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The assessment of genetic diversity and population structure of endemic *Scutellaria yildirimlii* (Lamiaceae) for conservation purposes

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Abstract: *Scutellaria yildirimlii* is an endangered perennial endemic species from Turkey and belongs to the family Lamiaceae, which has a high number of medicinal and aromatic plants. It is crucial to determine genetic diversity and population structure of this species for its conservation measures; therefore, 111 individuals from five natural populations of *S. yildirimlii* were investigated by using 15 inter simple sequence repeats (ISSR) primers. The percentage of polymorphic loci (PPL), Nei's gene diversity (H), and Shannon's information index (I) at the species and population-level were determined as 93.9%, 0.183, 0.292 and 56.5%, 0.158, 0.242, respectively. The analysis of molecular variance (AMOVA) showed that there is more genetic variation within populations (84%) than among populations (16%). Nei's differentiation coefficient (G_{ST} = 0.144) was determined to be moderate, which was confirmed by the level of genetic differentiation among populations. Gene flow (N_m = 2.984) showed high genetic exchange among populations. Pairwise genetic distance values among populations ranged from 0.0219 to 0.0512. The Mantel test revealed a statistically significant positive correlation between genetic and geographical distance (r = 0.493, p < 0.001). The Unweighted Pair Group Method with Arithmetic Averages (UPGMA) dendrogram and Principal Coordinate Analysis (PCoA) showed similar results. STRUCTURE analysis (ΔK = 3) revealed that the K test yielded maximum peaks for 3 clusters. Based on the findings, measures for genetic conservation and management of this species were presented.

Key words: Conservation, endemic, ISSR, population genetics, *Scutellaria yildirimlii*

1. Introduction

Scutellaria L. (Lamiaceae), also known as skullcap, is one of the largest genera of the family, with 470 species.[1] The principal diversity centers of *Scutellaria* are mostly in Central Asia, Afghanistan, and the Irano-Turanian phytogeographical region, while the East Mediterranean region and the Andes are considered secondary diversity hotspots (Paton, 1989, 1990). Turkey is rich in *Scutellaria* species diversity since the members of this species are distributed all over the country. However, some of these species are at high risk of extinction because of their partial and small populations (Minareci and Pekönür, 2017). Turkey is home to 41 *Scutellaria* taxa, of which 16 (39%) are (Edmondson, 1982; Davis et al., 1988; Duman, 2000; Çiçek and Yaprak, 2011, 2013; Çiçek and Ketenoğlu, 2011; Çiçek, 2012; Yıldırım et al., 2021; Tunçkol and Haşayacak, 2022). Although the extinction of species is considered a part of evolutionary processes related to ecological factors, today, the major reasons for the loss of biodiversity are human activities, climate changes, and habitat loss due to stochastic incidents (Primack, 2006). Genetic diversity plays a crucial role in species' long-term survival and adaptability. Obtaining data about the genetic structures of the species with multiple and separate populations is vital in case the species consist of separate populations because knowledge about genetic diversity within and among the populations has become a prerequisite for the establishment of a strategic plan for conservation program (Crema et al., 2009).

Molecular markers are commonly used to determine plant populations' genetic structure (Liu, 1998; Abbasi et al., 2021). Inter simple sequence repeat (ISSR), which is a Polymerase Chain Reaction (PCR)-based molecular marker, primers with double, triple, quadruple, and quintuple repetitive nucleotides are used, and the region between two microsatellites can be amplified with these primers (Zietkiewicz et al., 1994). ISSR markers have some advantages, like primer design without knowledge of genomic sequence, the requirement of a small amount of DNA, and low costs compared with restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR), and

[1] WCSP (2022) onward (continuously updated). World Checklist of Selected Plant Families. Facilitated by the Royal Botanic Gardens [online]. Website http://wcsp.science.kew.org/ [accessed 15 October 2022]

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amplified fragment length polymorphism (AFLP) techniques. Also, it produces more reliable and reproducible bands due to its higher binding temperature and extended primers compared to random amplification of polymorphic DNA (RAPD) (Qian et al., 2001). On the other hand, it has disadvantages such as the inability to distinguish heterozygosity in loci because it is a dominant marker, the determination of binding temperatures of the primers separately, and the possibility that particles of similar sizes are not homologous (Kesawat and Das Kumar, 2009). Given that it was more appropriate for the research topic and the setup of our research facility, ISSR was chosen for this investigation.

Under the scope of biodiversity conservation, it is crucial to draw attention to the future of endangered species for us and the next generations. *Scutellaria yildirimlii* is an edaphic endemic species, first introduced in 2013 from the marly-gypsaceous soils of Central Anatolia. It has a narrow distribution area with fragmented populations (Çicek and Yaprak, 2013). It was included in the Endangered (EN) category according to Red List Criteria of the IUCN (Yıldırım et al., 2019). The purpose of this study is to determine the genetic diversity level of natural populations of *S. yildirimlii* using the ISSR fingerprinting technique. Thus, it is aimed to evaluate the genetic differentiation level among populations and develop strategies for conserving the species in light of the study's findings.

2. Materials and methods

2.1. Plant material

Literature and herbaria data (ANK, HUB, GAZI, and ESSE) were utilized to accurately identify the reported distribution areas of *S. yildirimlii* and revealed that the species inhabits only the provinces of Ankara and Eskişehir at six different localities that were all studied in 2016 (Figure 1). Due to the negligible distance between them, the Aşağıkepen (approximately 28 ha) and Yeşilköy (approximately 0.5 ha) localities were considered to be a single population (Yıldırım et al., 2019). Based on the population sizes of these localities, a total of 22 samples, 17 from Aşağıkepen and 5 from Yeşilköy, were analyzed. In total, fresh leaf samples of 111 different individuals were collected from all indicated populations of the species (Table 1). Considering the size of each population in the sampling method, attention was paid to keeping the distance between the sampled individuals as far as possible. For this purpose, leaf samples were collected from large populations of the species (Kızlarkayası, Aşağıkepen, Kavuncu, and Oğlakçı) with at least 10 m distance by following the transect counting method. In Ayaş, one of the small populations, samples were collected with at least 4 m distance, while in Yeşilköy, the smallest population in terms of both the number of individuals and the size of the area, sampling was done as far as possible from each other for five individuals. The fresh leaf samples were labeled, dried in silica-gel-filled bags, and stored at –80 °C until DNA extraction.

Figure 1. The distribution areas of *S. yildirimlii*.

Table 1. The geographical locations of *S. yildirimlii*.

