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Genetic relationships of Thrace and Yığılca honey bee populations based on microsatellite structure

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Abstract: Thrace and Yığılca honey bees, two important honey bee ecotypes in apicultural activity of Turkey, are the subject of genetic conservation effort. In this study, the genetic structure and diversity of honey bee populations from Thrace and Yığılca were investigated using 27 microsatellites. Except Kırklareli and Yığılca (Fst: 0.14), it was observed lower genetic divergence between the populations based on the value of pairwise Fst. Although Thrace populations (Edirne, Tekirdağ and Kırklareli) were not fully separated from each other, Yığılca population was significantly separated from Kırklareli and separated slightly from the rest of other populations. The calculated gene diversity of the populations ranged from 0.44 in Kırklareli to 0.56 in Edirne and Tekirdağ. Despite the high genetic diversity within the populations, the significant heterozygous deficiency found in Kırklareli may be due to repeated and controlled swarming of the selected colonies by beekeepers. These factors could have contributed the observed genotypic homogenization within Kırklareli honey bee population. Our results demonstrate that genetic differantiation of Thrace and Yığılca populations is still conserved, but gene flow is not prevented by the current management strategies, creating urgent demand for an improved conservation management of honey bee populations.

Key words: *Apis mellifera* L., honey bee, genetic diversity, microsatellites, Turkey

1. Introduction

Honey bee (*Apis mellifera* L.) populations were found in Africa, Europe, and western Asia in their natural habitat. In this vast area, they have profoundly differentiated; 29 subspecies have been recognized through morphological and genetic analysis [1,2,3]. It is emphasized that *A. m. anatoliaca*, *A. m. carnica*, *A. m. caucasica*, *A. m. meda*, and *A. m. syriaca* naturally distribute in Turkey [1]. Ruttner [1] demonstrated dispersing pattern as *A. m. meda* in Southeast, *A. m. caucasica* in Northeast, *A. m. anatoliaca* throughout the rest of the country including Thrace (European part of Turkey) and *A. m. syriaca* in a small area, Hatay-Antakya in South of the Turkey according to morphometric analysis [1]. Latest examinations, based on both morphometric and mtDNA analyses, claimed that *A. m. carnica* or its ecotype was found in the Thrace region of Turkey [4,5,6,7,8,9]. In addition to those subspecies found in Turkey, there are unique ecological types including the Muğla and Yığılca honey bee ecotypes of *A. m. anatoliaca* [10,11,12,13,14,15,16,17,18,19,20,21]. Yığılca honey bee is claimed to be a special population, locally diverged and adapted to Black Sea Region, and discriminated by their scutellum color and the length of their wings and legs. This honey bee also has more honey production capacity than *A. m. anatoliaca* and *A. m. caucasica* hybrids [12]. Yığılca honey bee develop more broods before the main nectar flow and have a higher worker population during the nectar flow cycle than *A. m. anatoliaca* and *A. m. caucasica* hybrids, and colony expansion occurs suddenly in the spring [18]. Furthermore, Yığılca honey bee ecotype has higher propolis harvesting capacity than other honey bee races of Turkey [22]. Thrace honey bee, *A. m. carnica,* is in dark grey color, a gentle bee with high adaptation capacity on highland and cold climate, and has low swarm tendency [1,23]. It spends a long time with a relatively small winter group, and a different search behavior was observed with them [24,25]. All these counted characteristics can provide many advantages for the beekeeping industry. Therefore, native honey bee races and ecotypes, including particular differences, must be urgently identified and taken under protection to preserve gene pools of Anatolian honey bees. Otherwise, the biodiversity of the honey bee in Anatolia

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will face with losing the native gene pool due to the wrong beekeeping practices, queen bee transport, and migratory beekeeping [9,19,20,26,27,28,29,30]. In the Thrace region of Turkey, out of the native Carniolan honey bee race, Caucasican and Macedonian honey bee subspecies or their ecotypes were identified, as well as hybridization between Anatolian-Caucasian and Carniolan-Macedonian haplotypes based on restriction site patterns of mtDNA, sequencing, and microsatellite data [9,20]. Transporting of the queen bee of *A. m. caucasica* to the Thrace region poses significant threats to the conservation of native Thrace honey bee populations in their natural habitat. Genes from native populations and imported strains could have been constantly mixed, resulting in the extinction of native bee populations due to gene flow from migrated or imported stocks [9,19,20]

