

RESEARCH ARTICLE

ISSR-Based Population Genetic Structure of Some Turkish Honeybee (*Apis mellifera* L., 1758) Populations

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Introduction

Honey bees, *Apis mellifera* Linnaeus, 1758 are social insects belonging to Hymenoptera and live as colonies. Hymenoptera includes more than 19,000 identified bee species (Cane, 2008). *Apis* are named true honey bees and they have very important economic and ecological roles (Kilani, 1999). Bees pollinate plant species and produce honey, bee gum, pollen, and royal jelly. Early studies including morphometric and molecular characterization

of *Apis mellifera* L. showed that they are divided into five evolutionary strain groups.

The groups belonging to 33 subspecies are Group A (central and southern Africa), Group C (eastern Europe and the northern Mediterranean), Group M (northern Africa, northern and western Europe), Group O (the Middle East and the eastern Mediterranean) and Y (east Africa) (Ruttner, 1988; Franck *et al.*, 2000; Ilyasov *et al.*, 2020). Ruttner (1988) has determined different honey bee ecotypes and subspecies from Türkiye have a wide range of habitats

Abstract

Objective: *Apis mellifera*, in the order Hymenoptera, are social insects, also known as honey bees. Türkiye has many different honey bee ecotypes and subspecies. Examination of genetic diversity and evolutionary relationships of colonies in nine different locations in Türkiye was carried out using ISSR primers.

Materials and Methods: *Apis mellifera* samples were collected from Çorum, Elazığ, Eskişehir, Kütahya, İzmir, Manisa, Antalya, Samsun, and Muğla. The Lifton method was used for DNA extraction from 180 honey bees. DNA samples were amplified using six ISSR primers.

Results: From the amplification of 180 individuals with 6 ISSR primers, 283 polymorphic loci were identified. Genetic diversity data (N_a , N_e , h , I) were obtained for nine populations from 283 loci. In all data on the calculated genetic diversity, the lowest values were found in the Samsun population, and the highest values were found in the Manisa population. The gene flow level calculated from the genetic differentiation value was found to be 0.22.

Conclusion: Genetic diversity has a role in the adaptation of species to changing environmental conditions and it is one of the raw materials of evolution. Herein, we preferred ISSR markers to identify the genetic structure of honey bees. The genetic diversity of honey bees has been found to be lower compared to previous studies. This variability may be a result of the ecological, climatic conditions, and biogeographic differences of Anatolia. The studies to be carried out with more examples from more locations related to honey bees in Türkiye will contribute to the clear identification of the genetic structure of this organism. Additionally, using other marker systems will help to clarify the status of populations in Türkiye.

Keywords: *Apis mellifera*, Genetic diversity, ISSR, Türkiye

and climates. Almost all the Türkiye honey bees, except for the northeast and southeast, are *A. mellifera anatolica* Maa. (Anatolian), and the others are *A. mellifera caucasica* Gorbachev (Caucasian) and *A. mellifera meda* Skorikov (Iranian), respectively.

Amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), and simple sequence repeats (SSR) are PCR-based markers that are very suitable for determining phylogenetic relationships and genetic diversity of plants, animals, and particularly insects. ISSR markers have been essentially built up to distinguish plant genetic diversity but confirmed to be highly beneficial for population studies of other organisms, especially insects (Paplauskienė *et al.*, 2006; Shouhani *et al.*, 2014; Rahimi *et al.*, 2016). ISSR is a PCR-based dominant marker and it is used to investigate population structure analysis, phylogenetic analysis, identification of species, genetic mapping, and genetic variation of closely related species (Zietkiewicz *et al.*, 1994). This technique has a great advantage in that it does not need preliminary DNA sequence knowledge for primer design, and it gives enough variable and sensible data, so researchers can spend a short time and an acceptable amount of money (Sheppard & Smith, 2000). For these reasons, the ISSR marker system might be reasonable and beneficial for the determination of target genomic positions (Radjabi *et al.*, 2012). Some studies have been done to determine the population genetic structure of honey bees using ISSR markers (Ceksteryte *et al.*, 2012; Shouhani *et al.*, 2014; Rahimi *et al.*, 2016; Ahmad, 2018).

The present study primarily aimed to characterize the phylogenetic relationships and polymorphism of *A. mellifera* populations from nine different regions of Türkiye using ISSR markers, and for data analysis, POPGEN version 1.32 (Yeh *et al.*, 1999) and GenAlEx

version 6.5 (Peakall & Smous, 2012) computer programs were used.

