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Phylogenetic relationship of Turkish *Apis mellifera* subspecies based on sequencing of mitochondrial cytochrome C oxidase I region

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ABSTRACT. Mitochondrial DNA sequence variation can be used to infer honey bee evolutionary relationships. We examined DNA sequence diversity in the cytochrome C oxidase I (COI or *Cox*1) gene segment of the mitochondrial genome in 112 samples of *Apis mellifera* from 15 different populations in Turkey. Six novel haplotypes were found for the COI gene segment. There were eight variable sites in the COI gene, although only three were parsimony-informative sites. The mean pairwise genetic distance was 0.3% for the COI gene segment. Neighbor-joining (NJ) trees of the COI gene segment were constructed with the published sequences of *A. mellifera* haplotypes that are available in GenBank; the genetic variation was compared among the different honeybee haplotypes. The NJ dendogram based on the COI sequences available in GenBank showed that Eastern European races were clustered together, whereas the Mellifera and Iberian haplotypes were clustered far apart. The haplotypes found in this study were clustered together with *A. mellifera ligustica* and some of the Greek honey bees (accession Nos. GU056169 and GU056170) found in NCBI GenBank database. This study expands the knowledge about the mitochondrial COI region and presents the first comprehensive

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sequence analysis of this region in Turkish honeybees.

Key words: *Apis mellifera* L.; DNA sequence diversity; COI; Turkey

INTRODUCTION

Based on morphometric and molecular approaches, the honeybee, *Apis mellifera* L., has been divided into 4 or 5 evolutionary lineages: the West European lineage (M), including northern Africa; the south and central African lineage (A); the north Mediterranean and Eastern European lineage (C); the Near and Middle Eastern lineage (O), and based on molecular studies, the eastern African lineage (Y) in which *A. m. yemenitica* from Ethiopia is included (Ruttner, 1988; Hall and Smith, 1991; Garnery et al., 1992; Arias and Sheppard, 1996; Kauhausen-Keller et al., 1997; Franck et al., 2000, 2001).

Based on morphometrics, the Near Eastern subspecies - Anatolian (*A. m. anatoliaca*), Caucasian (*A. m. caucasica*), and Iranian (*A. m. meda*) - had been grouped within the O branch (Ruttner, 1988; Kauhausen-Keller et al., 1997). However, mitochondrial DNA (mtDNA) analysis has shown that they belong to the C lineage (Smith et al., 1997; Palmer et al., 2000; Franck et al., 2000, 2001; Özdil et al., 2009a,b; Bouga et al., 2011). Morphometric analyses by Ruttner (1988) have indicated that nearly all of Turkey is occupied with *A. m. anatoliaca* but that *A. m. caucasica* is found in the northeastern part of Turkey, and *A. m. meda* is found in the southeastern part of Turkey. More recent mitochondrial studies of Turkish honeybees have shown that *A. m. carnica* is also found in the European part of Turkey called Thrace (Palmer et al., 2000), and *A. m. syriaca* is found in the southern part of the country near the Hatay region (Kandemir et al., 2006).

Length and sequence variations within the mitochondrial genome are widely used to study genetic diversity among populations with a high level of nucleotide substitution, conserved gene order and content and maternal inheritance (Avise et al., 1987; Hall and Smith, 1991; Garnery et al., 1992, 1993; Rokas et al., 2003). The maternal inheritance of mtDNA is more important in honeybees because all individuals of a colony are progeny of the queen, which means that their mtDNA is identical. As a result, a colony can be treated as an individual in analysis (Garnery et al., 1992).

