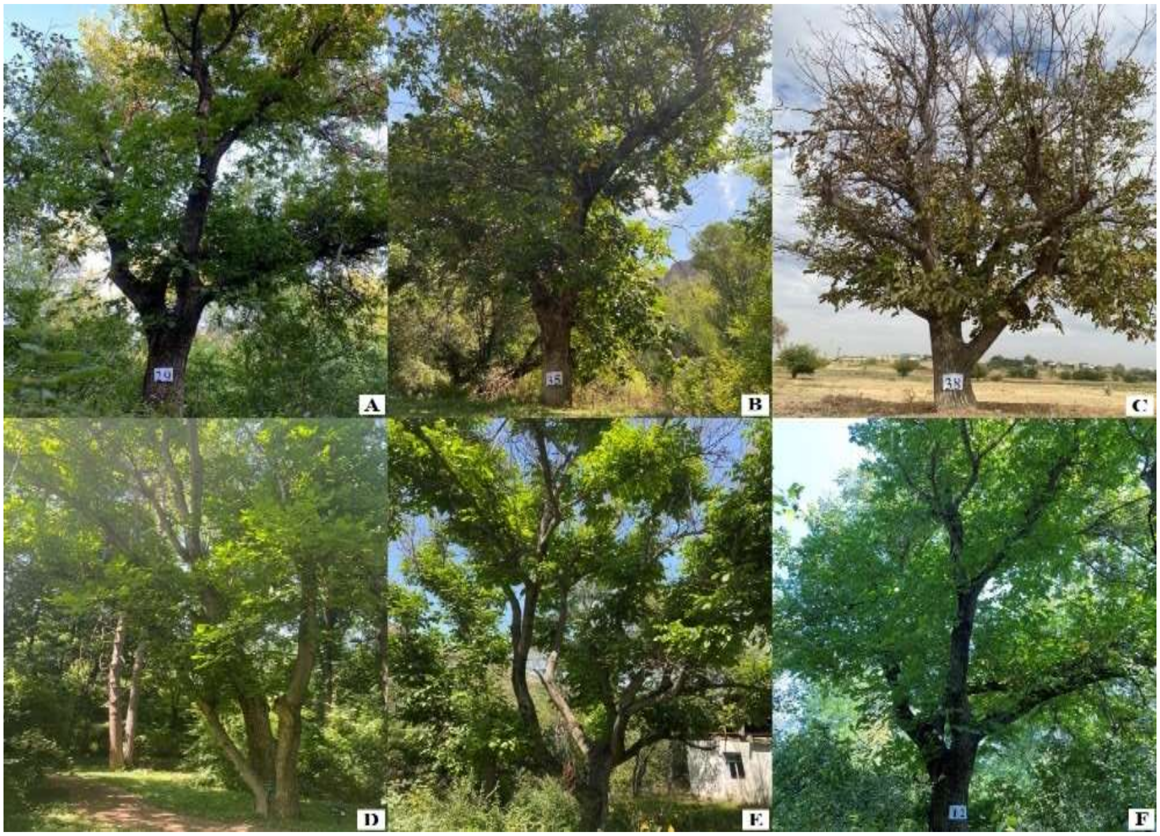


Figure 2. *Juglans regia* L. trees from various populations: (a) Sairam-Ugam State National Nature Park, (b) Mashat v., (c) Sarkyrama v. in Turkestan Region; (d) Almaty city, (e) Baidibek-bi, and (f) Esik in Almaty Region.

2.2. DNA Extraction and Molecular Analysis Using SSR Markers

For molecular genetic analysis in 2024, five young leaves were collected from each walnut tree during the active growth phase. Samples were immediately preserved in silica gel to prevent polyphenol oxidation and homogenized in liquid nitrogen using an optimized CTAB protocol with modern modifications [47]. DNA quality evaluation and quantification was performed by using a SmartSpecTMPlus Spectrophotometer (Bio-RAD Laboratories, Inc., 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 [48, 49]. The selection criteria included their even distribution across the walnut genome, high polymorphism levels (informative content), and consistent amplification performance in previous research. These markers were specifically selected to ensure robust and reliable genotyping results. Detailed information regarding each primer, including sequence, annealing temperature, and expected amplicon size, is presented in Table 2.

