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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Genetic Diversity of Kırklareli Honey Bee (*Apis mellifera* L.) Populations in Thrace Region of Turkey: Identification of Mitochondrial COI and ND5 Gene Regions*

Türkiye'nin Trakya Bölgesi Kırklareli Bal Arısı (*Apis mellifera* L.) Populasyonlarında Genetik Çeşitlilik: Mitokondriyel COI ve ND5 Gen Bölgelerinin İncelenmesi

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Abstract

The main goal of genetic resource conservation is to keep as much genetic diversity as possible within each species. In this respect, some difficulties in the protection of honey bee gene resources make it necessary to reveal the genetic structures of the subspecies and the genetic relationships between the subspecies. In this study, Kırklareli honey bee populations which were officially registered as an ecotype of Turkey's honey bee (*Apis mellifera* L.) gene resources by the Republic of Turkey, Ministry of Agriculture and Forestry, were examined in the COI and ND5 genes of mitochondrial DNA. The restriction fragment length polymorphism (RFLP) together with the polymerase chain reaction (PCR) was used to define *Apis mellifera* populations. A total of 117 worker bee samples were used which were collected from mostly the Kırklareli province. A newly found single nucleotide polymorphism (SNP), G→A transition in the COI gene region formed a novel *Nco*I restriction site resulting in a new haplotype. This new haplotype has been abbreviated as haplotype C. As a result of the COI/*Ssp*I digestion, the previously reported C haplotype was determined. No restriction was found with the treatment of COI/*Sty*I enzyme. On the other hand, as a result of ND5/*Alu*I restriction, 2 restriction site and previously reported haplotype C was obtained in all of the studied samples. No restriction was screened with ND5/*Fok*I and ND5/*Hinc*II enzymes in the whole samples, only a reported uncut B haplotype was observed. Within this study, novel genetic information has been revealed for the Kırklareli honey bee ecotype registered as the Thrace honey bee of Turkey's honey bee gene resources. Moreover, detailed studies with larger sample sizes should be conducted to characterize the origin and the subspecies of Kırklareli honey bees in detail. It is thought that this study will be useful in the identification and registration of the Kırklareli honey bees to be carried out in the future, and also in the creation of a database.

Keywords: *Apis mellifera*, COI gene, ND5 gene, Kırklareli honey bee, RFLP technique

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Öz

Genetik kaynakları korumanın temel amacı, her bir tür içinde mümkün olduğu kadar çok genetik çeşitliliği korumaktır. Bu açıdan bal arısı gen kaynaklarının korunmasındaki bazı güçlükler, alt türlerin genetik yapılarının ve alt türler arasındaki genetik ilişkilerin ortaya çıkarılmasını zorunlu kılmaktadır. Bu araştırmada, T.C. Tarım ve Orman Bakanlığı tarafından Trakya ekotipi olarak tescil edilen Kırklareli bal arısı populasyonlarını temsil eden bal arıları mitokondriyel genomda COI ve ND5 gen bölgelerinde farklı restriksiyon enzimleri ile incelenmiştir. *Apis mellifera* populasyonlarının tanımlanmasında restriksiyon parça uzunluk polimorfizmi (RFLP) yöntemi, polimeraz zincir reaksiyonu (PCR) ile birlikte çalışılmıştır. Ağırlıklı olarak Kırklareli ilinden örneklenen 117 adet işçi arı örneği materyal olarak kullanılmıştır. COI/*Nco*I kesiminde G→A transizyonu sonucu yeni bir kesim noktası ve ilk kez bu çalışmada bildirilen yeni bir haplotip elde edilmiştir. Bu haplotip daha önce bildirilen haplotipleri takip ederek C haplotipi olarak adlandırılmıştır. COI/*Ssp*I kesim sonucu olarak, birden fazla kesim noktası ve önceki çalışmalarda bildirilen C haplotipi elde edilmiş; COI/*Sty*I enzimi ile herhangi bir kesim noktası bulunamamıştır. Öte yandan, ND5/*Alu*I kesimi sonucunda, incelenen tüm örneklerde 2 kesim noktası ve daha önce bildirilen haplotip C elde edilmiştir. ND5/*Fok*I ve ND5/*Hinc*II enzimleri ile incelenen tüm populasyonda kesim tespit edilememiş ve tek bir bant profili sonucu B haplotipi görülmüştür. Bu çalışma ile Türkiye bal arısı gen kaynaklarından Trakya bal arısı olarak tescil edilen Kırklareli bal arısı ekotipi için yeni genetik bilgiler ortaya konmuş olup, Kırklareli bal arılarının orijin ve alttürlerinin detaylı olarak karakterize edilebilmesi için daha geniş örnek büyüklüğü ile detaylı çalışmalar yapılmalıdır. Bundan sonra yapılacak olan Kırklareli bal arısının tanımlanmasında ve tescil çalışmalarında ve ayrıca veri tabanı oluşturulmasında bu çalışmanın faydalı olacağı düşünülmektedir.