To date, many studies carried out to identified honey bee subspecies in Turkey by using morphometric and mtDNA analysis [4,5,6,7,9,14,15,16,19,28,31,32,33,34]. However, less microsatellite studies were carried on Anatolian honey bees [8,20]. Using microsatellites markers, we intended to add more information about their genetic variation, differentiation, and admixture. Microsatellites are important for population studies because they are conserved during evolutionary process and give more reliable results from other markers [35,36,37,38]. It is useful to determine population differentiation, gene flow, and hybridization between honey bee population [39,40,41,42,43,44,45]. In the present study, the microsatellites were used to investigate the genetic relationships of honey bee populations from Thrace (in the European part of Turkey) and Yığılca (in the Western Black Sea region of Turkey) where there exist two different honey bee species, *A. m. carnica* and *A. m. anatoliaca,* respectively [1,6,7,8,28,46]. However, recent studies have shown that migratory beekeeping and commercial queen bee predominant in Thrace region [9,20]. According to the results reported by Karabağ et. al., [20], there are alterations in the genetic structures of the populations in Kırklareli, where *A. m. caucasica* queen bees have been intensively sold. Considering the increasing importance of the conservation of native honey bees, this work aimed to determine further scientific investigation on the Thrace and Yığılca honey bee populations based on different microsatellites. It is expected that honey bee populations from two different geographic regions are differentiated naturally based on their genetic structure. It is also expected to find uncontrolled inbreeding, or controlled swarming could have been caused genetic diversity.

2. Materials and methods

2.1. Sampling

A total of 100 colonies (one individuals/per colony) were sampled from Tekirdağ (N=26), Kırklareli (N=14), Edirne (N=25) and Yığılca district of Düzce province (N=35), representing two different geographic regions of Turkey (Figure 1). Honey bee samples were collected from noncommercial beekeeping enterprises with a minimum genetic isolation distance of 20 km based on the maximum radius of the mating flight [40].

2.2. Molecular analysis

2.2.1. Microsatellite analyses

Total genomic DNA was extracted according to the protocol of DNA Kit: Tissue & Bacterial DNA purification cat (cat no: E3551-01 Lot No: F/240918).

Four multiplex PCR reactions were used to amplified Twenty-seven microsatellite loci. Multiplex 1: A014, A024, A088, AC088, AC139, AC306, Ap274, Ap015, A029, A043; multiplex 2: A079, A113, AT163, AT188;

Figure 1. Geographic distribution of the sampling area: Edirne, Kırklareli and Tekirdağ located in the Thrace part of Turkey, Yığılca district of Düzce Province located in the Western Black Sea of Turkey.

multiplex 3: A008, Ap068, AC011, Ap226; multiplex 4: Ap085, Ap090, Ap223, Ap224, Ap273, Ap238, Ap249, AT005, Ap288–(Supplementary material Table S1). PCR reactions were performed in a thermocycler in a total volume of 25 µL containing 2.5 µL 1X reaction buffer, 1 µL dNTP mixture (10 mM), 1 µL primer (10 pmol/µL), 1.5 unit DNA polymerase, 1 µL extracted DNA (100 ng), and $MgCl_2$ (25 mM), and the concentration was adjusted to 1.0–1.5 mM for the loci. The PCR conditions for all reactions were at initial denaturation at 95 °C for 7 min, 30 cycles denaturation at 95 °C for 30 s, annealing at 55 °C (multiplex 1), 60 °C (multiplex 2), 58 °C (multiplex 3) and 49 °C (multiplex 4) for 30 s, extension at 72 °C for 30 s and final extension for 6 min at 72 °C. The amplifications were performed with fluorescent dye-labeled primers, and the lengths of the 27 different microsatellite loci were observed on an automatic DNA sequencer (ABI 3700 Applied Biosystems).