Population	**Location**	**Latitude (N)** **Longitude (E)** **Altitude (m)**	**Sample size (individual code)**
Ayaş	A4 16th km of Ayaş–Ankara route, Aysantı pass, gypseous soils	**40° 05′ 37.8′032° 26′ 17.1′** **1205 m**	23 (1–23)
Kızlarkayası	B4 Ankara, Polatlı–Kızlarkayası	**39° 40′ 35.9′** **032° 00′ 12′** **755 m**	21 (24–44)
Oğlakçı	B3 24th km of Sivrihisar–Polatlı route, near Oğlakçı, Eskişehir, clayish hillsides	**39° 33′ 11.9′** **031° 43′ 35.9′** **849 m**	22 (45–66)
Aşağıkepen	B3 At the intersection of Yeşilköy road and Afyon–Sivrihisar route, near Aşağıkepen village Eskişehir, 872 m, gypseous hillsides	**39° 19′ 44.8′** **031° 27′ 27.7′** **877 m**	5 (67–71)
	B3 Near Aşağıkepen village in Sivrihisar, Eskişehir, gypseous soils.	**39° 22′ 15.2′** **031° 29′ 17.4′** **960 m**	17 (72–88)
Kavuncu	A3 North-East of Kavuncu village in Günyüzü, Eskişehir, gypseous step	**39° 24′ 39.9′** **031° 54′ 15.1′** **742 m**	23 (89–111)
Total			**111**

2.2. DNA extraction and ISSR-PCR amplification

Genomic DNA extraction was realized using the "Macherey-Nagel NucleoSpin® Plant II" kit with a denoted protocol. With the help of a Nanodrop spectrophotometer (Thermo Scientific Nanodrop One-W), the purities and concentrations of isolated DNA samples were determined. Afterwards, the samples were diluted to 10 ng/µL and stored at –20 °C for PCR applications. Fifty-nine universal UBC-ISSR primers (Set # 9 of University of British Columbia, Canada) were screened at PCR amplification using "Applied Biosystems Veriti 96 Well Thermal Cycler". Fifteen primers with the highest polymorphism, repeatability, and clearest bands were used among the scanned primers. PCR-ISSR amplification was performed for each primer in two parts due to the exceeding number of samples.

ISSR amplifications were realized with a reaction volume of 20 µL containing 1XTaq DNA polymerase buffer (with 2 mM $MgCl_2$), 200 µM dNTPs, 0.4 µM primer, 1 unit Taq DNA polymerase enzyme, and 10 ng genomic DNA applying the Touchdown PCR (TD) program (Don et al., 1991). The amplification reaction includes the following steps; initial denaturation step for 5 min at 95 °C followed by 1 cycle; denaturation 30 s at 95 °C, annealing 45 s at 65–55 °C, extension 1 min 30 s at 72 °C, followed by 15 cycles; denaturation 30 s at 95 °C, annealing 30 s at 45 °C, extension 1 min 30 s at 72 °C, followed by 20 cycles; final extension 7 min at 72 °C followed by 1 cycle. After combining with a 6X loading dye, the amplified DNA fragments were separated by 2% (w/v) agarose gel electrophoresis containing 0.5X TBE (Tris-Borate-EDTA) buffer at a specific voltage of 90 V for 5 h, and ethidium bromide (EB) was used to stain the gel. Band sizes were determined using "abm DNA ladder" molecular weight marker ranging from 100 pb to 3 kb. The gels were visualized and photographed under UV via BioRad Molecular Imager DocXR+.

2.3. Data analysis

To create a binary data matrix, clear and reproducibly amplified ISSR bands were scored as either present (1) or absent (0). All gathered data was combined and utilized to analyze genetic diversity parameters such as observed number of alleles (N_a), effective number of alleles (N_e) (Kimura and Crow, 1964), Nei's gene diversity (H) (Nei, 1973), Shannon's information index (I) (Lewontin, 1972), the number and percentage of polymorphic loci (NPL and PPL), total genetic diversity (H_T), genetic diversity within a population (H_S), and coefficient of genetic differentiation among populations (G_{ST}=1–H_S/H_T) with POPGENE 1.32.[2] This software was also used to calculate gene flow among populations (N_m) based on G_{ST} value using the equation

[2] Yeh FC, Yang RC, Boyle TJB, Ye ZH, Mao JX (1997) POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada.
Website https://sites.ualberta.ca/~fyeh/faqs.html [accessed 15 October 2022]

N_m=0.5(1-G_{ST})/G_{ST} (McDermott and McDonald, 1993). To assess marker polymorphism, the polymorphism information content (PIC) for each ISSR marker was calculated by the formula $PIC_i = 2f_i (1- f_i)$ where f_i is the frequency of the amplified allele (Abuzayed et al., 2017).

The unweighted pair-group method with arithmetic averages (UPGMA) dendrogram, which illustrates genetic relationships among populations, was generated using the Molecular Evolutionary Genetics Analysis (MEGA) 6.06 software based on the matrix of Nei's genetic distance (Nei, 1978; Tamura et al., 2013). Additional UPGMA dendrogram depicting genetic distances between individuals was constructed using SYN-TAX 2000 software with Nei's genetic distance data on the basis of Jaccard similarity coefficient (Jaccard, 1908; Podani, 2001). Using a hierarchical analysis of molecular variance (AMOVA) software (Excoffier et al., 1992) generated by the GenAlEx 6.5 program, pairwise population comparisons were utilized to explain the partition of genetic diversity within and among populations. AMOVA also computed Φ_{PT} statistics, the analogue for binary data to F_{ST} statistics (Fixation index). The principal coordinate analysis (PCoA) was performed using GenAlEx 6.5 software to provide the spatial presentation of the relative genetic distances among individuals and populations (Peakall and Smouse, 2012). The correlation between geographic and genetic distance matrices was calculated at a population level using the Mantel test (Mantel, 1967).

The genetic structure analysis of *S. yildirimlii* populations was performed with STRUCTURE software v 2.3.4 which admixture model-based clustering assigning individuals to genetic clusters (K) (Pritchard et al., 2000). To determine the best model describing population structure, the program was applied from K = 2 to 20 (where K is the number of clusters), using a burn-in period of 50,000 iterations and 300,000 Markov Chain Monte Carlo (MCMC) repetitions, with twenty iterations for each K value (Wu et al., 2015). The delta K was calculated based on the work of Evanno et al. (2005), which gives the best estimate for an optimal number of clusters through STRUCTURE HARVESTER 2.3.4 software (Earl and VonHoldt, 2012). The model with the highest K value was deemed to best describe the data. Populations were assigned to clusters using a threshold value of ≥0.80. Individuals that did not match this criterion were deemed admixed (Celik et al., 2016).