Anahtar sözcükler: *Apis mellifera*, COI geni, ND5 geni, Kırklareli bal arısı, RFLP tekniği

1. Introduction

Beekeeping has been one of the most effective production activities in Anatolia since ancient times. It has been reported that honey bees developed by spreading firstly in Europe, Africa, and the Near East until the 17th century, and after the 17th century, it was carried by immigrants, and beekeeping was carried out in all settlement areas (Fıratlı, 1988). While it was used to produce only honey to meet family needs, it has now become a commercial line of business. It is known that beekeeping is an activity that is more dependent on nature than other agricultural lines, and Türkiye is in an advantageous position for beekeeping with its climate pattern and rich flora.

It is known that the western honey bee (*Apis mellifera* L., 1758), (Hymenoptera: Apidae), has spread to all parts of the world due to its high adaptability and has adapted to the ecological conditions of the regions where it is found. A large number of geographical races have formed different ecotypes within these races. Adam (1983) reported that Turkey, due to its different climatic characteristics, is naturally a transition point between Africa, Europe, and Asia, and therefore it is a major gene pool that contains many bee races and ecotypes. Turkey has been one of the most important countries in beekeeping not only in terms of its natural riches but also because of its bee gene resources. Previous studies defined 27 subspecies of *Apis mellifera* based on mostly morphometric characters, and finally 29 *Apis mellifera* races (subspecies) identified so far with the help of molecular techniques. Considering this geography, it was concluded that Anatolian (*Apis mellifera anatoliaca* Maa, 1953), Caucasian (*Apis mellifera caucasica* Gorbachev, 1916), Iranian (*Apis mellifera meda* Skorikov, 1929) and Syrian bees (*Apis mellifera syriaca* Buttel-Reepen, 1906) were found in Turkey. The existence of the fifth honey bee subspecies in the Thrace region according to morphological (Güler et al., 2010) and genetic marker studies was also reported (Smith et al., 1997; Palmer et al., 2000; Kekeçoğlu et al., 2007, 2009; Özdil et al., 2009, 2022; Ünal and Özdil, 2018).

It is known that there has been an increase in the number of nomadic beekeepers in Turkey recently. For this reason, gene flow may increase between honey bee populations and uncontrollable genetic hybridizations may occur. Identification of the morphological and genetic variation in the honey bee subspecies and ecotypes, such as Thrace, Yığılca, Muğla, etc. ecotypes plays an important role in the formation of honey bee populations (Güler et al., 2010; Kekeçoğlu et al., 2007, 2009; Özdil et al., 2009, 2022; Güder et al., 2017; Gür et al., 2018; Ünal and Özdil, 2018).

In this study, in order to determine the genetic structure of honey bees in mostly Kırklareli and also in Tekirdağ provinces, possible mtDNA variations of COI and ND5 genes were analyzed and genetic markers were tried to be determined. *Nco*I, *Ssp*I, and *Sty*I restriction enzymes in the COI gene and *Alu*I, *Fok*I, and *Hinc*II restriction enzymes in the ND5 gene were studied. Thrace honey bee populations were considered as a different ecotype in Türkiye's honey bee gene resources and registered no need by the Republic of Türkiye, Ministry of Agriculture and Forestry (Anonymous, 2020). With this study, novel information has been revealed and updated for the ecotype registered as the Thrace bee.

2. Materials and Methods

2.1. Sample collection

In this study, a total of 117 DNA samples, representing the Thrace region, from mostly Kırklareli (107 samples) and Tekirdağ (10 samples) honey bee populations were examined.

2.2. Selection of genomic DNA samples

Genomic DNA isolation was previously carried out within the scope of the Tübitak 3001 Research Project. In this study, the honey bee DNA samples were selected according to the quantity and quality of the DNAs, samples were both checked on 1% agarose gels and controlled on a UV spectrophotometer.

