

GENETIC STRUCTURE OF HONEY BEE (*APIS MELLIFERA* LINNAEUS, 1758) SUBSPECIES BASED ON tRNA^{leu}-COX2 AND ND5 REGIONS OF mtDNA

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Abstract. Diversity of ecological conditions in Anatolia enables many species to adapt to different environments. Thus Turkey is inhabited by various strains of *A.m. mellifera* Linnaeus, 1978; *A.m. mellifera carnica*, *A.m. mellifera anatoliaca*, *A.m. mellifera caucasica*, *A.m. mellifera syriaca*, and *A.m. mellifera meda*. Analysis of mtDNA variants is a widely used tool to determine the phylogenetic relationships at the species and subspecies levels. In this study sequencing results of tRNA^{leu}-COX2 and ND5 gene segments of mtDNA were presented in comparison with some previously published mitochondrial haplotypes. According to the UPGMA dendrogram and estimates of evolutionary distances, divergence among the subspecies and ecotypes were not verified strongly. Reproductive isolation barriers could be ineffective and lead to the exchange of the genetic materials between the populations. Intensive care should be taken while managing the colonies of different subspecies in bee yards. Degradation of biodiversity of honey bee subspecies through hybridization was also reported at wider geographic areas rather than small breeding units in some studies. Broad ranges of isolated colonies of subspecies should be established with proper selection studies to reduce the hazards of migratory beekeeping activities. Successful management practices are needed through better beekeeping technologies and beekeeper training programmes to prevent the homogenization of the genetic structure of different subspecies and to conserve honey bee diversity.

Keywords: *hybridization, gene introgression, phylogenetic analysis, artificial sympatry, biodiversity*

Introduction

The natural dispersion areas of *A. mellifera* L. include Central Asia, Europe, Near East and sub-Saharan Africa and the species has also been introduced to East and Southeast Asia, Australia and the Americas (Ruttner, 1988). At least 29 subspecies of *A. mellifera* have been acknowledged and separated into four main lineages based on their morphometric, genetic, ecological, physiological, and behavioral features (Han et al., 2012).

A. m. capensis, *A. m. intermissa*, *A. m. litorea*, *A. m. monticola*, *A. m. sahariensis* and *A. m. scutellata* are African subspecies from north and south Sahara belonging to mtDNA group A (2) mainly *A. m. mellifera* and some *A. m. iberiensis* are the subspecies of western and northern European populations of group M (3) group C is composed of southeastern European and northern and eastern Mediterranean populations, containing *A. m. carnica*, *A. m. ligustica*, Turkish and Georgian *A. m. caucasica*, *A. m. meda* and Turkish *A. m. anatoliaca*; and (4) group O includes *A. m. syriaca*, and perhaps other subspecies from Turkey and the Middle East and the last group is Y from the east African country of Ethiopia. Divergence among some subspecies within each lineage was also recognized (Ruttner, 1988; Arias and Sheppard, 1996; Franck et al., 2000).

Although it was not possible to distinguish the classes C and O by sequencing and restriction analysis, they were classified as C (Arias and Sheppard, 1996). Group O verification was performed by examination of mitochondrial DNA and analysis of

microsatellites (Arias and Sheppard, 1996; Franck et al., 2000; Palmer et al., 2000). Anatolian ecological diversity helps adaptive species to evolve in various environments. Thus Turkey hosts five subspecies of *A. mellifera*; *A. mellifera carnica*, *A. mellifera anatoliaca*, *A. mellifera caucasica*, *A. mellifera syriaca*, and *A. mellifera meda* (Ruttner, 1988). No other country today has so many different breeds of honey bee. Most of the geographical regions of Turkey is comprised of *A. m. anatoliaca* except for the northeastern part where *A. m. caucasica* dominates and *A. m. meda* exists in the southeastern part of the country. Thrace region of European Turkey is inhabited by *A. m. carnica* (Palmer et al., 2000), while the southern part of the country near Hatay is occupied by *A. m. syriaca* (Kandemir et al., 2000).

It is understood that morphological characteristics are vulnerable to the effects of selection and environmental changes, therefore they are not accurate and adequate to reconstruct phylogenetic relationships. The variation of mtDNA can be used as an additional and more reliable genetic marker for both classification and phylogenetic analysis of honey bee subspecies (Avisé et al., 1987; Franck et al., 2000).

Honey bee lineages can be discerned by sequence analyses of the mitochondrial DNA (mtDNA) region between the cytochrome oxidase subunits I and II genes (CoxI–CoxII intergenic region). Throughout population genetics studies, evolutionary lineages and groups of subspecies can be distinguished by differences throughout length and sequence within the mitochondrial genome of honey bees (Garnery et al., 1992; Franck et al., 2000; Palmer et al., 2000). Numerous mitochondrial regions, such as 16s rDNA (Marino et al., 2002b; Bouga et al., 2005; Kekeçoğlu et al., 2009), ND2 (Arias and Sheppard, 1996), ND5 (Bouga et al., 2005; Martimianakis et al., 2011), cytochrome C oxidase I (COI or Cox1) (Bouga et al., 2005; Martimianakis et al., 2011), and the tRNA^{leu}-COII region (COI-COII intergenic region) (Garnery et al., 1992; Franck et al., 2000; Palmer et al., 2000; Susnik et al., 2004; Munoz et al., 2009; Özdil et al., 2009a,b) have been studied to determine honey bee subspecies' diversity and phylogenetic relationships. tRNA^{leu}-COX2 intergenic region is a noncoding region found between a leucine tRNA gene and the cytochrome oxidase II gene and variation in this region may help to distinguish some mitochondrial haplotypes (Canovas et al., 2008).