Table 2. 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)	Product Size Range (bp)
WGA001	ATTGGAAGGGAAGGGAAATG	CGCGCACATACGTAAATCAC	56	180–210
WGA027	AACCCTACAACGCCTTGATG	TGCTCAGGCTCCACTTCC	57	225-260
WGA042	GTGGGTTCGACCGTGAAC	AACTTTGCACCACATCCACA	55	210–260
WGA118	TGTGCTCTGATCTGCCTCC	GGGTGGGTGAAAAGTAGCAA	60	186–200
WGA009	CATCAAAGCAAGCAATGGG	CCATTGCTCTGTGATTGGG	56	231–245
WGA202	CCCATCTACCGTTGCACTTT	GCTGGTGGTTCTATCATGGG	62	259–295
WGA276	CTCACTTTCTCGGCTCTTCC	GGTCTTATGTGGGCAGTCGT	60	168–194
WGA376	GCCCTCAAAGTGATGAACGT	TCATCCATATTTACCCCTTTCG	56	230–265

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, 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 minutes; 35 cycles consisting of denaturation at 94°C for 30 seconds and extension at 72°C for 30 seconds; and a final extension step at 72°C for 5 minutes.

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 [50, 51]. 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 [52]. 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 [53]. The results generated by STRUCTURE were further analysed using STRUCTURE HARVESTER v.0.6.1 to identify the optimal number of genetic clusters (K) [54].

3. Results

3.1. Analysis of the Genetic Diversity of Juglans Regia

Using eight pairs of SSR markers, the genetic diversity and population structure of 75 *Juglans regia* 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 3. 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.

The effective number of alleles (Ne) ranged from 1.930 to 6.664, with an average of 4.219 (Table 3). Expected heterozygosity (He) varied between 0.482 to 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 от 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.

Table 3. 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 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 4.

Table 4. Comparison of six *Juglans regia* 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: pop 1- pop3 – walnut populations from the Turkestan region (Sairam-Ugam, S-U; Mashat, MA; Sarkyrama, SA); pop 4- 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 to 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 до 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. 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 *Juglans regia* genotypes was analysed (Figures 3 and 4). The STRUCTURE software evaluated the likelihood of classifying individuals into different numbers of clusters. The optimal number of groups was identified based on the ΔK statistic.