3. Results

Analysis was conducted on 111 samples of *S. yildirimlii* from five populations. Among the 59 ISSR markers analyzed, the 15 primers that had the highest polymorphism were used for further analysis (Table 2). A total of 311 PCR fragments (93.9%) generated by these primers were polymorphic. The primers UBC 807 and 812 had the highest polymorphism rates (100%), while the primer UBC 818 had the lowest polymorphism rate (81.2%). The total num-

Table 2. Polymorphism analysis of *S. yildirimlii* from ISSR primers.

Marker		Sequence (5'-3')	T_m(°C)	TNB	PPB%	PIC value	Band size range (bp)
1	UBC 807	$(AG)_8$T	TD-56	26	100	0.208	200–2000
2	UBC 808	$(AG)_8$C	TD-56	26	96.1	0.186	500–2000
3	UBC 810	$(GA)_8$T	TD-56	17	88.2	0.199	500–2000
4	UBC 812	$(GA)_8$A	TD-56	20	100	0.212	300–2000
5	UBC 818	$(CA)_8$G	TD-56	16	81.2	0.158	200–2000
6	UBC 826	$(AC)_8$C	TD-56	25	96.0	0.184	400–2000
7	UBC 835	$(AG)_8$YC	TD-56	25	96.0	0.188	300–2000
8	UBC 836	$(AG)_8$YA	TD-56	26	96.1	0.210	200–2000
9	UBC 840	$(GA)_8$YT	TD-56	26	96.1	0.189	200–2000
10	UBC 842	$(GA)_8$YG	TD-56	19	94.7	0.169	300–2000
11	UBC 856	$(AC)_8$YA	TD-56	24	95.8	0.204	300–2000
12	UBC 888	BDB$(CA)_7$	TD-65	25	92.0	0.165	300–2000
13	UBC 889	DBD$(AC)_7$	TD-65	18	83.3	0.149	600–2000
14	UBC 890	VHV$(GT)_7$	TD-56	17	88.2	0.155	400–2000
15	UBC 891	HVH$(TG)_7$	TD-56	21	95.2	0.205	500–2000
Total				**331**	**93.9**	**0.185**	**200–2000**

T_m: annealing temperature, **TNB:** total number of bands, **PPB:** percentage of polymorphic bands, PIC: Polymorphic information content, **Y**=(C,T), **B**=(C,G,T), **D**=(A,G,T), **V**=(A,C,G), **H**=(A,C,T)

ber of bands per primer ranged from 16 to 26. The average number of bands per primer was 22.1, with a total of 20.8 polymorphic bands. PIC values of markers ranged from 0.149 to 0.212, with a mean value of 0.185. In general, PIC values greater than 0.50 indicate that these markers were highly informative (Botstein et al., 1980). In the present study, the PIC value was moderately informative. The length of the amplified bands ranged from 200 to 2000 bp.

Both population-level and species-level genetic diversity parameters for *S. yildirimlii* were analyzed. At the population level, 187 polymorphic loci (NPL) were detected with a percentage of 56.5% (PPL), the mean number of effective alleles (N_e) was 1.263, while the mean number of observed alleles (N_a) was 1.565. For Nei's gene diversity (H) and Shannon's information index (I), mean values of overall populations were computed as 0.158 and 0.242, respectively. In addition, the following species-level values were obtained; polymorphic loci number (NPL) was found as 311 with a percentage of 93.9% (PPL), and the numbers of observed alleles (N_a) and effective alleles (N_e) were revealed to be 1.934 and 1.296, respectively. Species-level value of Nei's gene diversity (H) was 0.183, while Shannon's information index (I) was 0.292 (Table 3).

The values of Nei's analysis of gene diversity were obtained based on all loci data and given in Table 4. N_m values can be grouped into three categories as follows: $N_m \geq 1$ is considered a high, $0.250 < N_m < 0.990$ is considered an intermediate, and $0 < N_m < 0.249$ is considered a low gene flow (Slatkin, 1981, 1985; Caccone, 1985; Waples, 1987). Relatedly, Wright suggested that generally, if $N_m < 1$, local differentiation of populations will result, whereas if $N_m > 1$, population differentiation will be minimal among them (Wright, 1949). For Φ_{PT} (analogous to F_{ST}) interpretation, it has been proposed that values between 0 and 0.05 indicate low genetic differentiation, values between 0.05 and 0.15 indicate moderate differentiation, values between 0.15 and 0.25 indicate great differentiation, and values above 0.25 indicate very great genetic differentiation (Wright, 1978; Hartl and Clark, 1997). N_m result (2.984) also indicated a high level of gene flow and the values Φ_{PT} (0.159) and G_{ST} (0.144) indicate that genetic differentiation among populations is moderate. The results of AMOVA revealed that 84% of the total genetic variation occurred within populations, and only 16% was noted among populations which were statistically significant ($p < 0.001$) with the permutations based on 999 iterations (Table 5).

Nei's unbiased genetic distance was investigated between pairs of *S. yildirimlii* populations (Nei, 1978). Genetic distance ranged from 0.0219 between Aşağıkepen and Oğlakçı populations, to 0.0512 between Aşağıkepen and Ayaş (Table 6). The Mantel test showed a statistically significant positive correlation between genetic and geographic distance ($r = 0.493$ $p < 0.001$) (Nei, 1978).

Using Nei's binary genetic distance matrix (Nei, 1978), UPGMA and PCoA cluster analyses were used to study the relationship among *S. yildirimlii* popula-

Table 3. Genetic analysis of *S. yildirimlii* at population and species levels.

Pop. name	**N**	**N_a ± S**	**N_e ± S**	**H ± S**	**I ± S**	**PPL (%)**
Ayaş	23	1.547 ± 0.499	1.262 ± 0.354	0.155 ± 0.190	0.237 ± 0.271	54.7
Kızlarkayası	21	1.538 ± 0.499	1.252 ± 0.339	0.152 ± 0.185	0.234 ± 0.266	53.8
Oğlakçı	22	1.613 ± 0.488	1.280 ± 0.344	0.169 ± 0.187	0.262 ± 0.266	61.3
Aşağıkepen	22	1.601 ± 0.490	1.275 ± 0.353	0.165 ± 0.189	0.254 ± 0.268	60.1
Kavuncu	23	1.526 ± 0.501	1.244 ± 0.346	0.145 ± 0.186	0.223 ± 0.266	52.6
Average		1.565 ± 0.496	1.263 ± 0.348	0.158 ± 0.188	0.242 ± 0.268	56.5
Species level	111	1.934 ± 0.250	1.296 ± 0.336	0.183 ± 0.178	0.292 ± 0.246	93.9

N: number of individuals, **N_a:** number of observed alleles, **N_e:** number of effective alleles, **H:** Nei's (1973) genediversity, **I:** Shannon's information index (Lewontin, 1972), **NPL:** number of polymorphic loci, **PPL:** percentage of polymorphic loci, **S:** Standard deviation

Table 4. Nei's analysis of gene diversity for all loci (Nei, 1987).