The objective of this research was to find out the genetic structure and phylogenetic relationships of five *A. mellifera* subspecies and ecotypes of Turkey through direct sequencing of tRNA^{leu}-COX2 and ND5 mitochondrial regions with the comparison of reference mtDNA haplotypes that were published in previous studies.

Materials and methods

Sampling and DNA isolation

Subspecies and ecotypes were taken from their native habitats representing different regions of Turkey and then the colonies were located in a common garden at Middle East Technical University campus in the centre of Ankara. Subspecies were categorized according to their origin and distinguishable morphological characteristics prior to DNA isolation. Workers were collected in 95% ethanol and air-dried prior to the extraction. Four samples from each of the subspecies were used for DNA extraction. The extraction of DNA from thorax tissue by Qiagen DNeasy® Blood & Tissue Kit (www.qiagen.com).

The quantity of eluted DNAs was checked by Nanodrop ND-1000 (Thermo Fisher Scientific, Inc. Wilmington, Delaware, USA). By loading 2 µl of each sample, the

absorbance ratios of A260/280 and A260/230 were measured and DNA concentrations were quantified in ng/ul. To examine the phylogenetic relationships between the subspecies, E2-H2 primer pairs were used to amplify tRNA^{leu}-COX2 intergenic region and ND5 primer pairs were used to amplify ND-5 mitochondrial region. The sequence for forward primer, E2 was 5'-GGC AGA ATA AGT GCA TTG-3', and the reverse primer, H2 was 5'-CAA TAT CAT TGA TGA CC-3' whereas the sequence for ND5_F was 5'-TCG AAA TGA ATA GGA TAC AG-3' and ND5_R was 5'-GGT TGA GAT GGT TTA GGA TT -3'.

PCR was run in a total volume 30 µl of the following reaction mixture: 10 mM of each primer 3 µl of 10X reaction buffer with MgCl₂, 2mM of dNTP mix, 2 u of Tag polymerase, 20 ng DNA. For E2-H2 primer pair, the following reaction profile was used: initial denaturation at 94°C for 5 min, followed by 35 cycles of; 94°C for 45 sec, 48°C for 45 sec, 62°C for 2 min, and final elongation step of 20 min at 65°C. For ND5 primer pair, initial denaturation 94°C for 4 min, 35 cycles of 94°C for 1 min, annealing at 50°C (ND5) for 1 min, and extension at 72°C for 2 min, followed by a final extension step at 72°C for 15 min.

The amplified products obtained were next electrophoresed on 1.5% agarose gel to verify the size of the fragment length of 520 bp for tRNA^{leu}-COX2 intergenic region and 721 bp for ND5 mitochondrial region. PCR products were subsequently purified with QIAquick PCR purification kit (QIAGEN) and directly sequenced with E2 and ND5-F sequencing primers commercially by Macrogen (Rockville, MD, USA).

Statistical details of the phylogenetic analysis

For the phylogenetic analysis, DNA sequences from this study and additional reference sequences from Genbank were trimmed and aligned using Clustal W (Thompson and Higgins, 1994). The resulting sequences were organized and compared with the published mitochondrial tRNA^{leu}-COX2 intergenic region and ND5 sequences available in Genbank. The sequences obtained in this study were deposited to Genbank with accession numbers MN701721 to MN701744 for ND5 region and MN701745 to MN701763 for tRNA^{leu}-COX2 region.

BLAST searches of the international sequence database (NCBI [National Center for Biotechnology Information] BLASTN search [<http://www.ncbi.nlm.nih.gov/>]) were used to include the sequences of the outgroup and the sequences of species most closely related subspecies to the analysis. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 (Tamura et al., 2013).

UPGMA dendrogram was built and estimates of evolutionary divergence between sequences were calculated using the same software. The analytical formulas and the bootstrap approach used to measure standard estimation errors. Comparisons were done nucleotide-by-nucleotide (Nei and Kumar, 2000).

Results

Relationships between the subspecies within tRNA^{leu}-COX2 intergenic region

Fragment length was 520 bp for tRNA^{leu}-COX2 intergenic region (*Fig. 1*). tRNA^{leu}-COX2 region within the samples of this study consisted of 2 variable sites, 2 parsimony informative sites and conserved region was 493 bp. According to the dendrogram based on the statistical analysis of sequencing results of tRNA^{leu}-COX2

intergenic region (Fig. 2), Mugla and Yigilca ecotypes were grouped with *A. mellifera* haplotype C12 (FJ037777), *A. m. anatoliaca* C1g (FJ357804), *A. mellifera* haplotype C2d (JQ977701) and *A. m. anatoliaca* clone 5973 (AY618912) and they were closer to *A. m. anatoliaca* C1e (FJ357802) with a branch length of 0.001.

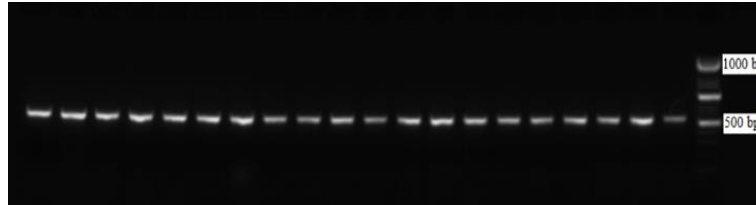


Figure 1. The amplified PCR products of $tRNA^{leu}$ -COX2 intergenic region of mitochondrial DNA. (Bands represent *A. m. anatoliaca*-Mugla-1-2-3-4; *A. m. caucasica* 1-2-3-4; *A. m. carnica* 1-2-3-4; *A. m. anatoliaca*-Yigilca 1-2-3-4; *A. m. syriaca* 1-2-3-4, respectively)