Our knowledge of the mitochondrial genome of *A. mellifera* has been mainly focused on restriction fragment length polymorphism (RFLP) variability or sequencing of the transfer RNA $(tRNA)^{leu}$-cytochrome C oxidase, COII region (formerly the COI-COII intergenic region). *Dra*I restriction of the $tRNA^{leu}$-COII region has revealed more than 60 different haplotypes, mainly in lineages A and M and fewer in lineage C (Garnery et al., 1992, 1993; Palmer et al., 2000; Franck et al., 2000, 2001; De La Rúa et al., 2002, 2004; Sušnik et al., 2004; Kozmus et al., 2007; Muñoz et al., 2009; Nedić et al., 2009; Özdil et al., 2009a; Magnus and Szalanski, 2010; Szalanski and Magnus, 2010). Although barcoding, a DNA-based identification system (Hebert et al., 2003), has mainly focused on sequence diversity in the COI region, we have little knowledge about the mitochondrial COI region beyond the $tRNA^{leu}$-COII region in *A. mellifera* subspecies. Only the RFLP profile of the COI region (Bouga et al., 2005; Kekeçoğlu et al., 2009; Özdil et al., 2012) and the phylogenetic relationships of *A. mellifera* subspecies based on sequencing of this region have been reported (Tanaka et al., 2001; Marino et al., 2002a,b; Sheffield et al., 2009; Martimianakis et al., 2011).

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The objective of this research was to determine the genetic diversity and phylogenetic relationships of *A. mellifera* subspecies of Turkey through the COI gene segment sequencing. Length variations and nucleotide substitutions were compared with those found in other mitochondrial surveys. These results could be also useful in determining the genetic structure of *A. mellifera*.

MATERIAL AND METHODS

Sampling and DNA extraction

A total of 112 honeybees, each representing a different colony, were collected from 15 widespread locations in Turkey (Figure 1, Table 1). Workers were collected in 95% ethanol and subsequently air-dried. Total DNA was extracted from the thorax of each bee following the process of Hall (1990). The concentration and purification of genomic DNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Inc., Wilmington, DE, USA) and 20 ng genomic DNA was used for the polymerase chain reaction (PCR).

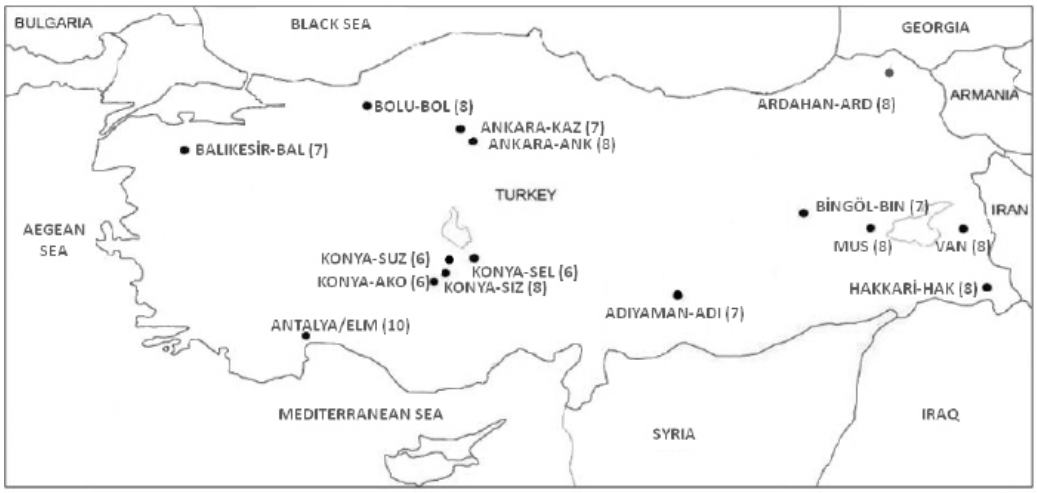

Figure 1. Sampling locations in Turkey. Number in parentheses show the number of colonies sequenced at each site.

Sequence analysis

The COI gene segments were amplified according to the method of Bouga et al. (2005). The PCR products were purified using a gel purification kit (QIAGEN) and sequenced in both directions on an ABI Prism 3130 automated sequencer (Applied Biosystems) using standard protocols. Sequences were aligned using Clustal X (Thompson et al., 1997). Molecular Evolutionary Genetics Analysis 5 (MEGA5) was used to estimate the similarity index and

Table 1. Sampling localities, geographical positions and number of colonies used for sequencing.