All loci	N	H_T ± S	H_S ± S	G_{ST}	N_m
Average	111	0.183 ± 0.031	0.157 ± 0.024	0.144	2.984

N: number of individuals, **H_T:** total genetic diversity, **H_S:** genetic diversity within population, **G_{ST}:** coefficient of genetic differentiation; $G_{ST} = 1 - H_S/H_T$, **N_m:** estimate of gene flow; $N_m = 0.5(1 - G_{ST})/G_{ST}$, **S:** Standard deviation

Table 5. Statistics of molecular variance analysis (AMOVA) of *S. yildirimlii*.

Source	**df**	**SS**	**MS**	**VC**	**V%**	Φ-statistics	**p-value**
Among populations	4	563.967	140.992	5.128	16%	Φ_{PT} 0.159	0.001
Within populations	106	2881.655	27.185	27.185	84%		0.001
Total	110	3445.622	-	32.313	100%		

df: degrees of freedom, **SS**: sum of square, **MS**: mean square, **VC**: variance components, **V%**: percentage of variance, Φ_{PT}(PhiPT): genetic differentiation among populations, **P:** statistical significance

Table 6. Genetic identity (above diagonal) and genetic distance (below diagonal) between populations of *S. yildirimlii*.

Population	Ayaş	Kızlarkayası	Oğlakçı	Aşağıkepen	Kavuncu
Ayaş	****	0.9748	0.9588	0.9501	0.9554
Kızlarkayası	0.0255 60 km	****	0.9715	0.9644	0.9733
Oğlakçı	0.0421 84 km	0.0290 27 km	****	0.9784	0.9630
Aşağıkepen	0.0512 113 km	0.0362 55 km	0.0219 29 km	****	0.9621
Kavuncu	0.0457 88 km	0.0270 31 km	0.0377 22 km	0.0387 36 km	****

Geographic distances (km) between population pairs were also indicated below diagonal in parentheses

tions (Figures 2 and 3). The dendrogram generated by the UPGMA algorithm was separated into two main clusters, the first cluster of which includes Ayaş and Kızlarkayası populations in Ankara Province, as well as Kavuncu population in Eskişehir Province. The second major cluster consisted of Oğlakçı and Aşağıkepen populations from Eskişehir Province (Figure 2). According to the PCoA results, PCoA axis 1 explained 46.99% of the variance, axis 2 explained 28.03% of the variance, whereas axis 3 explained 15.42% of the variance. Along the axis 1 of the PCoA graph, the populations clustered into two major groups: Ayaş, Kızlarkayası, Kavuncu populations, and Oğlakçı, Aşağıkepen populations (Figure 3). The PCoA graph and the UPGMA dendrogram support each other (Figures 2 and 3).

The genetic relationship among 111 individuals of the species was established based on the Jaccard similarity coefficient by UPGMA clustering analysis using the SYN-TAX 2000 program (Podani, 2001). The dendrogram generated by the UPGMA algorithm was separated into two main clusters, the first of which included Ayaş and Kızlarkayası populations in Ankara Province, as well as Kavuncu population with 2 individuals from Aşağıkepen (87 and 88) and 3 individuals from Oğlakçı population (46, 47, and 54) in Eskişehir Province. The second major cluster consisted of Oğlakçı and Aşağıkepen with Yeşilköy populations from Eskişehir Province (Figure 4). Another genetic relationship among individuals was created based on a distance matrix by PCoA using the GenAlEx 6.5 program (Peakall and Smouse, 2012). The first three components explained 7.73%, 5.46%, and 3.52% of the total variation, respectively. Along the first axis of the PCoA graph, the individuals were clustered into three major groups. The first cluster consisted of the individuals of Ayaş and Kızlarkayası populations, the second one consisted of the individuals from Oğlakçı and Aşağıkepen populations, and the third one consisted of the individuals from Kavuncu population (Figure 5). The PCoA and UPGMA cluster analyses were compatible with each other (Figures 4 and 5).

STRUCTURE analysis was conducted to determine the genetic structure among 111 *S. yildirimlii* genotypes. The best K value for representing *S. yildirimlii* genotypes was K = 3, with the highest peak, and the second peak was observed at K = 5 (Figure 6). These results for K=3 are consistent with those of UPGMA and PCoA. Concerning K = 3, the first cluster A contained 21 genotypes (18.9%) from Kavuncu population with a probability of membership (threshold) value qi > 80. The second cluster B also had 21 genotypes (18.9%) from Ayaş population with the same value of qi > 80%. There were 27 genotypes (24.3%) in cluster C, of which 11 were from Oğlakçı and 16 from Aşağıkepen (with Yeşilköy) populations. The highest number of genotypes of overall was found in Admixed group as 42 (37.8%) and with the value of qi < 80%, including all

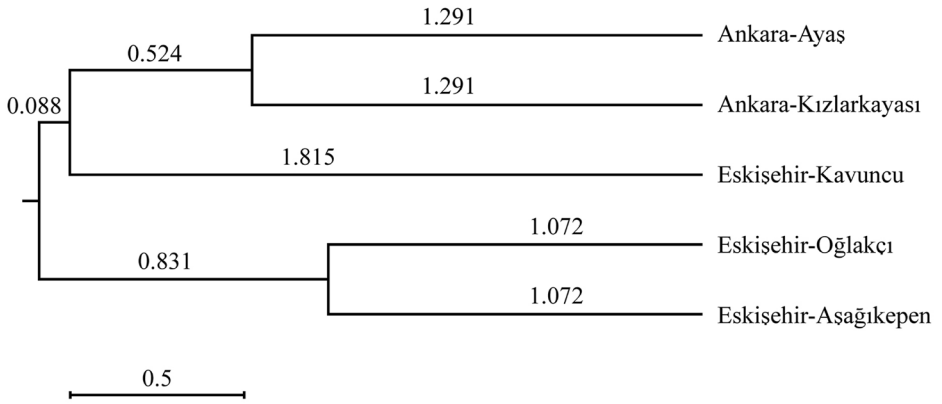

Figure 2. UPGMA dendrogram based on genetic distances among *S. yildirimlii* populations.