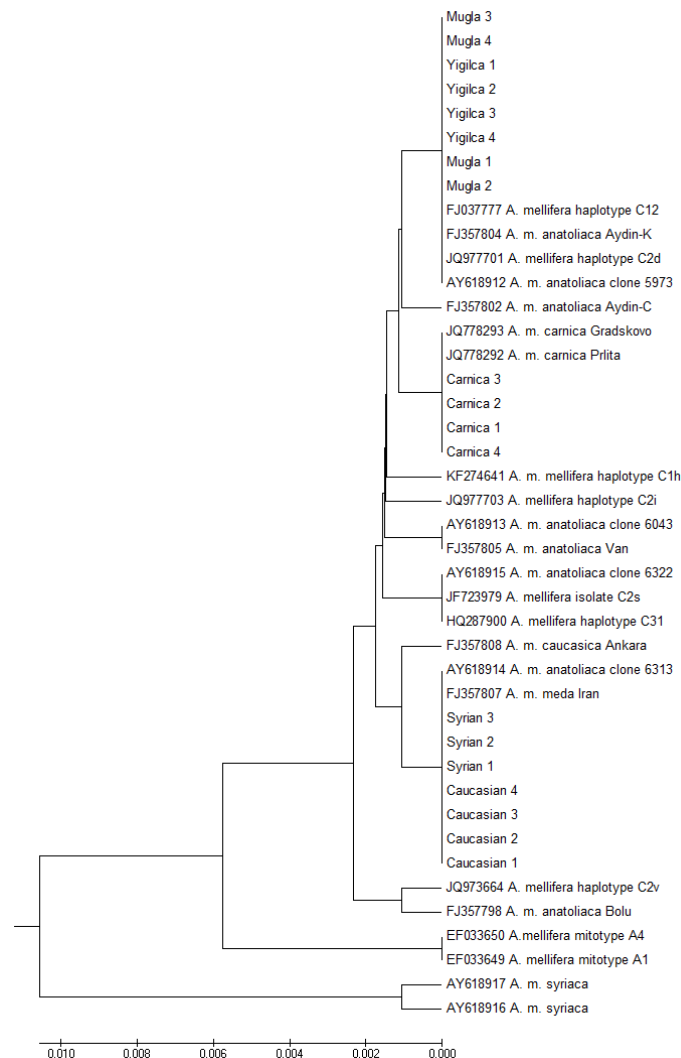


Figure 2. The phylogenetic tree based on $tRNA^{leu}$ -COX2 intergenic region. The evolutionary history was inferred using the UPGMA method. Samples of this study were labeled as Mugla 1-2-3-4; Yigilca 1-2-3-4; Carnica 1-2-3-4; Syrian 1-2-3; Caucasian 1-2-3-4

Table 1. Estimates of evolutionary divergence between sequences of tRNA^{leu}-COX2 intergenic region (left). Identification of the numbers representing the nucleotide sequences were given (right). (Samples of this study were represented by numbers 24, 25, 26, 27, 28)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28		
[1]																													[1]	#KF274641_A. m. <i>mellifera</i> _haplotype_C1h
[2]	1.00																												[2]	#AY618912_A. m. <i>anatoliaca</i> _clone_5973
[3]	10.00	9.00																											[3]	#AY618917_A. m. <i>syriaca</i>
[4]	11.00	10.00	1.00																										[4]	#AY618916_A. m. <i>syriaca</i>
[5]	2.00	1.00	10.00	11.00																									[5]	#AY618915_A. m. <i>anatoliaca</i> _clone_6322
[6]	2.00	1.00	10.00	11.00	2.00																								[6]	#AY618914_A. m. <i>anatoliaca</i> _clone_6313
[7]	2.00	1.00	10.00	11.00	2.00	2.00																							[7]	#AY618913_A. m. <i>anatoliaca</i> _clone_6043
[8]	2.00	1.00	8.00	9.00	2.00	2.00	2.00																						[8]	#JQ973664_A. <i>mellifera</i> _haplotype_C2v
[9]	2.00	1.00	10.00	11.00	2.00	2.00	2.00	2.00																					[9]	#JQ977703_A. <i>mellifera</i> _haplotype_C2i
[10]	1.00	0.00	9.00	10.00	1.00	1.00	1.00	1.00	1.00																				[10]	#JQ977701_A. <i>mellifera</i> _haplotype_C2d
[11]	2.00	1.00	8.00	9.00	2.00	2.00	2.00	2.00	2.00	1.00																			[11]	#JQ778293_A. m. <i>carnica</i> _Gradskovo
[12]	2.00	1.00	8.00	9.00	2.00	2.00	2.00	2.00	2.00	1.00	0.00																		[12]	#JQ778292_A. m. <i>carnica</i> _Prlita
[13]	2.00	1.00	10.00	11.00	0.00	2.00	2.00	2.00	2.00	1.00	2.00	2.00																	[13]	#JF723979_A. <i>mellifera</i> _isolate_C2s
[14]	2.00	1.00	10.00	11.00	0.00	2.00	2.00	2.00	2.00	1.00	2.00	2.00	0.00																[14]	#HQ287900_A. <i>mellifera</i> _haplotype_C31
[15]	2.00	1.00	10.00	11.00	2.00	0.00	2.00	2.00	2.00	1.00	2.00	2.00	2.00																[15]	#FJ357807_A. m. <i>meda</i> _Iran
[16]	3.00	2.00	11.00	12.00	3.00	1.00	3.00	3.00	3.00	2.00	3.00	3.00	3.00	3.00	1.00														[16]	#FJ357808_A. m. <i>caucasica</i> _Ankara
[17]	2.00	1.00	10.00	11.00	2.00	2.00	0.00	2.00	2.00	1.00	2.00	2.00	2.00	2.00	2.00	3.00													[17]	#FJ357805_A. m. <i>anatoliaca</i> _Van
[18]	1.00	0.00	9.00	10.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00	1.00	2.00	1.00												[18]	#FJ357804_A. m. <i>anatoliaca</i> _Aydin-K