Samples with good DNA quantity and quality were selected and used in this study. After checking the purity of DNA samples, PCR (Polymerase Chain Reaction) optimization was performed to amplify the targeted mitochondrial regions. All the analyses were performed in Molecular Genetics Laboratory in Tekirdağ Namık Kemal University in 2021.

2.3. PCR amplification of mitochondrial DNA COI and ND5 regions

PCRs were performed to amplify the 1028 bp of the COI (between the 2095-3123th mtDNA nucleotide sequence) and the 822 bp of the ND5 (between the 7395-8217th mtDNA nucleotide sequence) gene regions. COI (*Nco*I, *Ssp*I, *Sty*I) and ND5 (*Alu*I, *Fok*I, *Hinc*II) gene regions were amplified with the primers and digested with the restriction enzymes given in *Table 1*.

Table 1. Primers, restriction enzymes and the references used in this study

mtDNA Region	Primers (5'→3')	Enzymes	References
COI (1028 bp)		*Nco*I	Bouga et al., 2005
COI−*F	GATTACTTCCTCCCTCATTA	*Ssp*I	Stevanoviç et al., 2010
COI−*R	AATCTGGATAGTCTGAATAA	*Sty*I	Meixner et al., 2013
ND5 (822 bp)		*Alu*I	Bouga et al., 2005
ND5−*F	TCGAAATGAATAGGATACAG	*Fok*I-*Bts*CI	Ivanova et al., 2010
ND5−*R	GGTTGAGATGGTTTAGGATT	*Hinc*II	Meixner et al., 2013

Primers that were used to amplify the COI and ND5 gene regions, were designed based on the honey bee whole genome reference sequence (Access No: NC-001566) available in the NCBI GenBank database. PCR reactions for the amplification of COI and ND5 gene regions; prepared as 40 µl mixture, 20 ng genomic DNA, 0.5 µM of primer, 10X PCR buffer ($MgCl_2$), 2 mM dNTP, and 1U Taq DNA Polymerase.

The PCR cycling conditions were 94ºC for an initial denaturation for 5 min; 35 cycles of 94ºC for 1 min denaturation, 1,5 min at the primer annealing temperature, and 72ºC for 2 min extension; and a final 72ºC for 15 min. 1028 bp of the COI and 822 bp of the ND5 PCR products were digested with *Nco*I, *Ssp*I, *Sty*I, and *Alu*I, and *Fok*I and *Hinc*II (ER0571, ER0771, ER0411, ER0011, ER0871, ER0491, Thermo Fisher Scientific), respectively. The restricted products were controlled on 2% agarose gels.

3. Results and Discussion

In this study, mtDNA variations in COI and ND5 genes were determined and the genetic markers were tried to be presented. *Nco*I, *Ssp*I, and *Sty*I restriction enzymes were studied in the COI gene, and *Alu*I, *Fok*I, and *Hinc*II restriction enzymes were studied in the ND5 gene, and previously reported and newly found restriction sites were revealed.

3.1. Amplification of COI gene region and RFLP results

The COI region was amplified by PCR using the primers given in *Table 1* and 1028 bp PCR products were obtained. *Nco*I, *Ssp*I, and *Sty*I restriction enzymes were used to detect variation in this gene region.

After digesting the COI gene with the *Nco*I (ER0571 Thermo Fisher Scientific) restriction enzyme, a novel restriction site was formed as a result of a new SNP (G→A transition) at position 2345, and a new *Nco*I restriction site formed a profile of 246 and 782 bp on the gel (*Figure 1*). This newly found profile was abbreviated as the C haplotype in addition to the haplotypes reported previously (Bouga et al., 2005, Özdil et al., 2012). This haplotype was found in 2 out of 10 samples, belonging to the Tekirdağ region, and 12 samples of the Kırklareli population (10.26%). No restriction site was found in all the other studied samples and haplotype B (1028 bp) was found.

Cleavage of the COI gene region with the *Ssp*I (ER0771 Thermo Fisher Scientific) restriction enzyme revealed 4 restriction sites and 523, 213, 175, 85, and 32 bp bands on the gel (*Figure 2a*). This profile was previously reported as the C haplotype (Özdil et al., 2012). C haplotype was found in all of the studied samples.