2.2.2. Statistical analyses

GENALEX 6.3 Microsoft Office Excel macro software [47] was used to determine the number of average alleles per locus (Na), the mean number of effective alleles (Ne), observed heterozygosity (Ho) and expected (He) heterozygosity, inbreeding coefficients (Fıs and Fıt) and fixation indices (Fst) of per population, as well as the allelic richness and private alleles. With ARLEQUIN v3.1, genetic differences between honey bee populations were calculated from population pairwise genetic differentiation and analysis of molecular variance (AMOVA) [48]. GENEPOP v.4.0 version was used to estimate linkage disequilibrium between all possible pairs of loci, pairwise Fst value, and Fisher exact test for conformity to Hardy Weinberg performed by Markov chain method [49]. STRUCTURE v2.3.4 software was used to assess the structure among populations [50]. For the estimation, the admixture model and associated allele frequencies were used to assign individuals to populations. The Markov Chain Monte Carlo iterations with a burn-in period of 500,000 run were performed to estimate the most probable K (number of clusters). To decide the most likely value of K, the algorithm of Evanno et al. [51] was used (http://taylor0.biology.ucla.edu/structureHarvester/) [52]. The CLUMPP program was used to implement the clustering pattern, which was then visualized using DISTRUCT software, version 1.1 [53].

GENETIX 4.02 [54] was used to construct a factorial correspondence analysis (FCA). Relationships among individuals reconstructed using neighbor-Joining method on a matrix of allele sharing distances (ASD) with bootstrap values were generated using the Nei et al.'s Da distances (1983) method [55]. This was done using programs from the POPULATION 1.2.28 software (https://bioinformatics.org/populations/) [56]. Phylogenetic relationships of the population were determined by neighbour-joining method. NJ tree was drawn based on allele sharing distances (ASD) between the individuals [57]

3. Results

All of the 27 microsatellite loci, except Ap274, were found polymorphic in all honey bee populations from Kırklareli, Edirne, Tekirdağ, and Yığılca. The numbers of alleles in the studied populations ranged from 1 (AP274) to 16 (A029). The mean number of alleles for each population (Edirne, Tekirdağ, Kırklareli and Yığılca) were 5.25, 5.29, 3.96, and 5.14, respectively (Table 1).

Private alleles were scored for all honey bee populations. A total of 32 private alleles were observed. In the Kırklareli and Yığılca honey bee populations, 8 of these private alleles have frequencies of over 5 % (Table 2).

The He and Ho for each population and locus were shown in Table 3. The expected heterozygosities for each locus varied between 0.00 (AP274) and 0.9045 (A029) and the observed heterozygoties between 0.000 (AP274) and 1.00 (A029). A higher number of observed heterozygosity was found in the Edirne and Tekirdağ populations and a lower number of observed heterozygosity in the Kırklareli population (Table 3).

It was shown highly significant deviations from Hardy–Weinberg disequilibrium in 13 loci (A008, A014, A079, AP085, AP224, AP226; A029, A113; A008, A088, AP085, AP288, AT005) out of 108 population-locus combinations ($p < 0.05$).

Microsatellite pairwise Fst values were calculated between populations from Yığılca in Western Black Sea and Edirne, Tekirdağ and Kırklareli in Thrace region to determine the genetically differentiated populations. Accordingly, pairwise Fst values varied between 0.012 (Edirne-Tekirdağ) and 0.14 (Kırklareli-Yığılca). As the pairwise Fst levels were lower than 0.05 for Tekirdağ-Edirne and Tekirdağ-Kırklareli, no significant differences were determined between these pairwise populations. Both the Fst estimates and Ne'i genetic distance revealed weak differentiation among the Thrace populations and moderate level differentiation exist between Kırklareli and Yığılca (Table 4).

The structure of each population was calculated based on admixture levels for each honey bee individual. The population's admixture level was expressed by mixed colors with proportional lengths. After correction based on Evanno et al. [51], the optimum number of clusters in the STRUCTURE simulations with the four sample sets was found as three. (K = 3, mean LnP(K) = 5492.49, Delta K = 4.111). The highest Delta K value was determined at K = 3 indicating the optimal number of genetic clusters (Figure 2).

Table 1. Number of alleles and avarage number of alleles per locus in honey bee population.