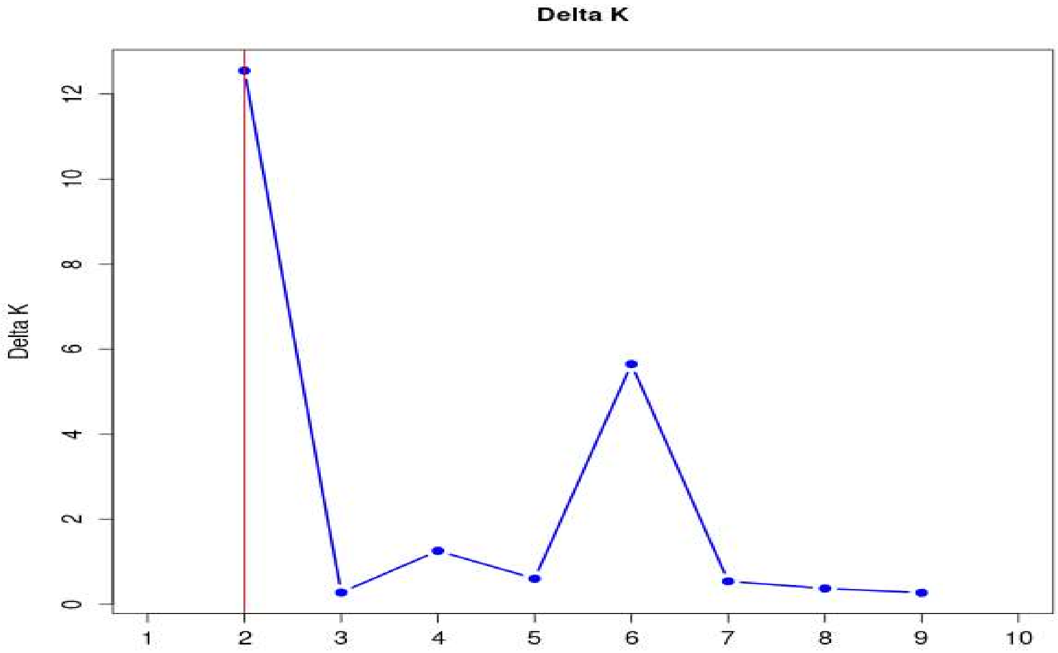


Figure 3. Population structure of 75 *Juglans regia* genotypes based on 8 SSR markers, as determined by STRUCTURE analysis, assuming K = 2.

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 [54]. A clear peak was detected at K = 2, confirming the most probable number of genetic clusters, as shown in Figure 3.

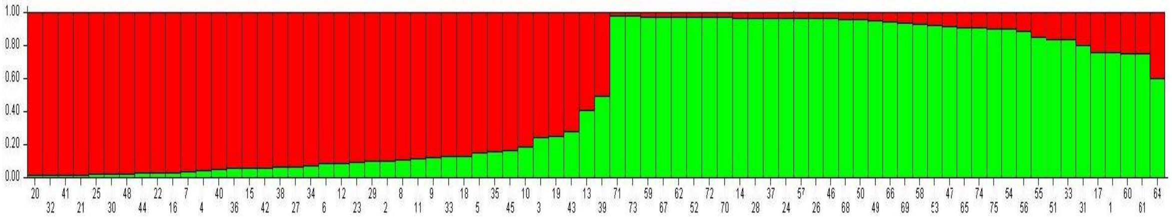


Figure 4. Genetic structure of 75 *Juglans regia* genotypes analysed using 8 SSR markers. Each color (red, green) represents a different genetic cluster among the samples.

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 pop 1 (S-U), pop 2 (MA), pop 3 (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: pop 1 (S-U), pop 2 (MA), and pop 3 (SA), pop 4 (AL), pop 5 (BA), and pop 6 (ES)). The average genetic distance (expected heterozygosity) within this cluster was 0.747 (Figure 4).

3.3. Cluster Analysis Using UPGMA and Principal Coordinates Analysis (PCoA

Genetic distances among *Juglans regia* groups based on origin were assessed using cluster analysis with the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and Principal Coordinates Analysis (PCoA). The list of studied samples, along with their population designations and geographical locations, is provided in Tables 1 and 4. The dendrogram of 75 *Juglans regia* 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 *Juglans regia* (49.3%), including populations from Almaty and Turkestan region (S-U pop 1, MA pop 2, SA pop 3, AL pop 4, BA pop 5, and ES pop 6). 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.