As a result of the restriction of the COI gene with the *Sty*I (ER0411 Thermo Fisher Scientific) enzyme, two haplotypes were found in the studied populations. No digestion was seen in most of the samples resulting the haplotype A, which was reported previously, on the other hand, a point mutation at position 2150 (G→A transition) changed the restriction site to CCWWG**A** from CCWWG**G**, and 628 and 400 (*Figure 2b*) bp was obtained on the gel as a result of a single cut, and this profile was abbreviated as the B haplotype (Bouga et al., 2005).

B haplotype was determined in 52 (44.4%) of the Kırklareli samples studied. No restriction site was found in the remaining Kırklareli and Tekirdağ samples, only A haplotype (55.6%) was found.

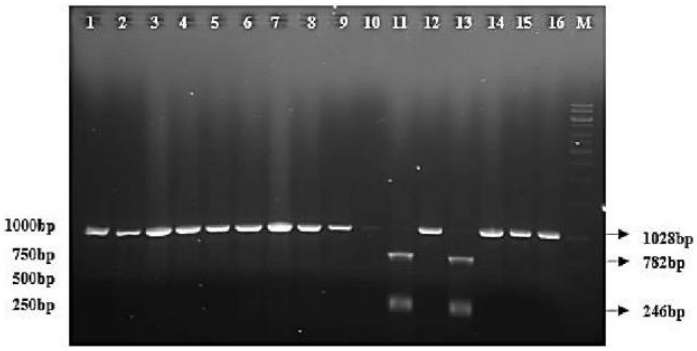

Figure 1. Cleavage of the COI gene region with the NcoI restriction enzyme on 2% agarose gel electrophoresis (1-10, 12, 14-16: B haplotype (1028 bp-PCR product); 11 and 13: C haplotype (782-246 bp); M: Fermentas GeneRuler^{TM} 1kb DNA Ladder.

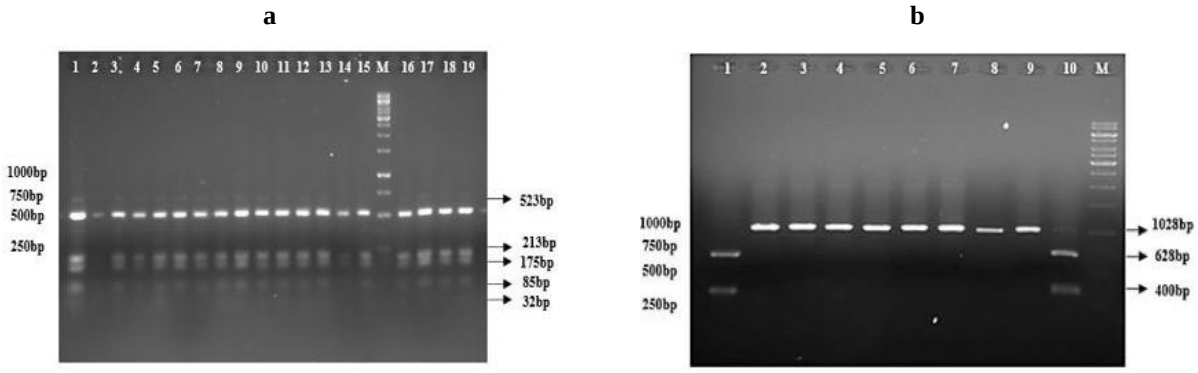

Figure 2. Cleavage of the COI gene region with the SspI (a) and StyI (b) restriction enzymes on 2% agarose gel electrophoresis.* (a) *1-19: C haplotype (523-213-175-85-35 bp); M: Fermentas GeneRuler^{TM} 1kb DNA Ladder.* (b) *2-9: A haplotype (1028 bp); 1 and 10: B haplotype (628-400 bp); M: Fermentas GeneRuler^{TM} 1kb DNA Ladder.

The cleavage of the COI gene region in different honey bee populations was performed in the previous studies. For example; in honey bee populations native to Greece (*Apis mellifera cecropia* Kieseweiter, 1860) and Cyprus (*Apis mellifera cypria* Pollman, 1879), Bouga et al. (2005) reported a different *Nco*I cleavage in this gene which is different from our study and abbreviated this haplotype as A (595 and 433 bp). In this study, A haplotype was not detected but a novel restriction was reported as the C haplotype. Kekeçoğlu et al. (2007) reported only the B haplotype (1028 bp-uncut) in the honey bee populations of Türkiye in the same gene region. And also, Ivanova et al. (2010) and Stevanovic et al. (2010), reported that Bulgaria, Serbia, Bosnia-Herzegovina (*Apis mellifera carnica* Pollman, 1879), and Macedonia (*Apis mellifera macedonica* Ruttner, 1988) honey bee populations, did not have the recognition site of the *Nco*I enzyme in the COI gene, only the B haplotype (1028 bp-uncut) was found in these populations.