Microsatellite loci	**Edirne** (N = 25)	**Tekirdağ** (N = 26)	**Kırklareli** (N = 14)	**Düzce (Yığılca)** (N = 35)	**Total allel number** (N = 100)
A008	7	5	4	5	7
A014	4	3	3	3	4
A024	5	4	3	5	5
A029	15	16	8	14	19
A043	4	4	3	4	5
A079	6	6	5	5	7
A088	5	7	5	6	7
A113	11	10	7	13	14
AC011	5	5	4	7	8
AC088	3	4	3	3	7
AC139	5	6	4	5	6
AC306	5	4	4	4	5
AP015	2	3	2	4	4
AP068	6	7	5	4	7
AP085	5	7	4	5	8
AP090	6	6	3	5	7
AP223	5	5	4	4	6
AP224	6	6	4	5	6
AP226	4	4	4	3	5
AP238	2	2	3	2	3
AP249	7	5	6	5	8
AP273	2	3	3	3	4
AP274	1	1	1	0	2
AP288	5	4	3	5	5
AT005	3	3	2	4	4
AT163	2	3	2	3	3
AT188	11	10	8	11	13
Total	142	143	106	138	179
Mean/pop	5.25	5.29	3.96	5.07	6.63

Factorial correspondence analyses (FCA) of all colonies plotted demonstrated that the populations were separated into three groups. The FCA plot showed the Kırklareli bees to be slightly distinct from the other Thrace population. Yığılca was well separated from the Kırklareli honey bee population (Figure 3).

Although genetic interaction was observed among all colonies from Thrace (Edirne, Tekirdağ and Kırklareli) and the Western Black Sea (Yığılca district of Düzce province), Neighbor-Joining (NJ) tree [57], based on Allele Sharing Distances (ASD) between the individuals, slightly grouped the entire collection of honey bee samples into two main groups. The first group consisted of colonies from the Thrace region, while the second group generally included the Western Black Sea samples (Figure 4).

4. Discussion

In the current study, the honey bee populations of Thrace (Edirne, Tekirdağ and Kırklareli) and Yığılca in the Western Black Sea region of Anatolia were studied in detail using 27 microsatellites. In terms of genetic diversity, mean heterozygosities per locus and over loci for all the studied populations were ranged from 0.492 to 0.551. Our values (He) are similar to those values found for C lineage (from 0.478 to 0.529), lower than those for A and O lineage and higher than M lineage value [58]. Our

Table 2. Private alleles, allele size (in base pairs) and allele frequencies per locus for 4 populations are shown in each column (E: Edirne, K: Kırklareli, T: Tekirdağ, Y:Yığılca).

Pop	Locus	Allele	Freq	Pop	Locus	Allele	Freq
E	A008	165	0.025	**T**	AC011	121	0.021
E	A008	171	0.025	**T**	AC088	217	0.021
E	A014	116	0.022	**T**	AC088	230	0.021
E	A079	109	0.024	**T**	AC139	339	0.021
E	A113	212	0.043	**T**	AP090	147	0.021
E	AC088	216	0.022	**T**	A079	113	0.021
E	AC306	171	0.022	**T**	AP273	102	0.043
E	AP085	208	0.024	**Y**	AT188	207	0.038
E	AP249	207	0.024	**Y**	AP015	210	0.036
E	AT188	203	0.024	**Y**	A043	143	0.025
E	AP223	177	0.023	**Y**	AC088	222	0.025
K	A029	187	0.167*	**Y**	AC088	210	0.075*
K	AP226	233	0.125*	**Y**	A113	218	0.056*
K	AP238	266	0.125*	**Y**	AP273	108	0.091*
K	AP249	211	0.125*	**Y**	AT005	268	0.077*
T	AP223	185	0.053*	**Y**	AP274	112	0.025

E: Edirne, K: Kırklareli, T: Tekirdağ, Y:Düzce (Yığılca) *Allel frequency is over 0.05.

values for the heterozygosity of Thrace populations were higher than those as seen in previous studies, Kırklareli by Tunca [59] (He = 0.483–0.331) and Kükrer [60] (He = 0.448) but lower than those of Bodur et al. [8] (He = 0.611) and Karabağ et al. [20] (He=0.80). The number of alleles (Na) per locus found in this study was higher than those reported by Tunca [59] and Kükrer [60] for Edirne and Tekirdağ in contrast to that Kırklareli honey bees was characterized by the low Na value (3.96). This low value observed in Kırklareli might have been due to the small sample size (n = 14), although the mean number of alleles (3.96) was similar to that found in other populations with higher sample sizes [59,40,42]. Other explanation for this result may be due to swarm colonies from a stock.