Locations	Abbreviation of the locations	Geographical position		Colonies analyzed for sequence analysis
ADIYAMAN	ADI	37°46'N	38°16'E	7
ARDAHAN	ARD	41°03'N	42°42'E	8
ANKARA/KAZAN	KAZ	39°58'N	32°52'E	7
ANKARA	ANK	40°12'N	32°41'E	8
ANTALYA/ELMALI	ELM	36°44'N	29°56'E	10
BALIKESIR	BAL	39°39'N	27°53'E	7
BINGÖL	BIN	39°00'N	40°41'E	7
BOLU/YIĞILCA	BOL	40°58'N	31°27'E	8
HAKKARI	HAK	37°35'N	43°34'E	8
KONYA/AKÖREN	AKO	37°27'N	32°22'E	6
KONYA/SELÇUKLU	SEL	37°57'N	32°26'E	6
KONYA/SIZMA	SIZ	38°05'N	32°24'E	8
KONYA/SUZ*	SUZ	38°02'N	32°30'E	6
MUŞ/VARTO	MUS	39°17'N	41°12'E	8
VAN/GEVAŞ	VAN	38°18'N	43°06'E	8
Total				112

*SUZ = The Apiary of the Selçuk University.

evolutionary divergence between DNA sequences. Maximum parsimony (MP) and neighbor-joining (NJ) analyses were performed using the same software, resulting in a consensus of the phylogenetic tree (Tamura et al., 2011).

We used a *Bombus ignitus* sequence (accession No. DQ870926) retrieved from GenBank as outgroup to root the trees for the construction of the phylogenetic trees. The various sequences obtained in this study have been deposited to GenBank with accession Nos. JN168656 to JN168661. The resulting sequences were compared to published sequences of the COI gene available in the NCBI database.

RESULTS

The sizes of the PCR-amplified COI segment of all samples studied were found to be approximately 989 bp (primers excluded). Six novel haplotypes were revealed for the COI gene segment. The number of variable sites was 8 for COI, but only 3 were parsimony informative sites. The average pairwise genetic distance was 0.3% for the COI gene segment (Kimura, 1980).

Table 2 lists the various haplotypes of the COI segment that are available in the NCBI GenBank database and summarizes the sequence information, GenBank accession numbers, variable sites of these haplotypes, and additional haplotypes found in this study. The mtDNA nucleotide positions are taken from Crozier and Crozier (1993). The distribution of the haplotypes across honeybee populations is given Table 3.

The trees drawn using the MP and NJ analyses exhibited nearly the same topology for the COI gene; therefore, only the NJ trees are presented here. Figure 2 depicts the phylogenetic relationships based on the COI sequences of all *A. mellifera* haplotypes that are available in GenBank. Less sequencing information is available about the mitochondrial COI region in *A. mellifera* L. than about the $tRNA^{leu}$-COII region, although genetic-based systems, primarily DNA barcoding studies, depend on the sequencing of the COI mitochondrial region. Figure 3 gives the NJ tree of the haplotypes obtained in this study.

Table 2. Haplotypes, GenBank accession numbers and variable sites of the COI region of *Apis mellifera* L.