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28		
[19]	2.00	1.00	10.00	11.00	2.00	2.00	2.00	2.00	2.00	1.00	2.00	2.00	2.00	2.00	2.00	3.00	2.00	1.00											[19]	#FJ357802_A. m. <i>anatoliaca</i> _Aydin-C
[20]	3.00	2.00	9.00	10.00	3.00	3.00	3.00	1.00	3.00	2.00	3.00	3.00	3.00	3.00	3.00	4.00	3.00	2.00	3.00										[20]	#FJ357798_A. m. <i>anatoliaca</i> _Bolu
[21]	1.00	0.00	9.00	10.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00	1.00	2.00	1.00	0.00	1.00	2.00									[21]	#FJ037777_A. <i>mellifera</i> _haplotype_C12
[22]	4.00	5.00	8.00	9.00	6.00	6.00	6.00	6.00	6.00	5.00	4.00	4.00	6.00	6.00	6.00	7.00	6.00	5.00	6.00	7.00	5.00								[22]	#EF033650_A. <i>mellifera</i> _mitotype_A4
[23]	4.00	5.00	8.00	9.00	6.00	6.00	6.00	6.00	6.00	5.00	4.00	4.00	6.00	6.00	6.00	7.00	6.00	5.00	6.00	7.00	5.00	0.00							[23]	#EF033649_A. <i>mellifera</i> _mitotype_A1
[24]	2.00	1.00	10.00	11.00	2.00	0.00	2.00	2.00	2.00	1.00	2.00	2.00	2.00	2.00	0.00	1.00	2.00	1.00	2.00	3.00	1.00	6.00	6.00						[24]	#Syriaca samples
[25]	2.00	1.00	8.00	9.00	2.00	2.00	2.00	2.00	2.00	1.00	0.00	0.00	2.00	2.00	2.00	3.00	2.00	1.00	2.00	3.00	1.00	4.00	4.00	2.00					[25]	#Carnica samples
[26]	1.00	0.00	9.00	10.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00	1.00	2.00	1.00	0.00	1.00	2.00	0.00	5.00	5.00	1.00	1.00				[26]	#Mugla samples
[27]	1.00	0.00	9.00	10.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	1.00	1.00	2.00	1.00	0.00	1.00	2.00	0.00	5.00	5.00	1.00	1.00	0.00			[27]	#Yigilca samples
[28]	2.00	1.00	10.00	11.00	2.00	0.00	2.00	2.00	2.00	1.00	2.00	2.00	2.00	2.00	0.00	1.00	2.00	1.00	2.00	3.00	1.00	6.00	6.00	0.00	2.00	1.00	1.00		[28]	#Caucasica samples

Samples of Carnica subspecies clustered with two *A. m. carnica* breeding lines (JQ778293) and JQ778292 with 69% bootstrap value. Because they have C→T transition at position 354. They also showed close proximity to *A. m. anatoliaca* (FJ357802) and *A. m. mellifera* haplotype C1h (KF274641) and *A. mellifera* haplotype C2i (JQ977703) with a distance of 0.001.

Samples of Syrian and Caucasian subspecies were grouped within the same branch with *A. m. anatoliaca* clone 6313 (AY618914) and *A. m. meda* (FJ357807) with a bootstrap value of 66% because they all have A→T transversion at site 158. They were also closely related to *A. m. caucasica* (FJ357808) with a branch length of 0.001. *A. m. caucasica* (FJ357808) included additional C→T transition at site 362.

A. m. anatoliaca clone 6043 (AY618913), *A. m. anatoliaca* (FJ357805) *A. m. anatoliaca* clone 6322 (AY618915), *A. mellifera* isolate C2s (JF723979), *A. mellifera* haplotype C31 (HQ287900) were clustered together apart from the samples of this study with the branch length of 0.002 (Fig. 2). *A. m. syriaca* (AY618917), *A. m. syriaca* (AY618916) and *A. mellifera* mitotype A4 (EF033650) and *A. mellifera* mitotype A1 (EF033649) formed separate branches distant from the remaining groups with 74% and 62% bootstrap values.

According to nucleotide number of differences model, pairwise differences between Yigilca and Mugla ecotypes were 0.00. These ecotypes differed from the other subspecies with a distance value of 1.00. Pairwise distance between Syrian and Caucasian subspecies were also 0.00 and a dissimilarity was observed between this group and Carnica subspecies with a magnitude of 2.00. *A. m. meda* (FJ357807) which was clustered with Syrian and Caucasian samples, was distant from Carnica subspecies with a pairwise distance of 2.00 and dissimilar to Mugla and Yigilca ecotypes with a pairwise distance of 1.00 (Table 1).

A. m. anatoliaca (FJ357805) Van and *A. m. anatoliaca* (FJ357802) Aydin-C shared the same distance of 1.00 to Mugla-Yigilca group and the distance of 2.00 to Carnica, Syriaca and Caucasian samples. *A. m. anatoliaca* (FJ357804) Aydin-Kusadasi showed pairwise difference of 1.00 with Carnica, Syrian and Caucasian samples while this haplotype indicated similarity with Mugla and Yigilca ecotypes (pairwise distance = 0.00). The distance between *A. m. anatoliaca* (FJ357798) Bolu and Mugla-Yigilca group was 2.00 while this haplotype was more distant from Carnica, Syriaca and Caucasian samples with a magnitude of 3.00 (Table 1). The branching pattern had a bootstrap value of 81% for UPGMA method.