Material and Methods

Honey bee samples: 180 worker honey bees were collected from different regions (20 individuals from each of the Çorum, Elazığ, Eskişehir, Kütahya, İzmir, Manisa, Antalya, Samsun, Muğla populations) of Türkiye in nine *A. mellifera* colonies from June to August 2021.

DNA extraction: Total DNA was obtained from honey bee samples collected from 9 different locations by the “Lifton DNA extraction” method (Bender *et al.*, 1983).

PCR amplification: PCR was performed in a total volume of 25 µl. The reaction content was established with 12.3 µl dH₂O, 10.5 µl of PCR Master Mix (Thermo Scientific™ Catalog number: K0171), 1 µl ISSR primer (base sequences are given in Table 1), 0.2 µl Tween®-20 and 1 µl DNA. A reaction of 35 cycles was prepared. Each cycle was optimized with denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and elongation at 68°C for 3 minutes. In addition, pre-denaturation at 95°C for 3 minutes and final elongation at 72°C were added to the reaction cycles. The amplified products were run at a 70 V constant current for three hours on a 1% agarose gel containing ethidium bromide prepared with 1X TBE buffer. PCR products were electrophoresed on a 1% agarose gel in 1X TBE buffer and stained with ethidium bromide.

Statistical analysis: Thermo Scientific™ GeneRuler 1 kb DNA Ladder was used as a reference to interpret the gel image obtained as a result of the electrophoresis of PCR products. The band sizes of each individual were

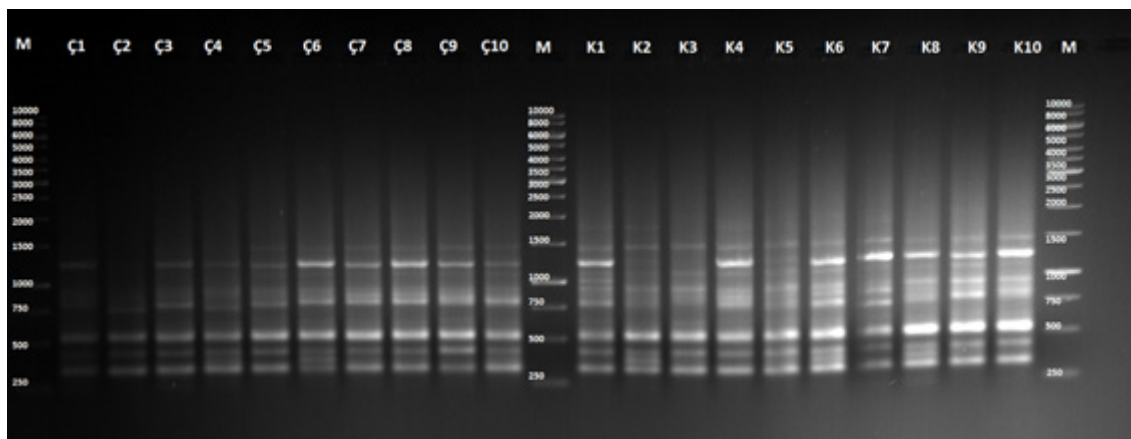


Figure 1. PCR amplification products obtained in 1% agarose gel from ten individuals belonging to two honey bee populations with CCA(TG)₇T primer [Ç1-10, ‘Çorum 1-10’; K1-10, ‘Kütahya 1-10’; M, 1kb ladder (GeneRuler™)].

scored according to the band sizes created by the Thermo Scientific™ GeneRuler 1 kb DNA Ladder. By examining the corresponding band lengths on the DNA ladder, the presence of a band was scored with 1, and absence with 0 on Microsoft Excel (Fig. 1). Bands that did not show a clear image were not included in the scoring. The generated Microsoft Excel data were analyzed with PopGene ver. 1.32 software. As a result of the analysis, genetic diversity parameters, genetic differentiation values, and gene flow level values were obtained. In addition, the phylogenetic tree structure between populations was revealed with the UPGMA dendrogram by using the genetic distance value between the populations. Finally, the generated scoring table was analyzed with GenAIEx 6.503 software, and the main sources of genetic variation were revealed by molecular variance analysis.