Haplotype/ Reference	GenBank accession Nos.	Variable sites																												
		1851	1933	2085	2108	2117	**2135****	2144	**2148**	2261	2276	2291	2324	2366	2370	2382	2412	2438	2447	2468	2475	2477	2495	2516	2555	2594	2598	2601	**2606**	2615
Crozier and Crozier 1993	NC_001566[1]	C	G	A	C	T	T	T	A	T	C	T	C	T	T	T	C	A	C	T	C	T	A	T	T	T	T	A	A	A
Ligus9	AY114452[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ligus7	AY114453[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ligus6	AY114454[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
Ligus5	AY114455[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ligus3	AY114456[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ligus4	AY114457[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ligus2	AY114458[2]	*	*	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
Melli4	AY114459[2]	*	*	G	.	A	.	C	.	.	T	.	.	.	C	.	.	.	T	.	T	A	.	.	.	A	C	G	.	.
Ligus8	AY114460[2]	*	*	.	.	A	.	C	.	.	T	.	T	.	.	.	.	.	T	.	T	A	.	.	.	A	C	G	.	.
Carni3	AY114461[2]	*	*	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carni6	AY114462[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carni4	AY114463[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carni5	AY114464[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Buckf1	AY114465[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cauca1	AY114466[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cauca4	AY114467[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
Cauca3	AY114468[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Anato1	AY114469[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Anato2	AY114470[2]	*	*	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.
Anato3	AY114471[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cauca2	AY114472[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Maced2	AY114473[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Maced3	AY114474[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
Adami1	AY114475[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Adami2	AY114476[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
Adami3	AY114477[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Iberi2	AY114478[2]	*	*	.	.	.	.	C	.	.	.	.	.	A	.	C	.	.	.	.	T	A	.	.	C	.	C	G	.	.
Iberi3	AY114479[2]	*	*	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	T	A	.	.	.	.	C	G	.	.
Sicul4	AY114480[2]	*	*	.	.	A	.	C	.	.	T	.	T	.	.	.	.	.	T	.	T	A	.	.	.	A	C	G	.	.
Sicul2	AY114481[2]	*	*	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	T	A	.	.	.	.	C	G	.	.
Sicul5	AY114482[2]	*	*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Sicul6	AY114483[2]	*	*	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	C	G	.	.

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Table 2. Continued.

Haplotype/ Reference	GenBank accession Nos.	Variable sites continued																											
		2639	**2647**	2651	2660	2671	2687	2720	2768	**2771**	2789	2894	**2900****	2903	2918	**2935****	2939	**2960****	3015	3077	3103	3107	3152	3176	3204	3259	3275	3281	3285	3300
Crozier and Crozier 1993	NC_001566[1]	T	T	A	C	T	T	C	T	T	A	T	T	C	T	T	T	A	T	T	G	T	T	T	T	T	T	A	C	T
Ligus9	AY114452[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
Ligus7	AY114453[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	T	.
Ligus6	AY114454[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	C
Ligus5	AY114455[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
Ligus3	AY114456[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
Ligus4	AY114457[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
Ligus2	AY114458[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
Melli4	AY114459[2]	.	.	.	T	.	C	T	.	.	G	.	.	T	.	.	A	.	.	C	.	.	C	.	.	.	C	.	T	.
Ligus8	AY114460[2]	.	.	.	T	.	C	T	.	.	G	C	.	T	.	.	A	.	.	.	.	.	C	.	.	.	C	.	T	.
Carni3	AY114461[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
Carni6	AY114462[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carni4	AY114463[2]	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Carni5	AY114464[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Buckf1	AY114465[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
Cauca1	AY114466[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cauca4	AY114467[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cauca3	AY114468[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Anato1	AY114469[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Anato2	AY114470[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
Anato3	AY114471[2]	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cauca2	AY114472[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Maced2	AY114473[2]	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
Maced3	AY114474[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
Adami1	AY114475[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
Adami2	AY114476[2]	G	G	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
Adami3	AY114477[2]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Iberi2	AY114478[2]	.	.	.	T	.	.	T	.	.	.	.	.	T	.	.	A	.	.	.	.	C	.	C	.	.	.	.	T	.
Iberi3	AY114479[2]	.	.	.	T	.	.	T	.	.	.	.	.	T	.	.	A	.	.	.	.	C	.	C	.	.	.	.	T	.
Sicul4	AY114480[2]	.	.	.	T	.	C	T	.	.	G	C	.	T	.	.	A	.	.	.	.	.	C	.	.	.	C	.	T	.
Sicul2	AY114481[2]	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	A	.	.	C	.	C	.	C	.	.	.	.	T	.
Sicul5	AY114482[2]	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Sicul6	AY114483[2]	.	.	.	**T**	.	.	**T**	.	.	.	.	.	.	**C**	.	**A**	.	**C**	.	.	**C**	.	**C**	.	.	.	**G**	**T**	.