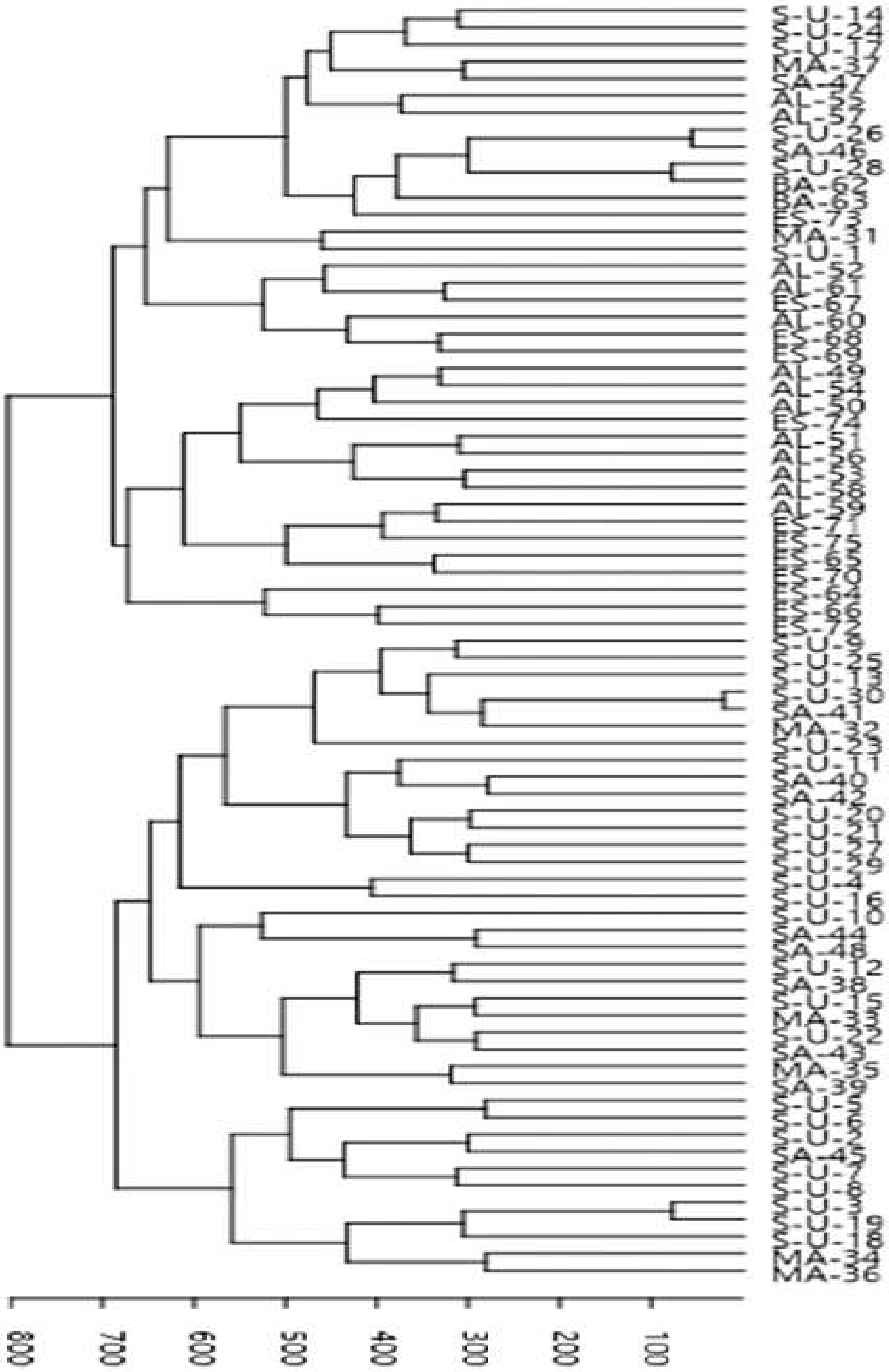


Figure 5. Clustering analysis for 75 walnut individuals collected from the 6 locations of Kazakhstan using the UPGMA method.

The results of the Principal Coordinates Analysis (PCoA) confirmed the findings of the UPGMA cluster analysis. Based on PCoA, two distinct groups of samples were identified (Figure 6). 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).