Results

Molecular finger printings of 180 worker honey bees collected from different regions of Türkiye were carried out using six ISSR primers. All primers used produced polymorphic bands in DNA amplification. The number of total bands was 283 and their fragment size ranged from 150 to 10000 bp. Using six ISSR primers, 283 polymorphic loci were identified. The average number of polymorphic loci detected per primer used is approximately 47. All 283 loci identified with the six primers used were polymorphic, and no monomorphic locus was detected (Table 1).

All primers used produced the observed number of bands in populations between 107 (Manisa) and 65 (Samsun). On the other hand, polymorphic locus percentages were between 30.04% (Manisa) and 8.48%

Table 1. Information on primer sequences provided by Baysal *et al.* (2011) and information on band profiles created by these primers.

Primer Numbers	ISSR Primer Sequence (5' 3')	Number of total bands	Percent of polymorphic bands	Range of fragment size (bp)
1	(ACC) ₆ CC	53	100	300-8100
2	CCA(TG) ₇ T	67	100	400-10000
3	CCA(TGA) ₅ TG	29	100	400-2900
4	GCA(AC) ₇	44	100	400-8000
5	GGG(AC) ₇	52	100	300-6500
6	(GA) ₆ GG	38	100	150-4000
	Total	283	-	-
	Mean	47.16	100	325-6583

Table 2. Band profiles and percent polymorphism rates of *A. mellifera* populations.

Population	Sample Size	Number of bands	Number of polymorphic bands	Number of private bands	% Polymorphism
Corum	20	81	58	4	20.49
Elazığ	20	104	70	12	24.73
Eskişehir	20	100	70	12	24.73
Kütahya	20	85	55	11	19.43
İzmir	20	102	76	16	26.86
Manisa	20	107	85	23	30.04
Antalya	20	83	62	1	21.91
Samsun	20	65	24	0	8.48
Muğla	20	71	39	5	13.78
Mean	20	88.66	59.88	9.33	21.16

Table 3. Genetic diversity values of *A. mellifera* populations. *Na*: the mean number of observed alleles; *Ne*: the mean number of effective alleles; *He*: Nei's gene diversity; *I*: Shannon's information index.

Population	<i>Na</i>	<i>Ne</i>	<i>He</i>	<i>I</i>
Çorum	1.20±0.40	1.12±0.28	0.06±0.15	0.10±0.22
Elazığ	1.24±0.43	1.13±0.29	0.08±0.16	0.12±0.23
Eskisehir	1.24±0.43	1.13±0.29	0.08±0.16	0.12±0.23
Kütahya	1.19±0.39	1.10±0.26	0.06±0.14	0.09±0.21
İzmir	1.26±0.44	1.15±0.29	0.08±0.16	0.13±0.23
Manisa	1.30±0.45	1.17±0.31	0.10±0.17	0.15±0.25
Antalya	1.21±0.41	1.12±0.26	0.07±0.15	0.11±0.22
Samsun	1.08±0.27	1.04±0.16	0.02±0.09	0.03±0.14
Muğla	1.13±0.34	1.09±0.25	0.05±0.14	0.08±0.20
Mean	1.20±0.39	1.11±0.26	0.06±0.14	0.10±0.21

(Samsun). The number of polymorphic and specific bands in the populations ranged from 24 to 85 and 0 to 23, respectively (Table 2).

The average number of effective alleles (N_e) was found to be 1.12, 1.13, 1.13, 1.10, 1.15, 1.17, 1.12, 1.04, 1.09 in Çorum, Elazığ, Eskişehir, Kütahya, İzmir, Manisa, Antalya, Samsun, and Muğla, respectively. The average number of observed alleles (N_a) were found to be 1.20, 1.24, 1.24, 1.19, 1.26, 1.30, 1.21, 1.08, 1.13 in Çorum, Elazığ, Eskişehir, Kütahya, İzmir, Manisa, Antalya, Samsun, and Muğla, respectively. Nei's gene diversity (H_e) values ranged between 0.10 (Manisa) and 0.02 (Samsun). Shannon's information index (I) values ranged from 0.15 (Manisa) to 0.03 (Samsun). Generally, the highest values of genetic diversity were found in the Manisa population, and the lowest values in the Samsun population (Table 3).

Total genetic variation (H_T) was 0.22 ± 0.02 based on the POPGENE ver. 1.32 analysis in all populations studied. The total genetic variation originated from approximately 32% within the populations (H_S : 0.07 ± 0.003) and approximately 68% among populations (D_{ST} : 0.15). Tests of homogeneity among the studied nine populations were made using

analysis of molecular variance (AMOVA). The results are summarized in Figure 2. Similar to the POPGENE ver. 1.32 analysis, in the AMOVA analysis, a major part of the total genetic variation is due to variation among populations, while a minor part is due to variation within populations.