Relationships between the subspecies within ND5 region

The amplified PCR product of NADH dehydrogenase subunit 5 (ND5) gene was 721 bp (Fig. 3). The dendrogram based on the phylogenetic analysis of sequencing data for NADH dehydrogenase subunit 5 (ND5) gene, the samples included to this study did not reveal any genetic divergence and they were grouped in the same branch with *Apis mellifera* ND5 gene haplotype 3 (GU060468), *Apis mellifera* ND5 gene haplotype 1 (JN410833) and haplotype 5 (JN410837) and did not show much difference from *Apis mellifera* ND5 gene haplotype 2 (JN410834), haplotype 3 (JN410835), haplotype 4 (JN410836), haplotype 1 (GU060466), haplotype 2 (GU060467), haplotype 3 (GU060468), haplotype 4 (GU060469), haplotype 6 (GU060471) and haplotype 7 (GU060472) with a bootstrap value of 66% (Fig. 4). They indicated a pairwise difference of 0.01 with haplotype 8 (GU060473).

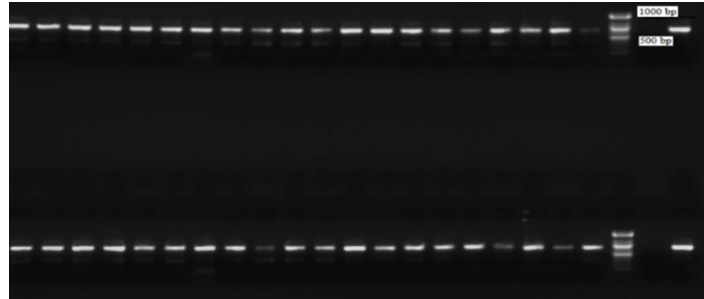


Figure 3. The amplified PCR products of NADH dehydrogenase subunit 5 (ND5) gene of mitochondrial DNA (Bands represented *A.m. anatoliaca*-Mugla-1-2-3-4; *A.m. caucasica* 1-2-3-4; *A.m. carnica* 1-2-3-4; *A.m. anatoliaca*-Yigilca 1-2-3-4; *A.m. syriaca* 1-2-3-4, respectively)

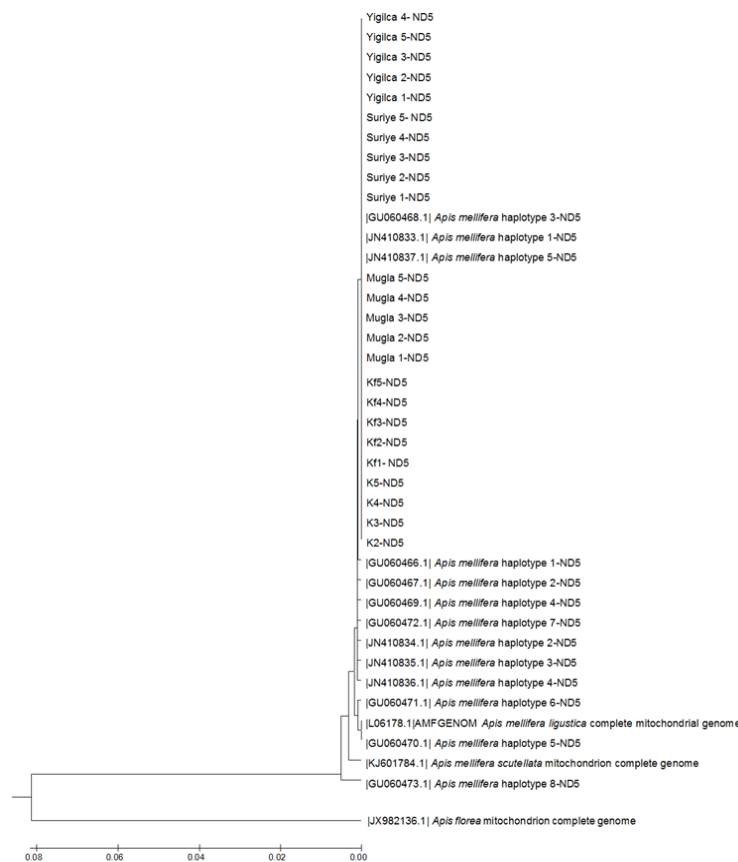


Figure 4. The phylogenetic tree based on NADH dehydrogenase subunit 5 (ND5) gene. The evolutionary history was inferred using the UPGMA method. *A.m. syriaca* samples of the study were labeled as Suriye (1 to 5), *A.m. caucasica* samples as Kf (1 to 5), *A.m. carnica* samples as K (2 to 5), *A.m. anatoliaca* as Mugla ecotype (1 to 5) and *A.m. anatoliaca* as Yigilca ecotype (1 to 5)

Only Yigilca ecotype was different from ND5 haplotype 5 (GU060470) and *Apis mellifera ligustica* ND5 segment (L06178) with a pairwise value of 0.01. All of the samples were distant from *Apis mellifera scutellata* ND5 gene (KJ601784) with a magnitude of 0.01 and from *Apis mellifera florea* ND5 gene JX982136 with a magnitude of 0.014 (Table 2).