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Table 2. Continued.

Haplotype/ Reference	GenBank accession Nos.	Variable sites continued																												
		1851	1933	2085	2108	2117	**2135****	2144	**2148**	2261	2276	2291	2324	2366	2370	2382	2412	2438	2447	2468	2475	2477	2495	2516	2555	2594	2598	2601	**2606**	2615
Tanaka et al., 2001	AF214668[3]	*	*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Sheffield et al., 2009	FJ582088[4]	*	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*	*	*
	FJ582089[5]	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*
	FJ582090[5]	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*
	FJ582091[5]	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*
	FJ582092[5]	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*
Martimianakis et al., 2011	GU056169[6]	*	*	*	*	*	·	·	-	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	GU056170[6]	*	*	*	*	*	·	·	-	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	GU056171[6]	*	*	*	*	*	·	·	-	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·
	GU056172[6]	*	*	*	*	*	·	·	-	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	GU056173[6]	*	*	*	*	*	·	·	-	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·
	GU056174[6]	*	*	*	*	*	·	·	-	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	GU056175[6]	*	*	*	*	*	·	·	-	·	T	·	T	·	·	·	·	·	T	·	T	A	G	·	·	·	A	C	G	·	·
New-ANATO1	JN168656[7]	*	*	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·
New-ANATO2	JN168657[7]	*	*	·	·	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
New-ANATO3	JN168658[7]	*	*	*	*	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
New-CAUCA1	JN168659[7]	*	*	*	*	·	·	·	-	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
New-MEDA1	JN168660[7]	*	*	*	*	·	·	·	-	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
New- MEDA2	JN168661[7]	*	*	*	*	·	·	·	-	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·

Continued on next page

Table 2. Continued.

Haplotype/ Reference	GenBank accession Nos.	Variable sites continued																													
		2639	**2647**	2651	2660	2671	2687	2720	2768	**2771**	2789	2894	**2900****	2903	2918	**2935****	2939	**2960****	3015	3077	3103	3107	3152	3176	3204	3259	3275	3281	3285	3300
Tanaka et al., 2001	AF214668[3]	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*	*	*	*
Sheffield et al., 2009	FJ582088[4]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	FJ582089[5]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	FJ582090[5]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	FJ582091[5]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	FJ582092[5]	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Martimianakis et al., 2011	GU056169[6]	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*	*	*	*
	GU056170[6]	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*	*	*	*
	GU056171[6]	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*	*	*	*
	GU056172[6]	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*	*	*	*
	GU056173[6]	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*	*	*	*
	GU056174[6]	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*	*	*	*
	GU056175[6]	·	·	·	T	·	C	T	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*	*	*	*
New-ANATO1	JN168656[7]	·	C	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	G	·	·	·	*	*	*	*	*	*	*	*	*
New-ANATO2	JN168657[7]	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	*	*	*	*	*	*	*	*	*
New-ANATO3	JN168658[7]	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*
New-CAUCA1	JN168659[7]	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*
New-MEDA1	JN168660[7]	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	·	·	*	*	*	*	*	*	*	*	*
New-MEDA2	JN168661[7]	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	*	*	*	*	*	*	*	*	*