In cleavage of the COI gene region with the *Ssp*I enzyme, different restriction patterns were reported as A haplotype (487-277-264 bp) (Bouga et al., 2005) and B haplotype (580, 439 bp) (Kekeçoğlu et al., 2007) but in our study, only C haplotype (523-213-175-85-32 bp) which were reported previously (Özdil et al., 2012) was found in all of the studied populations. Our results were found in accordance with Özdil et al. (2012). Ivanova et al., (2010) reported that the *Ssp*I and *Sty*I enzymes did not have a recognition site and a single PCR product (1028 bp) occurred in Bulgarian honey bee populations.

The digestion of the COI gene region with the *Sty*I enzyme revealed two different haplotypes, the A haplotype (1028 bp-uncut) (Ivanova et al., 2010; Stevanovic et al., 2010), and the haplotype B (626, 402) in honey bee populations.

It was emphasized that diagnostic patterns were revealed in the Macedonian populations after the digestion of the COI gene segment with the restriction enzymes *Nco*I (haplotype A) and S*ty*I (haplotype B) (Bouga et al., 2005). In this study, the B haplotype was determined in 52 of the Kırklareli samples out of the 117 samples which can be the result of the Macedonian origin.

3.1. Amplification of ND5 gene region and RFLP results

The 822 bp of the ND5 gene region was amplified by PCR using the primers in *Table 1*, and *AluI*, *Fok*I (BtsCI), and *Hinc*II restriction enzymes were used to detect variation in this gene region. Only *Alu*I digestion resulted in a restriction site, the remaining enzymes have no restriction sites in the ND5 gene region of the studied honey bee populations.

In this study, cleavage of the ND5 gene region with the *Alu*I (ER0011 Thermo Fisher Scientific) restriction enzyme revealed two restriction sites, and a profile consisting of 3 bands of 554, 211, and 57 bp in all of the studied samples (*Figure 3*) which were reported as the C haplotype (Özdil et al., 2012). The 57 bp long band formed a faint band in the gel, and cannot be seen properly. Our results were found similar to Özdil et al. (2012). Bouga et al. (2005) reported 2 haplotypes (A: 554, 268 and B: 554, 171 and 97 bp long) which had different cleavage sites compared to the haplotype found in our study.

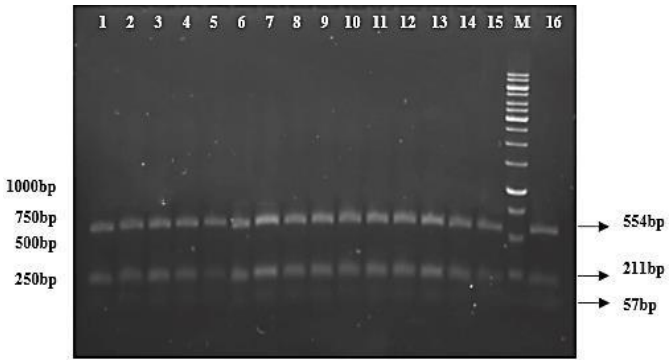

Figure 3. Cleavage of the ND5 gene region with AluI restriction enzyme on 2% agarose gel electrophoresis (1-16: C haplotype (554-211-57 bp), M: Fermentas GeneRuler^{TM} 1kb DNA Ladder.

No restriction sites were found with *Fok*I (*Bts*CI) (ER0871 Thermo Fisher Scientific) (*Figure 4a*) and *Hinc*II (ER0491 Thermo Fisher Scientific) (*Figure 4b*) restriction enzymes in the ND5 gene resulting in a single band profile of 822 bp in our study. This result was found in accordance with Ivanova et al. (2010). Bouga et al (2005) reported a single cleavage site in both of the restriction enzymes; the *Hinc*II enzyme, A haplotype (418-404 bp), and the *Fok*I enzyme, A haplotype (430-392 bp).