Table 2. Estimates of evolutionary divergence between sequences of ND5 gene (left). Identification of the numbers representing the nucleotide sequences were given (right). (Samples of this study were represented by numbers 1, 2, 3, 4, 5)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
[1]																						[1]	#Carniolan samples
[2]	0.00																					[2]	#Caucasica samples
[3]	0.00	0.00																				[3]	#Mugla samples
[4]	0.00	0.00	0.00																			[4]	#Syrian samples
[5]	0.00	0.00	0.00	0.00																		[5]	#Yigilca samples
[6]	0.00	0.00	0.00	0.00	0.00																	[6]	#GU060466_ <i>Apis mellifera</i> _haplotype_1
[7]	0.00	0.00	0.00	0.00	0.00	0.00																[7]	#GU060467_ <i>Apis mellifera</i> _haplotype_2
[8]	0.00	0.00	0.00	0.00	0.00	0.00	0.00															[8]	#GU060468_ <i>Apis mellifera</i> _haplotype_3
[9]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00														[9]	#GU060469_ <i>Apis mellifera</i> _haplotype_4
[10]	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.01													[10]	#GU060470_ <i>Apis mellifera</i> _haplotype_5
[11]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00												[11]	#GU060471_ <i>Apis mellifera</i> _haplotype_6
[12]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00											[12]	#GU060472_ <i>Apis mellifera</i> _haplotype_7
[13]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01										[13]	#GU060473_ <i>Apis mellifera</i> _haplotype_8
[14]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01									[14]	#JN410833_ <i>Apis mellifera</i> _haplotype_1
[15]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00								[15]	#JN410834_ <i>Apis mellifera</i> _haplotype_2
[16]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00							[16]	#JN410835_ <i>Apis mellifera</i> _haplotype_3
[17]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00						[17]	#JN410836_ <i>Apis mellifera</i> _haplotype_4
[18]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00					[18]	#JN410837_ <i>Apis mellifera</i> _haplotype_5
[19]	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.13	0.14	0.14	0.14	0.14	0.14				[19]	#JX982136_ <i>Apis florea</i>
[20]	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.14		[20]	#KJ601784_ <i>Apis mellifera</i> _scutellata
[21]	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.00	0.14	0.01		[21]	#LO6178_ <i>Apis mellifera</i> _ligustica

Discussion

Relationships between the subspecies

In this study the genetic relationships of the subspecies was revealed by direct sequencing of mtDNA based on tRNA^{leu}-COX2 and ND5 genes. Fragment lengths can be determined with certainty but the identities of many of the mitochondrial haplotypes can't be specified by RFLP patterns alone. Genetic variation can be established with greater sensitivity of DNA sequencing.

For systematic and population biology, the mtDNA that is maternally inherited without recombination is a pivotal tool. DNA sequence data analysis can be performed in a simple phylogenetic context, making mitochondrial data advantageous over morphometric data. Sometimes there may be differences between morphometric and mitochondrial data sets, when previously isolated populations of honey bees come into contact through human transportation or range expansion and mtDNA haplotypes may be introgressed into new populations as a result of mating (Palmer, 2000). While mtDNA's uniparental inheritance appears to be a downside, it retains an notable source of data by preserving information on queen and queen lines that shed light on the history and phylogeography of *A. mellifera*. However, the mitochondrial genes are interrelated and the evolution of different regions of the mitochondrial genome at different rates leads to incompatible phylogenetic and phylogeographical patterns (Henriques et al., 2019).

Subspecies of C evolutionary lineage pinpoints a short sequence with a lack of length variability. Mitochondrial haplotypes can be discriminated via single nucleotide polymorphisms (Franck et al., 2000; Sušnik et al., 2004). Thus conservative mtDNA regions are more convenient than length polymorphisms to identify molecular variation within C lineage subspecies. The analysis of tRNA^{leu}-COX2 intergenic region indicated identical genetic information for Mugla and Yigilca sharing similarity with *A. mellifera* haplotype C2d (JQ977701) which was also frequent in *A. m. macedonica* Greek populations and observed in *A. m. carnica* Croatian subpopulations which meanted that these two ecotypes also carried mixed genetic information of different subspecies aside from *A. m. anatoliaca*. Mugla and Yigilca honey bees are ecotypes of *A. m. anatoliaca* thus they mostly shared similarity with *A. m. anatoliaca* haplotypes such as *A. m. anatoliaca* clone 5973 (AY618912), *A. m. anatoliaca* (FJ357804) from Aydin-Kusadasi. These ecotypes were also closer to *A. m. anatoliaca* haplotype C1e (FJ357802) from the center of Aydin which were studied by Özdil et al. (2009b). tRNA^{leu}-COX2 region of Mugla and Yigilca ecotypes also carried *A. mellifera* haplotype C12 (FJ037777). In the study of Solorzano et al. (2009), *A. mellifera* haplotype C12 was the most prevalent mitotype acknowledged as basal ancestral mitotype and it corresponded for 47% of the *Apis mellifera* 'C' lineage samples and was found in 13 of the 22 sampled locations including Aegean (Mugla, Izmir), Black Sea (Yigilca, Giresun), Marmara (Kirkclareli, Canakkale, Bursa), Mediterreanean (Hatay) regions.