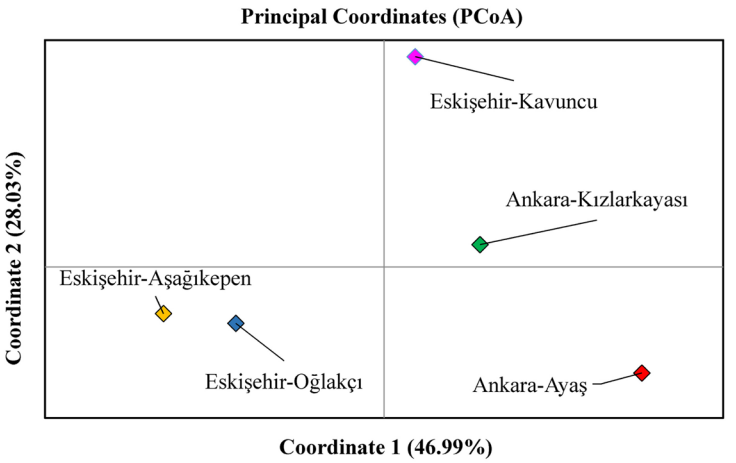

Figure 3. Principal coordinate analysis (PCoA) graph of *S. yildirimlii* populations.

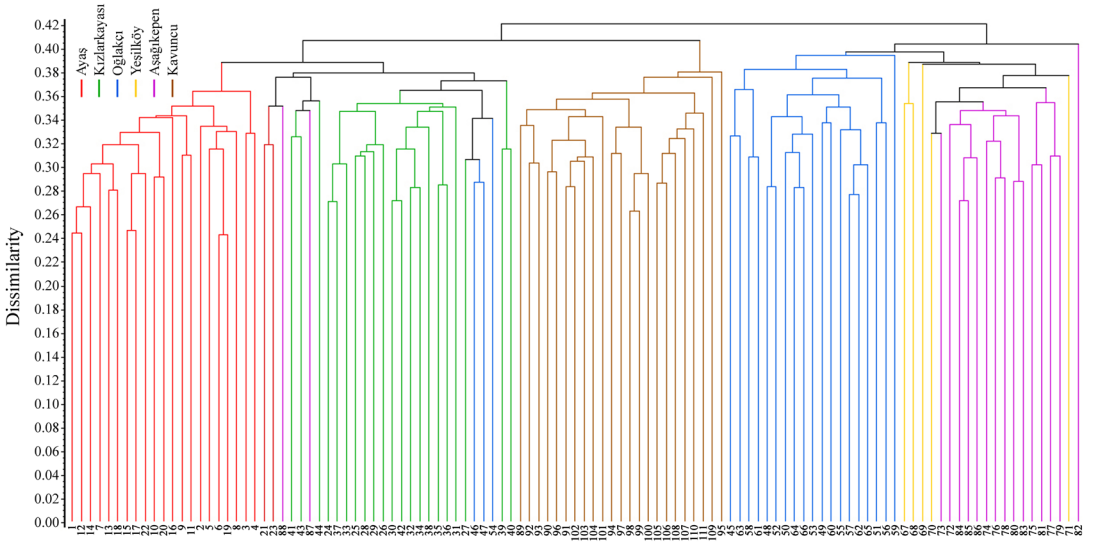

Figure 4. UPGMA dendrogram showing the genetic distance among 111 genotypes of *S. yildirimlii*.

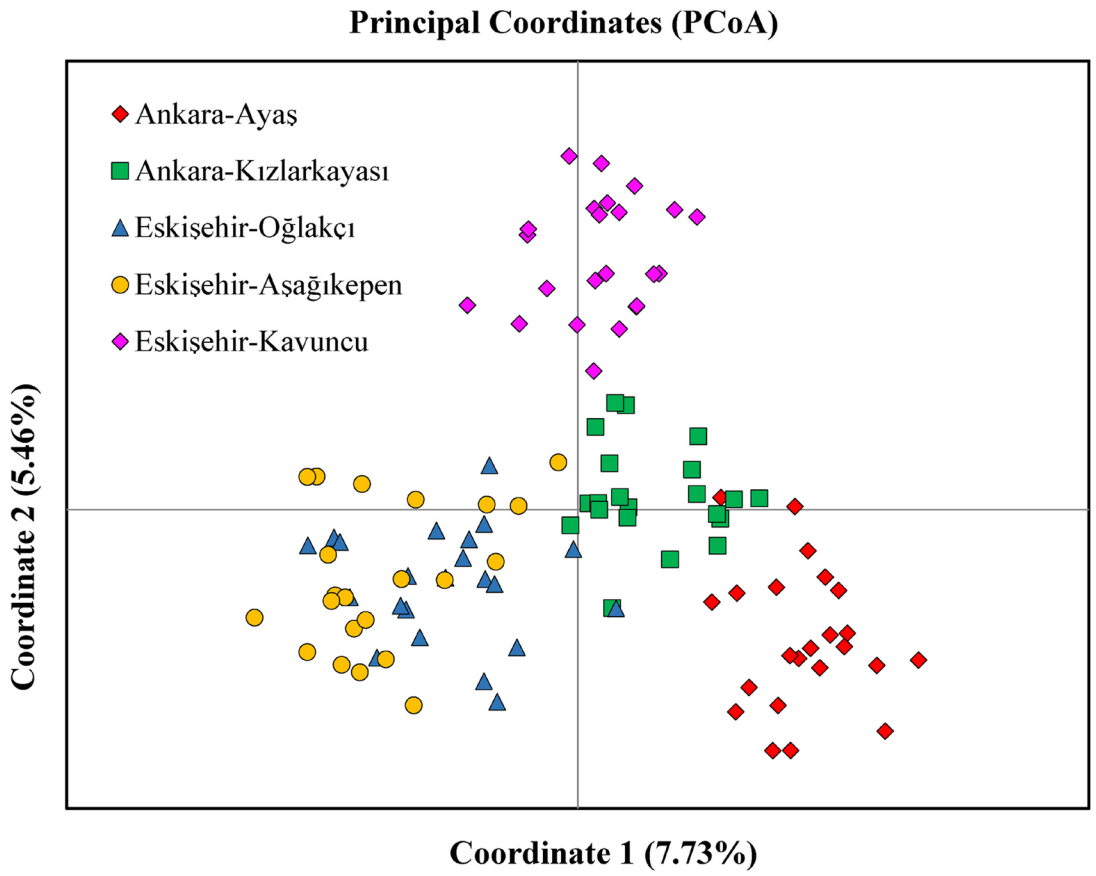

Figure 5. Principal coordinate analysis (PCoA) graph showing the spatial distribution of 111 *S. yildirimlii* genotypes.

genotypes (21) from Kızlarkayası population, 2 from Ayaş, 11 from Oğlakçı, 6 from Aşağıkepen, and 2 from Kavuncu populations (see Appendix).