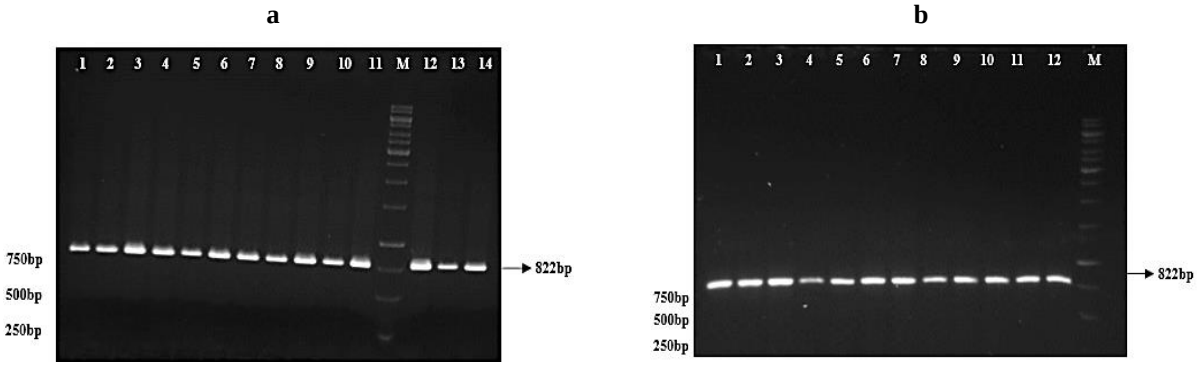

Figure 4. Cleavage of the COI gene region with the FokI (BtsCI)* (a) *and HincII restriction enzymes on 2% agarose gel electrophoresis.* (a) *1-14: B haplotype (822 bp); M: Fermentas GeneRuler^{TM} 1kb DNA Ladder.* (b) *1-12: B haplotype (822 bp); M: Fermentas GeneRuler^{TM} 1kb DNA Ladder.

In this study, COI and ND5 gene regions were restricted with the *Nco*I, *Ssp*I, *Sty*I and *Alu*I, *Fok*I, *Hinc*II restriction enzymes in order to determine possible new haplotypes in terms of mtDNA molecule in honey bees from Kırklareli and Tekirdağ representing Thrace honey bees.

The similarities and differences between races, ecotypes, and even populations can be investigated on the basis of nucleotide changes obtained as a result of cleavage with restriction endonuclease enzymes. In this way, genetic proximity and distance in all species can be revealed.

In this study, it is known that different mtDNA haplotype groups are formed with the restriction enzymes applied to the gene regions of the honey bees, and the following enzyme haplotype combinations were formed according to the enzyme recognition sites found in gene regions (*Table2*).

Table 2. The haplotype diversity of the populations in the mitochondrial genome of COI and ND5 genes

Gene Region	**COI**			**ND5**		
Enzyme/Haplotype	*Nco*I	*Ssp*I	*Sty*I	*Alu*I	*Fok*I	*Hinc*II
Type 1	B	C	A	C	B	B
Type 2	B	C	B	C	B	B
Type 3	C	C	A	C	B	B
Type 4	C	C	B	C	B	B

In this study, two different mtDNA regions (COI and ND5) were investigated and genetic similarities or differences in Kırklareli and Tekirdağ honey bee populations were revealed. As a result of the analysis, different variations were found as a result of cleaving with *Nco*I and *Sty*I restriction enzymes, which will be used to distinguish the populations as a result of treatment with different enzymes. As a result of the digestion of the COI region with the *Nco*I enzyme, variations were detected in 2 samples from Tekirdağ and 12 samples from Kırklareli populations observed and both populations were found to be polymorphic. Likewise, in the *Sty*I digestion of the COI gene region, 44.4% of Kırklareli populations were found as haplotype B, the rest as the haplotype A.

4. Conclusion

In this study, some of the haplotypes that were found in Kırklareli samples were found similar to the haplotypes detected in the Macedonian (*Apis mellifera macedonica* Ruttner, 1988) samples reported previously whereas some of them showed quite different haplotype profiles. The reason for this situation may be due to the low number of samples examined in this study, and also the intense use of Carniolan (*Apis mellifera carnica* Pollman, 1879) queen bees in the region. Intra-regional races and ecotypes should be revealed, these hypotheses should be confirmed or tested by conducting studies with different gene combinations in the Kırklareli population and throughout Thrace.

It is a necessity to define or protect the physiological, morphological, or behavioral characteristics of honey bee breeds in detail. In order to do this, genetic diversity must be determined. It is thought that this study will be useful in similar studies to be carried out in the future and also in the creation of a genetic database on Thrace honey bees in Turkey.

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