The pairwise Fst value of the current study showed that Yığılca samples well separated from Kırklareli but slightly distinguished from other Thrace populations. Yığılca is the ecotype of *A. m. anatoliaca* confined in Northwest Anatolia and genetically different from *A. m. caucasica* and *A. m. carnica* [14,15,16,19,20,34,61]. According to NJ tree and structure analysis, honey bee samples from Yığılca and Thrace were slightly formed as two separete groups. Moreover, there is no special branchs among them and no clear subdivision within the Thrace populations, which were visible as a single population on structure graph. However, the distinction of the Thrace population was much more apparent in the FCA graphic. Here, Kırklareli honey bee population appeared separate from the bees in the other locations of Thrace (Edirne and Tekirdağ) and Düzce/Yığılca.

Pairwise Fst values were estimated by Bodur et al. [8] as 0.12 between Kırklareli and Artvin honey bee populations; Artvin is in the Eastern Black Sea region and naturally including *A. m. caucasica* subspecies. In the last study, it was surprising that the results of pairwise Fst (0.04) and structure analyses of individuals appeared that there are no differences between Kırklareli and Artvin populations [20]. The pairwise Fst (0.14) of the present study for Kırklareli and Yığılca was similar to those estimated by Bodur et. al., [8]. This may be indicated that there are two different subspecies in Kırklareli and Yığılca [4,6,7,11,28], or other explanations for these results is due to the effect of using nonnative commercial queen bee in Kırklareli as reported by Ünal and Özdil [9] and Karabağ et. al., [20] based on the mtDNA and microsatellite analyses respectively.

There are contradictions with regard to genetic diversity of Turkish honey bees. Some previous studies indicated that Thrace populations were similar to Anatolian honey bees [1,5,33,62]. Bodur et al. [8] showed that Kırklareli honey bees differentiate from the rest of Turkey and have similarity to the *A. m. carnica.* Ünal and Özdil [9] demonstrated that *A. m. caucasica, A. m. carnica* and *A. m. anatoliaca* or their hybrids have been in Thrace region. Moreover, Karabağ et. al., [20] announced that Kırklareli

Table 3. Observed heterozigoties (H_O) Expected heterozigoties (H_e) for per microsatellite locus in honey bee (*Apis mellifera L.*) populations from the Thrace and Western Black Sea Region.

	Edirne (n = 25)		**Tekirdağ (n = 26)**		**Kırklareli (n = 14)**		**Düzce (Yığılca) (n = 35)**	
LOCUS	**Ho**	**He**	**Ho**	**He**	**Ho**	**He**	**Ho**	**He**
A008	0.678	0.662	0.692	0.678	0.678	0.593	0.468	0.461
A014	0.485	0.476	0.449	0.440	0.535	0.468	0.111	0.109
A024	0.618	0.604	0.498	0.488	0.535	0.468	0.566	0.558
A029	0.897	0.879	0.924	0.904	1.000	0.833	0.871	0.858
A043	0.387	0.379	0.279	0.274	0.250	0.218	0.457	0.451
A079	0.739	0.723	0.748	0.733	0.821	0.718	0.657	0.647
A088	0.726	0.710	0.747	0.732	0.866	0.722	0.589	0.580
A113	0.809	0.793	0.799	0.783	0.428	0.375	0.856	0.843
AC011	0.578	0.566	0.466	0.457	0.821	0.718	0.272	0.268
AC088	0.456	0.447	0.546	0.536	0.250	0.218	0.455	0.449
AC139	0.571	0.558	0.654	0.642	0.678	0.593	0.553	0.545
AC306	0.666	0.653	0.711	0.698	0.607	0.531	0.667	0.657
AP015	0.043	0.042	0.076	0.074	0.428	0.375	0.137	0.135
AP068	0.678	0.663	0.793	0.776	0.750	0.656	0.540	0.532
AP085	0.407	0.398	0.505	0.494	0.464	0.406	0.337	0.332
AP090	0.608	0.608	0.559	0.559	0.500	0.500	0.660	0.660
AP223	0.716	0.701	0.702	0.688	0.571	0.500	0.669	0.660
AP224	0.542	0.531	0.482	0.473	0.464	0.406	0.468	0.462
AP226	0.457	0.448	0.537	0.526	0.785	0.687	0.110	0.109
AP238	0.463	0.453	0.506	0.496	0.464	0.406	0.420	0.420
AP249	0.676	0.661	0.671	0.657	0.515	0.508	0.933	0.777
AP273	0.360	0.353	0.388	0.380	0.250	0.218	0.526	0.517
AP274	0.000	0.000	0.000	0.000	0.000	0.000	0.028	0.028
AP288	0.578	0.566	0.490	0.480	0.571	0.500	0.510	0.500
AT005	0.664	0.650	0.590	0.577	0.428	0.375	0.655	0.640
AT163	0.480	0.480	0.503	0.492	0.250	0.218	0.48	0.477
AT188	0.897	0.874	0.857	0.839	0.928	0.812	0.891	0.878
0rt/pop	0.562	0.551	0.562	0.551	0.445	0.492	0.499	0.492