4. Discussion

Nei's gene diversity (H), Shannon's information index (I), and percentage of polymorphic loci (PPL) are important parameters for assessing genetic diversity. Previous studies showed that in Lamiaceae family members like *Scutellaria baicalensis* (Bai et al., 2013), *Cunila spicata* (Echeverrigaray et al., 2016), *Thymus daenensis* subsp. *daenensis* (Rahimmalek et al., 2009), *Salvia miltiorrhiza* (Zhang et al., 2013), the percentage of polymorphic bands based on ISSR markers ranged from 85.32% to 96.04%. We can conclude that *S. yildirimlii* has a comparatively high proportion of polymorphic bands at the species level compared to these findings. When compared with the genetic diversity results of *Scutellaria baicalensis* (H_{sp} = 0.25, I_{sp} = 0.39, PPL_{sp} = 95.86%) (Bai et al., 2013), *Lamiophlomis rotate* (H_{sp} = 0.291, PPL_{sp} = 96.73% and H_{pop} = 0.166 PPL_{pop} = 51.81%) (Liu et al., 2006), *Satureja khuzistanica* (H_{sp} = 0.306, I_{sp} = 0.466, PPL_{sp} = 98.33% and H_{pop} = 0.262, I_{pop} = 0.390, PPL_{pop} = 74.88%) (Hadian et al., 2017), *Scutellaria montana* (H_{sp} = 0.374 and H_{pop} = 0.287, PPL_{pop} = 75.42%) (Cruzan, 2001), *Hemigenia exilis* (H_{sp} = 0.378, PPL_{sp} = 97.7% and H_{pop} = 0.311, PPL_{pop} = 71.5%) (Mattner et al., 2002) genetic diversity of *S. yildirimlii* presents comparable results at the species level but is generally lower at the population level. At the species level, the genetic diversity of *S. yildirimlii* is similar to that of endemic and threatened *Primula apennina* (H_{sp} = 0.242, I_{sp} = 0.318, PPL_{sp} = 96.95%) (Crema et al., 2009) and *Uechtritzia armena* (H_{sp} = 0.192, I_{sp} = 0.333, PPL_{sp} = 96.21%) (Yıldırım Doğan et al., 2016). Consequently, the genetic diversity of *S. yildirimlii*, based on genetic diversity parameters, is high (H_{sp} = 0.183, I_{sp} = 0.292, PPL_{sp} = 93.9%) at the species level, while it is relatively low (H_{pop} = 0.158, I_{pop} = 0.242, PPL_{pop} = 56.5%) at the population level.

According to the number of individuals from the most to the least, the populations are in the following

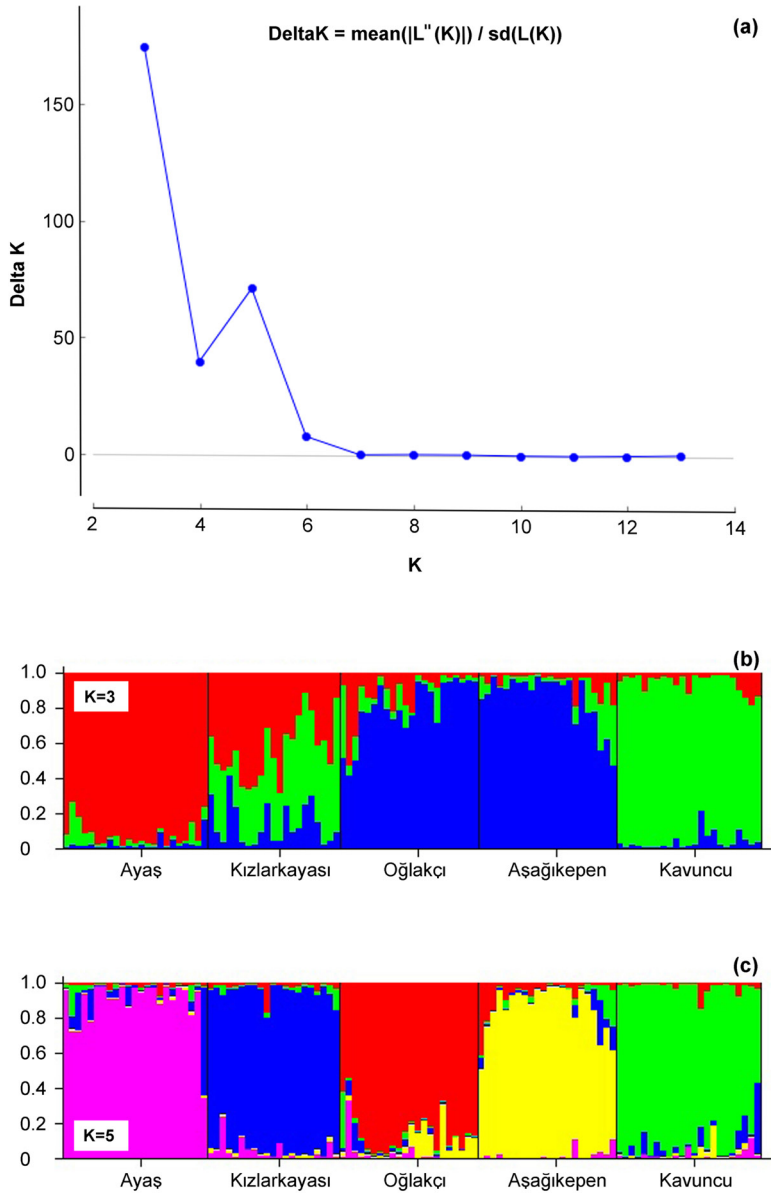

Figure 6. (a) STRUCTURE plot of population structure analysis and Delta-K values, (b) population structure analysis K = 3 and (c) population structure analysis K = 5.

order: Kızlarkayası, Aşağıkepen (Yeşilköy), Kavuncu, Oğlakçı, and Ayaş (Yıldırım et al., 2019), whereas the order of genetic diversity values (H, I, and PPL) is Oğlakçı, Aşağıkepen (Yeşilköy), Ayaş, Kızlarkayası, and Kavuncu. These findings demonstrate that the size of a population is not necessarily proportional to its genetic diversity. At PCoA, UPGMA, and STRUCTURE analysis, the two populations with the highest genetic diversity, Aşağıkepen and Oğlakçı, are clustered together (Figures 2, 3, and 6). Thus, it may be concluded that these two populations are not genetically isolated from one another and that gene flow between them is ongoing. Ayaş population is the third most genetically diverse among the other populations despite having the smallest population size, with only 587 individuals (Yıldırım et al., 2019). It is estimated that this population was substantial until recently. However, it is assumed that there has been a sharp decline since 2010 due to the expansion of agricultural areas, reforestation, habitat degradation and loss (Ayyıldız, 2010). Despite having a considerable number of individuals (48,000), Kızlarkayası population ranks only fourth in terms of genetic diversity (Yıldırım et al., 2019). This outcome can be attributed pri-

marily to genetic drift. Given that the habitat is steppe and the species is perennial, it is likely that a natural catastrophe such as a fire in the past induced genetic drift. Kavuncu population exhibits the lowest genetic diversity among the other populations of *S. yildirimlii*, and as evidenced in all cluster analyses, it was apart from the others. This finding is consistent with earlier isolation from the other populations. Although the geographical distance between Oğlakçı and Kavuncu population pair is less than that between Oğlakçı and Aşağıkepen populations, the lack of gene flow between them could be explained by different natural selection processes, different means of geographical barriers, low germination rates, limited seed dispersal, and ineffective long-distance pollen migration as well as the more intense anthropogenic threat on Kavuncu population compared to other populations.