The genetic differentiation value (G_{ST}) was 0.68 and using the G_{ST} value, the gene flow level (N_M) was calculated as 0.22. Genetic distance values (D_N) ranged from 0.0337 to 0.3252 among population pairs. Independent with geographic distances, the minimum distance was detected between Antalya and Samsun, and the maximum distance was detected between Elazığ and Samsun populations (Table 4).

The UPGMA dendrogram was created using Nei's (1987) genetic distance values (D_N). According to the dendrogram given in Figure 3, the tree structure is divided into three main branches. Similar to the UPGMA dendrogram in the baseline coordinate analysis (PCoA), all studied populations were clearly separated, and the relationship between the populations was shown once again using PCoA (Fig. 4).

Table 4. Estimates of Nei's (1987) genetic distance (D_N) coefficients between the *A. mellifera* populations.

Population	Çorum	Elazığ	Eskişehir	Kütahya	İzmir	Manisa	Antalya	Samsun	Muğla
Çorum	*****	0.7965	0.8049	0.9019	0.8062	0.7998	0.8228	0.7872	0.7864
Elazığ	0.2275	*****	0.8785	0.7953	0.8933	0.8589	0.7617	0.7224	0.7439
Eskişehir	0.2170	0.1295	*****	0.7959	0.8708	0.8624	0.7818	0.7446	0.7754
Kütahya	0.1033	0.2291	0.2283	*****	0.8008	0.7913	0.7988	0.7654	0.7871
İzmir	0.2154	0.1129	0.1383	0.2222	*****	0.8770	0.7640	0.7242	0.7458
Manisa	0.2235	0.1521	0.1480	0.2341	0.1312	*****	0.7722	0.7365	0.7582
Antalya	0.1951	0.2722	0.2462	0.2247	0.2691	0.2585	*****	0.9669	0.9299
Samsun	0.2392	0.3252	0.2949	0.2673	0.3227	0.3059	0.0337	*****	0.8908
Muğla	0.2403	0.2959	0.2544	0.2395	0.2933	0.2768	0.0726	0.1156	*****

Percentages of Molecular Variance

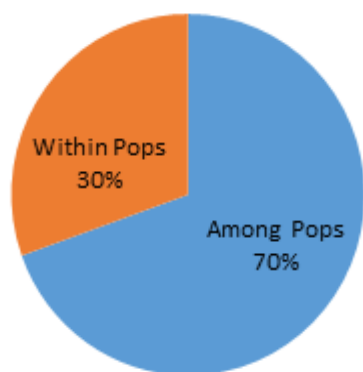


Figure 2. AMOVA results of all studied *A. mellifera* populations.

Discussion

The honey bee (*Apis mellifera* L.) is a globally important species of certain economic and ecological significance. Data we obtained as a result of the study indicated that the ISSR method is an appropriate technique for the determination of genetic polymorphism in *A. mellifera* populations. ISSR bands are highly repeatable in comparison with other dominant marker systems.

The aim of this study was the molecular characterization and determination of the genetic polymorphism of nine different *A. mellifera* populations using six ISSR primers. ISSR markers have been chosen in this study, because of giving a high level of polymorphic bands. When we compare data in our investigation with data in other honey bee studies in which the polymorphism levels were