Table 4. Pairwise Fst values between pairs of honey bee populations from the Thrace and the Western Black Sea F_{ST} (above main diagnol) and Genetic distance [Nei, 1996] (below main diagnol). NS: nonsignificant.

	Edirne	Tekirdağ	Düzce (Yığılca)	Kırklareli
Edirne	-	0.012 ^{NS}	0.055***	0.054**
Tekirdağ	0.043*	-	0.052***	0.015^{NS}
Düzce (Yığılca)	0.058***	0.077***	-	0.140***
Kırklareli	0.175***	0.116***	0.274***	-

* p > 0.05, ** p > 0.01, ***p > 0.001

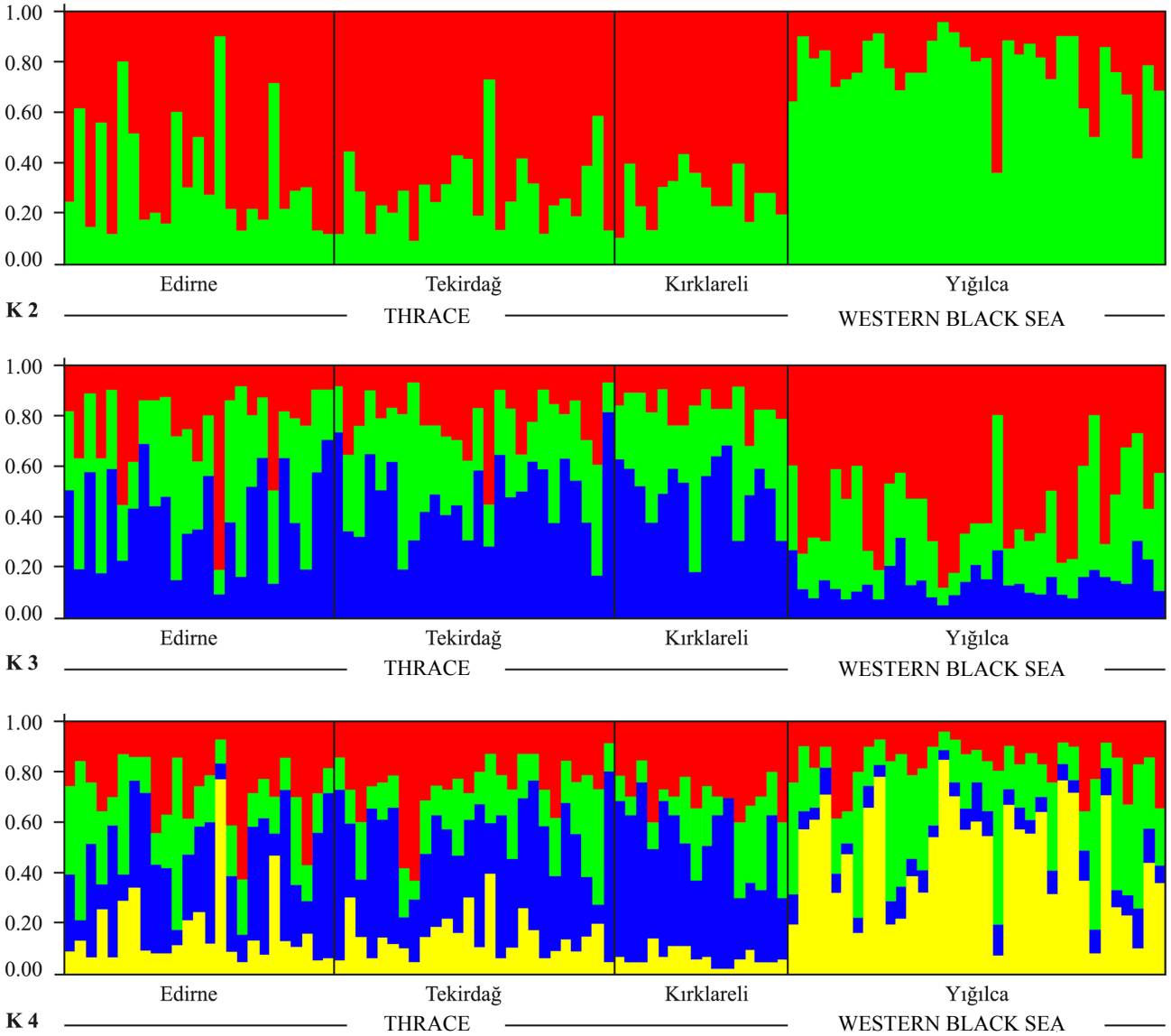

Figure 2. STRUCTURE analysis based on 27 microsatellite data of all Thrace honey bee populations and the Western Black Sea honey bee population from Turkey. According to Evanno et al. [2005], the most probable number of clusters (K =3) was estimated.

population close to Artvin population of *A. m. caucasica*. On the other hand, Tozkar [19] claimed that mtDNA analysis did not support the genetic distinctiveness of Kırklareli honey bees. Düzce/Yığılca honey bee is ecotype mostly shared similarity with *A. m. anatoliaca* haplotypes from Aegean and Central Anatolia [14,15,16,19,20,34]. According to mtDNA analysis, Kırklareli and Düzce/Yığılca ecotype had the C12 haplotype of *A. m. anatoliaca,* which was the most common mitotype acknowledged as basal ancestral mitotype, which was found predominatly through Anatolia [19,61]. Furthermore, some mtDNA researches show that identical genetic information for Thrace honey bees have similarity with *A. m. macedonica, A. m. carnica, A. m. caucasica*. It means that Thrace ecotype also carried mixed genetic information of different subspecies aside from *A. m. anatoliaca* [9].

The results of the current study appeared that the admixture in the managed populations such as Kırklareli does not necessarily lead to an increase in genetic diversity. These results occured in Kırklareli might be attributed to partial reproductive isolation among native honey bee colonies. High Fıs value and lower heterozygosity in Kırklareli population suggested that heterozygous deficiency in the pooled population may have due to the Wahlund effect leading to subpopulation occurence [38,42,63]. The existence of subpopulations within Kırklareli may be the result of at least two factors, the existence of foreigner queen bee purchased or the