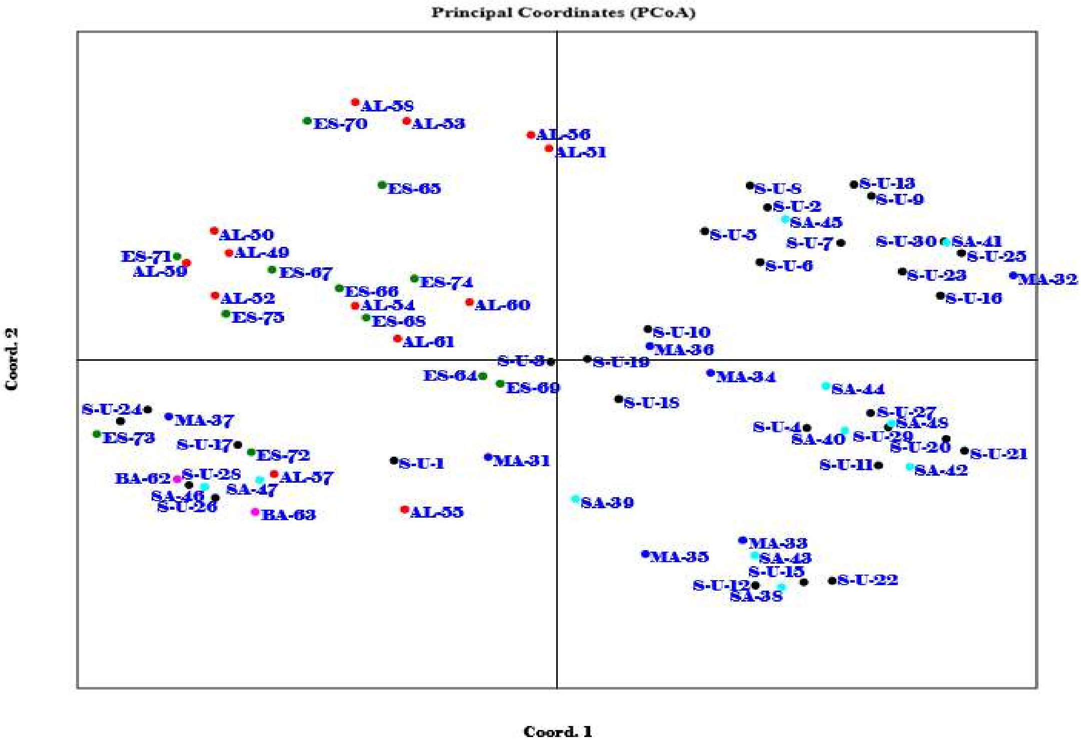


Figure 6. Principal Coordinates Analysis (PCoA) plot of 75 *Juglans regia* genotypes, illustrating a clear overlap between the two clusters, which reflects low genetic distances among the studied individuals.

Therefore, the analysis of the 75 *Juglans regia* 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 analysing genome structure and genetic variation. Among these, SSR (microsatellite) markers are most frequently used in walnut diversity studies. 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 analysed 31 samples and identified an average of 5.10 alleles per locus [55]. 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 [56]. 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, refecting historical isolation and adaptation, while cultivars exhibit a more dispersed genetic profle, indicative of admixture and artifcial selection. They showed, that Inbreeding coefcients (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 [20].

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 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 [57-60]. 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 [60, 61]. 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 [62]. 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 [58, 63]. 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

Overall, our findings indicate that Kazakh populations of *Juglans regia* possess a rich genetic resource, providing a solid foundation for future breeding initiatives. However, the low observed heterozygosity highlights the need for more detailed investigations into factors that may constrain genetic variability, such as inbreeding, selection pressures, and migration dynamics. Understanding these processes is crucial for developing effective conservation strategies and optimizing the utilization of the gene pool for sustainable breeding and preservation efforts.

5. Conclusions

This study confirmed the high genetic diversity of Kazakh *Juglans regia* populations, highlighting their potential for use in breeding programs. The pronounced polymorphism of SSR markers, particularly at locus WGA276, underscores their effectiveness in assessing intrapopulation 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 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.

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