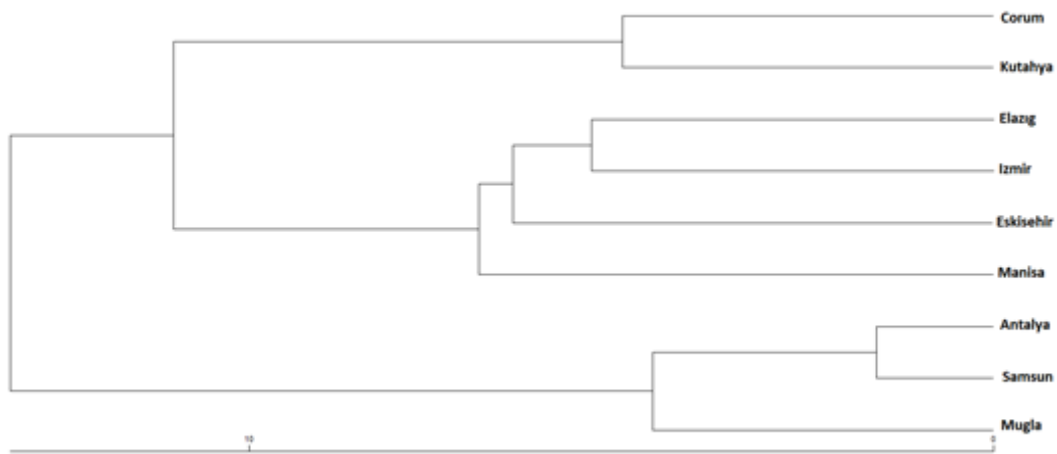


Figure 3. UPGMA dendrogram of *A. mellifera* populations revealed by Nei's (1987) genetic distance values (DN).

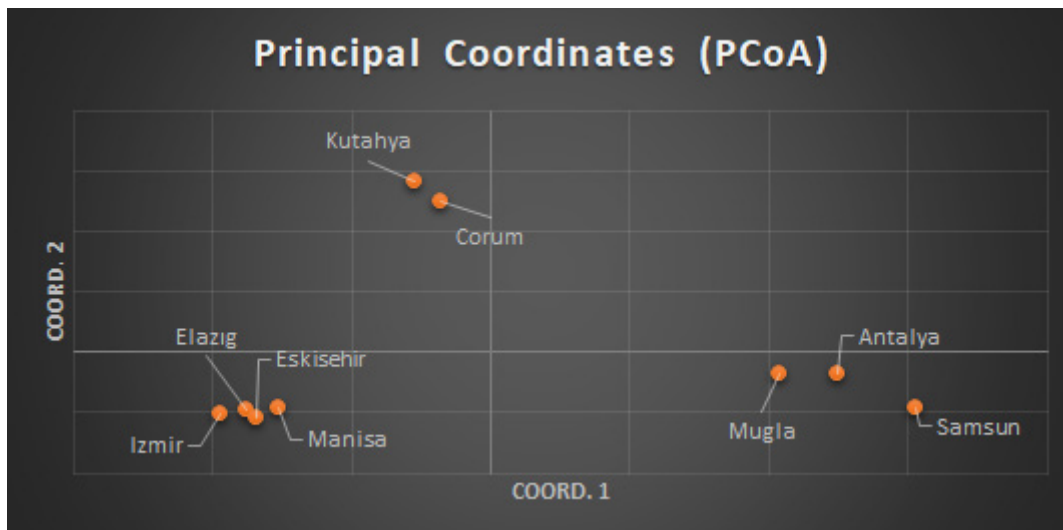


Figure 4. PCoA graph of *A. mellifera* populations

detected using ISSR markers (Al-Otaibi, 2008; Ceksteryte *et al.*, 2012; Karakaş, 2013; Shouhani *et al.*, 2014; Rahimi *et al.*, 2016; Ahmad, 2018), the polymorphism levels were found 81.6 %, 38.27 %, 51.33 %, 53.66 %, 61.56 %, and 55.52 %, respectively. Our polymorphism level (21.16%) was found to be less than in other studies.

We found 283 reproducible bands, ranging from 150 to 10000 bp. Al-Otaibi (2008), Rahimi *et al.*, (2016), and Ahmad (2018), reported different results. Rahimi *et al.* (2016) and Ahmad (2018) determined the genetic relationship of different honey bee populations using ten ISSR primers. Rahimi *et al.* (2016) obtained 40 polymorphic bands ranging from 150 bp to 1500 bp while Ahmad (2018) obtained 50 polymorphic bands ranging from 100 bp to 850 bp. On the other hand, Dušinsk *et al.* (2006) determined polymorphism of some blackflies species (*Simulium* sp.)

having many taxonomic problems using five ISSR primers and they obtained 55 polymorphic bands ranging from 230 bp to 1400 bp. The same differences were revealed in the results of other studies using ISSR primers for the determination of genetic polymorphism (Hundsdoerfer *et al.*, 2005; Al-Otaibi, 2008; Shouhani *et al.*, 2014).