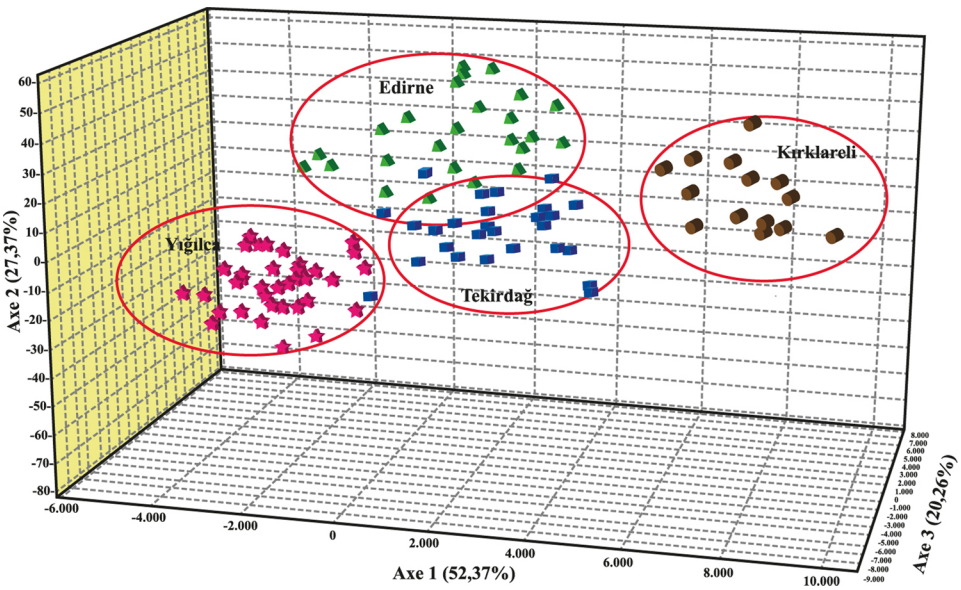

Figure 3. Distribution of four populations by factorial correspondence analysis (FCA) based on 27 microsatellite loci.

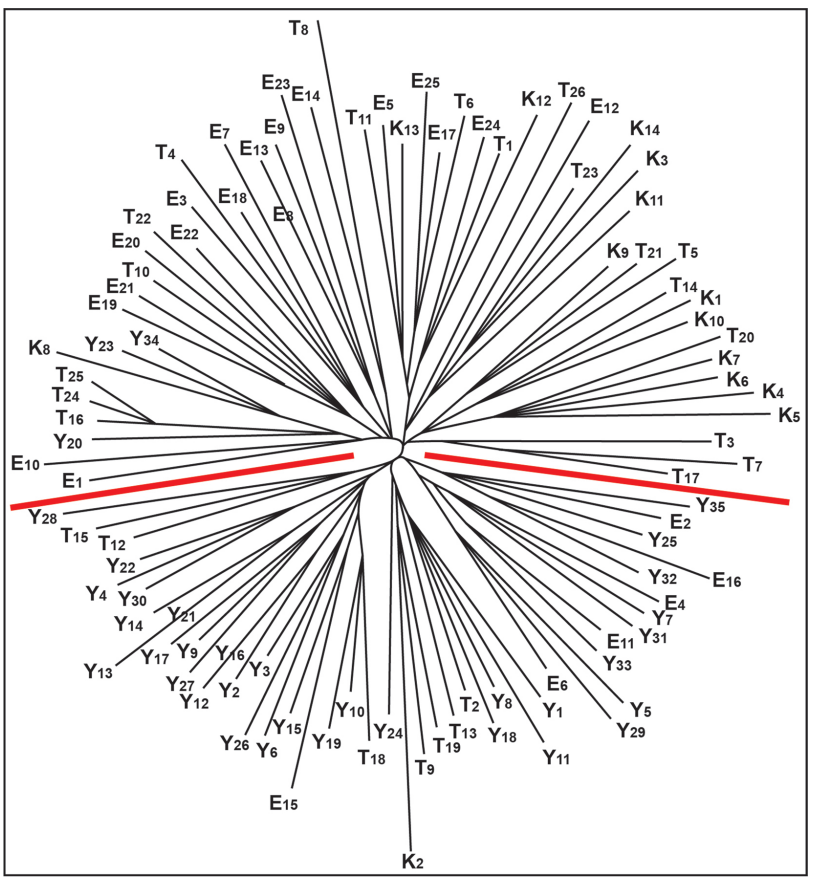

Figure 4. NJ tree based on Allele Sharing Distances (ASD) between the individuals [Nei, 1996] Phylogenetic relationships of the Thrace (Kırklareli: K, Edirne: E, Tekirdağ: T) and the Western Black Sea (Düzce/Yığılca: Y) honey bee individuals based on the neighbour-joining method.

propagation of selected colonies by the beekeepers through repeated and controlled swarming. Subsequent factor may have contribute to the observed heterozygote deficiency throughout Kırklareli. According to personal communication with local beekeepers, the conservation program was prompted, and no imported honey bees had been introduced to Kırklareli area, since 2010 [1]. As a result, while Thrace populations were not fully separated from each other, Yığılca population was significantly separated from Kırklareli and slightly from the rest of Thrace populations. Our findings provided valuable information that can be used for the effective management of the native honey bees in Turkey. This is an important study as it will provide the proof for the legal conservation protocol to address the identification of these populations.

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