Microsatellite analysis of honey bees from Turkey (Bodur et al., 2007) indicated that Kirkclareli region had the lowest levels of gene flow but mtDNA results of Solorzano et al. (2009) did not support the genetic distinctiveness of this region because all sampled colonies consisted of mitotype C12. It was also concluded by Palmer et al. (2000) and Kandemir et al. (2006) that Thrace populations were not divergent from Anatolian

populations. In the current study, Carnica samples branched out separately away from C12 mitotype with a pairwise distance of 1.00. According to Solorzano et al. (2009), the incompatibility between mitochondrial and nuclear markers may reflect the maternal inheritance of mitochondrial DNA, and nuclear markers might be representing the polyandrous matings of queens with genetically distinct drones that makes the population in this region diverse. Samples of Carnica subspecies clustered with two *A. m. carnica* breeding lines JQ778293 and JQ778292 from Eastern Serbia. They also showed close proximity to *A. m. anatoliaca* from Aydin and *A. m. mellifera* haplotype C1h of German honey bee and *A. mellifera* haplotype C2i from Eastern Serbia which could result from human and biological influences that mix populations and compromise their purity.

Samples of Syrian and Caucasian subspecies were grouped within the same branch with *A. m. anatoliaca* clone 6313. This clone (TrDra-2 haplotype) was found to be widely distributed in Eastern Anatolia and also observed in Black Sea (7.09%), Mediterranean (5.48%), Marmara (7.50%), Central Anatolia (3.92%) and Aegean (3.03%) regions (Kandemir et al., 2006). Syrian and Caucasian subspecies also shared the same branch with *A. m. meda* (FJ357807) and were closer to *A. m. caucasica* (FJ357808). The former was C2g haplotype from Iran and the latter was C2h from Ankara- Kazan (Özdil et al., 2009b). C2h haplotype from Ankara-Kazan were found to be similar to published sequences of *A. m. caucasica* (Garnery et al., 1992). Honey bee populations spreading along the northern coast from Kırklareli to Artvin were stated to be *A. m. anatoliaca* or an ecotype of it (Kandemir et al., 2000; Kekeçoğlu, 2007; Kekeçoğlu and Soysal, 2010; Çakmak et al., 2014). According to the results of Kambur and Kekeçoğlu (2018), *A. m. caucasica* was not found in each province of northeast of Turkey because of beekeeping activities. This resemblance of Caucasian subspecies to other subspecies could be attributed to the widespread use of Caucasian bees by many beekeepers in different provinces. The existence of close relationship between these subspecies (Syriaca and Caucasica) and a variety of haplotypes is coherent with the hypothesis that Anatolian zone is close to the origin of the speciation of *Apis mellifera* (Ruttner, 1988).

A. m. anatoliaca clone 6043 (AY618913), *A. m. anatoliaca* C2a haplotype from Van (Özdil et al., 2009b), *A. m. anatoliaca* clone 6322 (AY618915), *A. mellifera* isolate C2s haplotype from Spain (JF723979) were clustered together apart from the samples of this study. The frequency of haplotype *A. m. anatoliaca* clone 6043 (TrDra-1) ascended from east to west in Turkey, the highest frequency was discovered in Thrace and with the lowest in northeastern Anatolia where *A. m. caucasica* prevailed. *A. m. anatoliaca* clone 6322- TrDra-3 haplotype which accounted for C2b (Franck et al., 2000) was identified in eastern Anatolia, Ardahan and Van (Kandemir et al., 2006).

A. m. anatoliaca haplotype C1a from Bolu (Özdil et al., 2009b) and *A. mellifera* haplotype C2v from Romania were clustered together and genetically distinct from Mugla-Yigilca ecotypes as well as Caucasica, Carnica and Syrian subspecies by demonstrating more distance.

In the study of Kandemir et al. (2006), TrDra-4 haplotype (AY618916) which was detected in one province (Kastamonu) on the Black Sea coast and corresponds to C2a. *A. m. carnica* haplotype of Franck et al. (2000) was submitted as *A. m. syriaca* clone 6783 to the Genbank. In the same study, TrDra-5 haplotype (AY618917) was also submitted as *A. m. syriaca* clone 6800 to the Genbank. These clones as well as *A. mellifera* mitotypes A1 and A4 were far from Syrian and other samples of the

subspecies included in this study with a particular branching pattern. *A. mellifera* mitotypes A1 and A4 from Brazil and Uruguay also clustered apart from the other haplotypes included in the mtDNA analysis.

Conspicuous differences were not detected between honey bee subspecies and ecotypes in tRNA^{leu}-COX2 region in this study. Henriques et al. (2019) concluded in their study that care should be taken with regard to the results of analysis of tRNA^{leu}-COX2 region and this may not be a suitable marker for detecting relationships between honey bee colonies. The large numbers of indels and duplications of large fragments and homoplasmy in this region reduce the accuracy of this region in relation to the capturing of maternal history.

Relative to other mitochondrial regions, the ND5 region was studied less at population level. According to the analysis of mitochondrial NADH dehydrogenase subunit 5 (ND5) gene, the samples of this study belonged to the same clade with *Apis mellifera* ND5 gene haplotype 3 (GU060468), *Apis mellifera* ND5 gene haplotype 1 (JN410833) and haplotype 5 (JN410837). In the study about phylogenetic relationships among *A. mellifera* subspecies depended on sequencing of mtDNA segments, ND5 haplotype 3 (GU060468) was monitored in *A. m. carnica* (Martimianakis et al., 2011). ND5 haplotype 1 (JN410833) was Anatolian haplotype captured in the samples from Konya-Sızma and haplotype 5 (JN410837) was found in honey bees from the South-East part of Turkey where mostly inhabited by *A. m. meda* (Özdil and İlhan, 2012).

NADH dehydrogenase subunit 5 (ND5) gene segments of our samples also did not point out any discrepancy from *Apis mellifera* ND5 gene haplotype 2 (JN410834-from Antalya and Konya), haplotype 3 (JN410835-widely distributed Anatolian haplotype among samples of Balıkesir, Bolu-Yığılca, and Konya), haplotype 4 (JN410836-Caucasian haplotype from Ardahan and Ankara) within the study of Özdil and İlhan (2012). In that study, the races couldn't be separated from each other according to the NJ dendrogram based on ND5 sequences, because they belonged to East European (C) lineage.