Expected heterozygosity (H_e) and Shannon's information index (I') values, it was seen that Manisa population values were greater than the other populations. Moreover, the percentage of polymorphic loci and private bands have the highest in the Manisa population according to other populations. In addition to these results, the average number of observed alleles (1.30) and the average number of effective alleles (1.17) values were found high in the Manisa population. The average values of genetic diversity parameters were 1.20 for N_a , 1.11 for N_e , 0.06 for H_e , and

0.10 for I and these results were consistent with the previous studies. These values were found in 1.38, 1.22, 0.13, and 0.19 (Ceksteryte *et al.*, 2012), 1.51, 1.29, 0.17 and 0.25 (Karakas, 2013), respectively. These results show that the genetic diversity of the honey bees studied is relatively less. In all studied populations, H_T , H_S , G_{ST} , and N_M values were found as 0.22, 0.07, 0.68, and 0.22, respectively. These results are relatively lower than Ceksteryte *et al.*, (2012) (0.22, 0.42, 0.42, and 0.68 respectively) and Karakas (2013) (0.29, 0.17, 0.39, and 0.76 respectively).

G_{ST} reveals genetic differentiation and takes a value between 0 and 1. A G_{ST} value greater than 0.25 indicates high differentiation between populations. The genetic differentiation value determined in nine honey bee populations screened with six ISSR primers was 0.68. The gene flow level (N_M) calculated based on the genetic differentiation value was found to be 0.22. According to Wright (1951), a gene flow level higher than 0.5 is considered a limit to prevent genetic drift of populations. If the N_M value is above 1, it is accepted that it prevents the local differentiation of populations (Slatkin, 1987). The calculated level of genetic differentiation and gene flow indicates high inbreeding in the studied populations. This indicates that existing populations are at risk of genetic drift.

In the studies conducted by Ceksteryte *et al.* (2012) and Karakas (2013), the G_{ST} value is above the critical value and the genetic differentiation level is high. However, contrary to the work we have done in both studies, the gene flow level (N_M) is high. Therefore, the probability of genetic drift in the populations used in this study is higher than in the other two studies.

According to the data obtained as a result of POPGENE ver. 1.32 software and AMOVA analysis, it was determined that the main contribution to genetic variation was caused by population genetic variation. In parallel with low gene flow and high genetic differentiation values among populations, this situation may cause irreversible significant gene losses in the gene pool in case of colony losses.

According to the percentages of molecular variance obtained from the AMOVA analysis, it was determined that the main contribution to genetic variation originates among populations (%70). These results are relatively compatible with Karakas (2013) (54.4%) using the ISSR markers. Kükürer (2013) and Ahmad (2018) reported that, unlike this study, the main contribution to total genetic variation originates from within the studied populations.

PCoA analyses confirmed the grouping. Our principal component analysis (PCoA) results in our study were compatible with the results of Kükürer (2013), Karakas

(2013), and Tunca & Kence (2011). The UPGMA dendrogram was created using Nei's (1987) genetic distance values (DN). According to the dendrogram given in Figure 3, the tree structure is divided into three main branches. The first branch consists of Çorum and Kütahya populations, the second branch consists of Elazığ, İzmir, Eskişehir, and Manisa populations, and the third branch structure consists of Antalya, Samsun, and Muğla populations. This is an unexpected result because groups formed a tree structure independent of their geographical distance. This situation can be explained by the transportation of honey bee colonies living in a certain region to different cities and the creation of new bee colonies there. Also, Ruttner (1988), almost all of the honey bees located outside the northeastern and southeastern regions in Türkiye "*A. mellifera anatolica* Maa. (Anatolian)" was stated. Since there is no population in the northeast and southeast regions among the studied populations, it is thought that a branching structure independent of the geographical location may have occurred.

Conclusion

Knowledge of the genetic diversity of the populations is very important. Genetic diversity has a role in the adaptation of species to changing environmental conditions, and it is one of the raw materials of evolution. Herein, we preferred ISSR markers to identify the genetic structure of honey bees. The genetic diversity of honey bees has been found to be lower compared to previous studies. This variability may be a result of the ecological, climatic conditions, and biogeographic differences of Anatolia. The low gene flow identified and the high level of genetic differentiation between populations indicate inbreeding. This combined with low genetic diversity makes it likely that conditions such as disease that may occur will not be enough to save the colony and result in the loss of an entire colony. In this case, it will result in the disappearance of important gene resources. For this reason, it is essential to develop existing protection programs and implement additional protection programs. The studies to be carried out with more examples from more locations related to honey bees in Türkiye will contribute to the clear identification of the genetic structure of this organism. Additionally, using other marker systems will help to clarify the status of populations in Türkiye.

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