[1]NC_001566: Crozier and Crozier, 1993; 16,343-bp *Apis mellifera ligustica* complete mitochondrial genome; [2]AY114452-AY114483: Marino et al., 2002a,b; 1246-bp COI gene (bases 2070 to 3315); [3]AF214668: Tanaka et al., 2001; 1041-bp COI gene (bases 2004 to 3044); [4]FJ582088: Sheffield et al., 2009; 600-bp COI gene (bases 1867 to 2466); [5]FJ582089-FJ582092: Sheffield et al., 2009; 654-bp COI gene (bases 1839 to 2492); [6]GU056169-GU056175: Martimianakis et al., 2011; 916-bp COI gene (bases 2144 to 3061); [7]JN168656-JN168661: 989-bp the COI gene (bases 2115 to 3103) of Turkish honey bees found in this study. · = identical nucleotides at that site. *No sequence information at that site of the haplotype. Variable sites are indicated in bold numbers. **Variable sites that are newly found in this study.

Table 3. The distribution of the haplotypes across honeybee populations.

Haplotypes	GenBank accession Nos.	Populations																
		ADI	ARD	KAZ	ANK	ELM	BAL	BIN	BOL	HAK	AKO	SEL	SIZ	SUZ	MUS	VAN	Total	Frequency (%)
ANATO1	JN168656												5				5	4.5
ANATO2	JN168657						7		8								15	13.4
ANATO3	JN168658					10					6	6	3	6			31	27.7
CAUCA1	JN168659		8	7	8												23	20.5
MEDA1	JN168660	7						4							4	5	20	17.8
MEDA2	JN168661							3		8					4	3	18	16.1
Total		7	8	7	8	10	7	7	8	8	6	6	8	6	8	8	112	

For abbreviations, see Table 1.

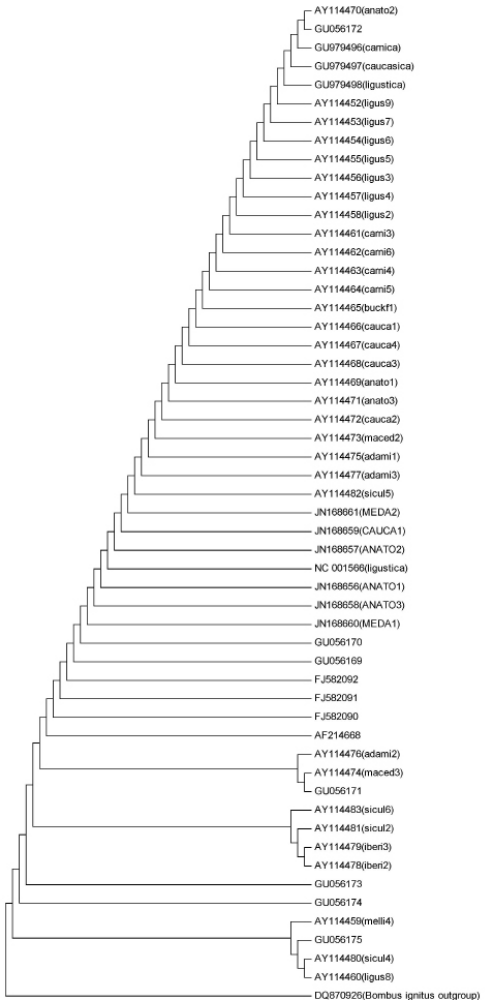

Figure 2. Neighbor-joining dendogram based on COI sequences of *Apis mellifera* haplotypes that are available in the NCBI GenBank database. Sequences obtained in this study are written in capital LETTERS.

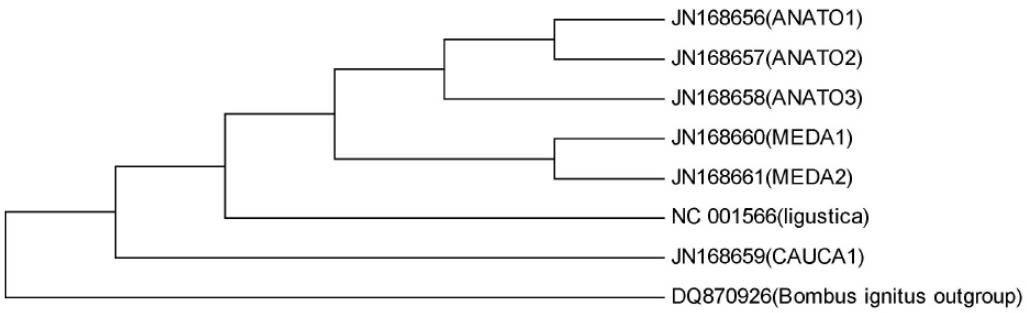

Figure 3. Neighbor-joining dendogram of COI sequences showing the relationships between the Turkish honeybee haplotypes obtained in this study.