S. yildirimlii exhibited higher genetic diversity when compared to *Scutellaria indica*, a perennial plant, on both allozyme (H_{sp} = 0.101, H_{pop} = 0.008 PPL_{pop} = 2.36%) and RAPD (H_{sp} = 0.139, H_{pop} = 0.027 PPL_{pop} = 8.94%) bases. On the other hand, there is a very high level of genetic differentiation among the populations of *S. indica* (allozyme G_{ST} = 0.92 and RAPD G_{ST} = 0.81). This *Scutellaria* species with floral dimorphism produces seeds through cross-pollination of its casmogamic flowers and self-pollination of its cleistogamic flowers (Sun, 1999). Therefore, these values were compatible with self-fertilized reproductive systems. These values suggest that, in comparison to perennial self-fertilized species, *S. yildirimlii* has a higher level of heterozygosity and a lower level of genetic differentiation (Hamrick and Godt, 1996). A high level of heterozygosity and polymorphism, along with a low level of population differentiation (G_{ST}), are generally associated with outbreeding plant species (Loveless and Hamrick, 1984). To comprehend the genetic differentiation of a species, it is necessary to have in-depth knowledge of pollination biology, seed dispersal systems, and the reproductive system, but there is no such study for *S. yildirimlii*. However, cross-pollination and pollination by insects or birds are common in the family Lamiaceae, members of this family have floral morphology that allows bees to suck nectarine and provide a good source of nectarine and pollen (Judd et al., 2015).

The results of AMOVA revealed that variance within populations (84%) was higher than among populations (16%). It was observed during fieldwork that *S. yildirimlii* has no trace of vegetative reproduction in any of its populations. This also explains the high level of genetic diversity within the populations. It can be said that there exists significant population differentiation among populations when gene differentiation parameters G_{ST} and Φ_{PT} are larger than 0.25, whereas gene flow parameter N_m is smaller than 1 (Slatkin, 1987). Gene-flow values equal to or higher than one are sufficient to prevent significant differentiation caused by genetic drift (Slatkin and Barton, 1989). The high value of gene flow is explained by the plant's ability for long-distance pollen or seed dispersal ability of plant (Sözen et al., 2017). In this study, the G_{ST} = 0.144, Φ_{PT} = 0.159, and N_m = 2.984 values of *S. yildirimlii* indicated moderate level of genetic differentiation among populations and high level of gene flow. Similar results to those of our study in high gene flow and moderate genetic differentiation were shown in *Verbascum gypsicola* (Keser and Yaprak, 2022) and *Teucrium leucophyllum* (Sözen et al., 2017), both species are insect-pollinated steppe perennials. This situation can be speculated by different natural selection processes among the populations. The genetic differentiation among the populations can be assumed due to geographic barriers, which segregated different gene pools. Inefficient long-distance pollen flow, close seed dispersal, and low germination rates could be the latent reasons, which have led to three distinct *S. yildirimlii* gene pools.

The UPGMA and PCoA cluster analyses also confirm the results of the Mantel test for *S. yildirimlii* indicated a positive correlation ($r = 0.493$, $p < 0.001$) between geographic distance and genetic distance with statistically highly significant p-value ($r = 0.493$, $p < 0.001$). For instance, Ayaş population is clustered with Kızlarkayası, which is geographically the nearest (60 km) as well as in terms of genetic identity (0.9748). Although Oğlakçı population is geographically closer to Kavuncu population by 22 km, it is genetically more similar to Aşağıkepen population (0.9784) by 29 km and clustered with it (Figure 1 and Table 6). In UPGMA dendrogram, which shows the genetic relationships between genotypes, individuals from each population clustered together with the exception of a few. In addition, the geographic distance between the populations of Kızlarkayası and Aşağıkepen is 55 km, while the geographic distance between the populations of Kızlarkayası and Oğlakçı is 27 km (Figure 4). This suggests the possibility of secondary seed dispersal between populations via birds. The results of STRUCTURE analysis clearly imply that the sampling locations behave as three clusters, with some examples of admixed individuals. These admixture traces indicate that gene flow still exists among the locations (which is corroborated by the high value of N_m greater than 1). Furthermore, STRUCTURE assignment helped to clarify the spatial distribution in PCoA graph and supported the UPGMA clustered tree. This shows that analyses by STRUCTURE software were accurate for this study. Since five natural populations were categorized into three clusters, which should possibly be considered three management units for the objective of conservation.

5. Conclusion

The present study provides an understanding of genetic diversity and population structure based on ISSR markers of endemic and endangered *S. yildirimlii*. It is possible to attract attention regarding the conservation of *S. yildirimlii* from the findings obtained in this study. Our results indicated that in situ conservation must be planned for all natural populations of *S. yildirimlii* to protect the species' existing genetic diversity. This is especially important for Ayaş and Oğlakçı populations, which have relatively small populations that are found in restricted areas with a high level of genetic diversity. Ex situ conservation techniques are also critical for future environmental challenges. Attempts can be undertaken to create new populations in similar habitats. Furthermore, preventative steps can be implemented to address issues such as habitat fragmentation and loss caused by agricultural expansion and road building that prevent gene flow among populations. Through leaflets and informative signage, locals should also be informed about conservation initiatives to reduce the pressure caused by unregulated and overgrazing in the species' distribution areas.

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Conflict of interest

The Authors declare that they have no conflicts of interest.

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Appendix

Table A1. Assignment of the genotypes of *S. yildirimlii* to the subgroups identified by STRUCTURE based on highest membership probability.