Our samples also did not differ from ND5 haplotypes of some other races such as haplotype 1 (GU060466) of *A. m. anatoliaca* (Bartin), *A. m. meda* (Osmaniye), *A. m. carnica*; haplotype 2 of *A. m. ligustica* (GU060467); haplotype 4 of *A. m. cecropia* (GU060469); haplotype 6 (GU060471) and haplotype 7 (GU060472) of *A. m. adami* (Martimianakis et al., 2011). Overall, a common branching pattern of the honey bee subspecies and ecotypes of this study with *A. m. anatoliaca*, *A. m. meda*, *A. m. carnica*, *A. m. ligustica* ND5 haplotypes of the other studies presented a coherent result by revealing the close relations at the species and subspecies level. It is known that the pattern typical of *A. m. carnica*/*A. m. ligustica* is considered to prevail across Turkey (97.9%) (Kandemir et al., 2006). It is not surprising to observe similarity to *A. m. ligustica* haplotype because its adaptability to various climatic conditions makes it a favored species to export globally. Consequently, the introduction of this species has usually been the main cause of genetic changes in local species (De la Rúa et al., 1998; Garnery et al., 1998a,b). Appearance of *A. m. meda* and *A. m. ligustica* in the same cluster was also congruent with the finding about the similarity between *A. m. meda* (Iranian) and *A. m. ligustica* (Italian) based on several characters of morphology and behavior (Ruttner et al., 1988). Settar (1983) showed that honey bee subspecies in Aegean region were transitional populations between *A. m. caucasica* and *A. m. ligustica*. In another study showing the haplotypes obtained in honey bee populations of Thrace region, some of the samples with variation were found to have *A. m. caucasica*

origin and hybridization between Caucasian/Anatolian and Carniolan/Macedonian haplotypes was detected. The reasons for this situation is thought to be the intense introduction of Caucasian queen bees and migratory beekeeping activities (Ünal and Özdil, 2018). Also some studies proved that there were mixed populations of *A. m. syriaca* and *A. m. meda* in both southeast and the eastern parts of Turkey (Palmer et al., 2000; Kandemir et al., 2000).

According to the study of Kambur and Kekeçoğlu (2018), the groups representing the Aegean and Marmara regions coincided and intertwined with the provinces representing the Eastern Anatolia. Those results indicated that bee biodiversity was significantly affected by commercial queen use activities.

Turkey is at the crossroads between Europe, Asia and the Middle East with a wide variety of climates and habitats. There are many subspecies and ecotypes of honey bees within these ranges such as *A. m. anatoliaca*, *A. m. caucasica*, *A. m. meda*, and *A. m. syriaca* as described by Ruttner (1988). Honey bees have been able to adapt to a variety of ecological environments including three phytogeographic regions; Euro-Syberian; Mediterranean; and Irano-Turanian where several honey bee ecotypes appeared with many morphological, biochemical, physiological, and behavioural adaptations. Widespread migratory beekeeping activities and the demand of exporting and establishing Caucasian bees (*A. m. caucasica*) in new locations contribute to transplantation and mixing of populations impacting the integrity of the ecotypes and the geographical variations among them.

Conclusion

Molecular data on mitochondrial DNA (mtDNA) tRNA^{leu}-COX2 and ND5 sequences inferred that all the subspecies and ecotypes in this study were not so diverse genetically. There is a possibility that reproductive isolation couldn't be achieved and gene introgression could have occurred between them because of the close proximity of the colonies in the bee yard or in the past, the genetic structure of the colonies could have already mixed and exchanged in their native habitats from where they were originated and sampled. Phylogenetic analysis of this study would be more effective and strong if sample size was increased by including more colonies of the subspecies. Degradation of honey bee biodiversity in some locations of Turkey were reported previously by many studies. Overlapping of samples representing subspecies from different regions could mean that honey bee biodiversity could not maintain its current status and the use of commercial queen bee has significantly affected the biodiversity of honey bee in Turkey. Each of the subspecies has its own unique behavioral and morphometric characteristics that make them well suited to their local environments, but hybridization and modification of the existing genetic pool among local bees can appear through fragmentation of land resources via urbanization, uncontrolled reproduction, the introduction of queen bees from other mtDNA lines and the preferences of the beekeepers to use different breeds for high yield (Güler, 2010). Thus local honey bee subspecies and ecotypes may lose their novel characteristics. Due to their social nature of the honey bees at the individual and colonial reproduction level, the distribution of mitochondrial markers are affected and diversified by commercial breeding and migratory beekeeping activities. Identification and protection of natural honey bee gene resources is vital for the development of beekeeping. Conventional methods are not suitable to define the hybrids and not sufficient to prevent gene

flow among subspecies. Thus identification of hybrids must be enhanced to improve conservation management. Also next-generation sequencing of bee transcriptomes and genomes will extend the number of genes and data and provide new approaches to resolve phylogenetic relationships. Pure breeding populations are also a major instrument for an improved conservation management. Detailed beekeeping information of different regions needs to be collected systematically before making future conservation programmes. The mechanisms of hybridization and mating distances may assist protection of honey bee subspecies. Apicultural researches about identifying and improving local genetic lines must be encouraged and granted. Beekeeping policies must be regulated to minimize human induced artificial sympatry of honey bee populations. Conservation of honey bee biodiversity is a very necessary step to preserve the biological equilibrium in ecological environment, to meet the agricultural demands of humans and to benefit from production potentials in breeding and hybridization studies in the future.

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