DISCUSSION

Several mitochondrial studies of Turkish honeybee populations have been conducted (Smith et al., 1997; Palmer et al., 2000; Kandemir et al., 2006, Özdil et al., 2009a). Nearly all Turkish colonies analyzed by other surveys with restriction digests were found to belong to the C (Eastern European and Mediterranean) lineage. This finding was expected because the Turkish populations consist mainly of *A. m. anatoliaca*, *A. m. caucasica*, and *A. m. meda*.

Our previous study defined the phylogenetic relationships and PCR-RFLP profiles of Turkish honeybees based on 16srDNA, COI, and ND5 gene segments using several restriction enzymes (Özdil et al., 2012). In this study, we performed a comprehensive sequencing analysis of the COI gene segment to verify the genetic divergence in Turkish honeybee populations. More than 35 haplotypes in the COI region of *A. mellifera* were recorded to the NCBI GenBank database. Additionally, 6 new haplotypes were added in this study (see Tables 2 and 3). Three came from the Anatolian geographical locations, 2 from the Iranian, and one from the Caucasian. The ANATO1 haplotype was the rarest (4.5%) in this study, found only in some of the Sızma (SIZ) population. The frequency of the ANATO2 haplotype was 13.4%, found in the northern populations of Anatolia, Balıkesir (BAL), and Bolu (BOL). The highest haplotype frequency obtained was ANATO3, with a percentage of 27.7%, in the populations in the southern and central region of Anatolia (Elmalı, Akören, Selçuklu, Sızma, and Konya (SUZ). The frequency of the CAUCA1 haplotype was 20.5% in northern and northeastern Anatolia (Kazan (KAZ), Ankara (ANK), and Ardahan (ARD), where *A. m. caucasica* predominates. The frequencies of MEDA1 and MEDA2 were 17.8 and 16.1%, respectively, found in populations in the southeastern part of Turkey, where *A. m. meda* predominates (see Table 3).

In this study, nucleotide substitutions and a deletion site were observed in the COI region. A base substitution, which was first observed in this study at position 2135 (T→A), was obtained from all Anatolian races. A deletion site at position 2148, which had been previously reported in all haplotypes examined by Martimianakis et al. (2011), were detected in Caucasian and Iranian haplotypes but not in the Anatolian haplotype (see Table 2). The NJ dendrogram (see Figure 2) based on the COI sequences available in GenBank showed that that Eastern European races were clustered together, whereas the Mellifera and Iberian haplotypes were clustered far apart. The haplotypes found in this study were clustered together with

A. m. ligustica and some of the Greek honeybees recorded in GenBank with accession Nos. GU056169 and GU056170.

To add to previous COI gene segment findings, we report herein the sequencing of the COI region in Turkish honeybees that belong to the C (Eastern European and Mediterranean) lineage and its comparison with that of various *A. mellifera* subspecies. Based on the sequence results, Turkish honeybee populations can be clearly classified into geographical locations in which *A. m. caucasica* and *A. m. meda* samples are classified in CAUCA1 and MEDA1-MEDA2 haplotypes, respectively. These results suggest that the haplotypes maintain their native origin, which might be pure. High migratory beekeeping activity and the commercial activities of queen rearing in Turkey may result in the loss of genetic diversity, so the identification of the genetic structure of Turkish honeybee races and improvement of strategies to conserve them are important.

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