Genotype	Location	A	B	C	Structure
1	Ayaş	0.072	**0.915**	0.013	B
2	Ayaş	0.245	0.727	0.027	admixed
3	Ayaş	0.168	**0.816**	0.016	B
4	Ayaş	0.070	**0.913**	0.017	B
5	Ayaş	0.070	**0.905**	0.025	B
6	Ayaş	0.013	**0.979**	0.008	B
7	Ayaş	0.018	**0.969**	0.014	B
8	Ayaş	0.014	**0.932**	0.055	B
9	Ayaş	0.060	**0.925**	0.015	B
10	Ayaş	0.010	**0.980**	0.01	B
11	Ayaş	0.032	**0.949**	0.019	B
12	Ayaş	0.019	**0.967**	0.014	B
13	Ayaş	0.024	**0.954**	0.022	B
14	Ayaş	0.017	**0.971**	0.013	B
15	Ayaş	0.017	**0.974**	0.009	B
16	Ayaş	0.025	**0.883**	0.092	B
17	Ayaş	0.009	**0.982**	0.009	B
18	Ayaş	0.021	**0.926**	0.053	B
19	Ayaş	0.017	**0.968**	0.015	B
20	Ayaş	0.011	**0.957**	0.032	B
21	Ayaş	0.135	**0.839**	0.026	B
22	Ayaş	0.032	**0.952**	0.015	B
23	Ayaş	0.073	0.760	0.167	admixed
24	Kızlarkayası	0.327	0.359	0.313	admixed
25	Kızlarkayası	0.396	0.516	0.088	admixed
26	Kızlarkayası	0.415	0.551	0.034	admixed
27	Kızlarkayası	0.055	0.524	0.422	admixed
28	Kızlarkayası	0.322	0.434	0.244	admixed
29	Kızlarkayası	0.328	0.637	0.034	admixed
30	Kızlarkayası	0.335	0.649	0.016	admixed
31	Kızlarkayası	0.332	0.645	0.023	admixed
32	Kızlarkayası	0.332	0.575	0.092	admixed
33	Kızlarkayası	0.435	0.304	0.261	admixed
34	Kızlarkayası	0.488	0.470	0.043	admixed
35	Kızlarkayası	0.270	0.680	0.050	admixed
36	Kızlarkayası	0.416	0.343	0.241	admixed
37	Kızlarkayası	0.543	0.366	0.091	admixed
38	Kızlarkayası	0.652	0.232	0.115	admixed
39	Kızlarkayası	0.637	0.112	0.251	admixed

Genotype	Location	A	B	C	Structure
40	Kızlarkayası	0.495	0.280	0.297	admixed
41	Kızlarkayası	0.441	0.407	0.152	admixed
42	Kızlarkayası	0.604	0.374	0.022	admixed
43	Kızlarkayası	0.441	0.516	0.043	admixed
44	Kızlarkayası	0.767	0.141	0.092	admixed
45	Oğlakçı	0.411	0.067	0.523	admixed
46	Oğlakçı	0.064	0.515	0.421	admixed
47	Oğlakçı	0.140	0.352	0.508	admixed
48	Oğlakçı	0.145	0.073	0.783	admixed
49	Oğlakçı	0.088	0.132	0.780	admixed
50	Oğlakçı	0.024	0.146	**0.831**	C
51	Oğlakçı	0.051	0.016	**0.932**	C
52	Oğlakçı	0.116	0.086	0.798	admixed
53	Oğlakçı	0.043	0.213	0.743	admixed
54	Oğlakçı	0.144	0.058	0.797	admixed
55	Oğlakçı	0.130	0.174	0.696	admixed
56	Oğlakçı	0.017	0.216	0.766	admixed
57	Oğlakçı	0.032	0.013	**0.955**	C
58	Oğlakçı	0.025	0.036	**0.939**	C
59	Oğlakçı	0.053	0.049	**0.898**	C
60	Oğlakçı	0.204	0.074	0.721	admixed
61	Oğlakçı	0.041	0.010	**0.949**	C
62	Oğlakçı	0.023	0.023	**0.954**	C
63	Oğlakçı	0.016	0.010	**0.973**	C
64	Oğlakçı	0.023	0.027	**0.950**	C
65	Oğlakçı	0.028	0.010	**0.962**	C
66	Oğlakçı	0.023	0.023	**0.954**	C
67	Yeşilköy	0.129	0.016	**0.855**	C
68	Yeşilköy	0.056	0.060	**0.884**	C
69	Yeşilköy	0.011	0.010	**0.979**	C
70	Yeşilköy	0.012	0.069	**0.918**	C
71	Yeşilköy	0.080	0.010	**0.910**	C
72	Aşağıkepen	0.015	0.015	**0.970**	C
73	Aşağıkepen	0.034	0.016	**0.950**	C
74	Aşağıkepen	0.024	0.020	**0.955**	C
75	Aşağıkepen	0.087	0.008	**0.905**	C
76	Aşağıkepen	0.013	0.007	**0.980**	C
77	Aşağıkepen	0.019	0.024	**0.957**	C
78	Aşağıkepen	0.031	0.015	**0.953**	C
79	Aşağıkepen	0.018	0.027	**0.956**	C
80	Aşağıkepen	0.029	0.032	**0.940**	C
81	Aşağıkepen	0.015	0.025	**0.960**	C
82	Aşağıkepen	0.103	0.176	0.721	admixed

Genotype	Location	A	B	C	Structure
83	Aşağıkepen	0.017	0.023	**0.960**	C
84	Aşağıkepen	0.193	0.026	0.781	admixed
85	Aşağıkepen	0.110	0.102	0.788	admixed
86	Aşağıkepen	0.273	0.164	0.563	admixed
87	Aşağıkepen	0.327	0.049	0.634	admixed
88	Aşağıkepen	0.346	0.174	0.480	admixed
89	Kavuncu	**0.924**	0.043	0.033	A
90	Kavuncu	**0.973**	0.017	0.009	A
91	Kavuncu	**0.950**	0.026	0.024	A
92	Kavuncu	**0.974**	0.010	0.016	A
93	Kavuncu	**0.888**	0.099	0.013	A
94	Kavuncu	**0.967**	0.025	0.008	A
95	Kavuncu	**0.961**	0.030	0.009	A
96	Kavuncu	**0.966**	0.019	0.015	A
97	Kavuncu	**0.968**	0.020	0.011	A
98	Kavuncu	**0.880**	0.062	0.058	A
99	Kavuncu	**0.972**	0.019	0.009	A
100	Kavuncu	**0.874**	0.112	0.014	A
101	Kavuncu	**0.964**	0.010	0.026	A
102	Kavuncu	0.776	0.022	0.202	admixed
103	Kavuncu	**0.905**	0.022	0.073	A
104	Kavuncu	**0.885**	0.010	0.105	A
105	Kavuncu	**0.963**	0.010	0.027	A
106	Kavuncu	**0.977**	0.011	0.012	A
107	Kavuncu	**0.951**	0.023	0.026	A
108	Kavuncu	**0.801**	0.090	0.109	A
109	Kavuncu	**0.815**	0.132	0.053	A
110	Kavuncu	0.798	0.176	0.025	admixed
111	Kavuncu	**0.839**	0.125	0.036	A

* According to the membership fractions of the Structure, accessions with a probability of ≥80% were assigned to corresponding subgroups "A, B, and C", while others were classified as "Admixture".