Genetic Divergence of Turkish *Apis mellifera* Subspecies Based on Sequencing of ND5 Mitochondrial Segment

by

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ABSTRACT

Mitochondrial DNA sequence variation can be used to infer honey bee evolutionary relationships. In this study, DNA sequence diversity in the ND5 region of the mitochondrial genome was investigated in 93 samples of *Apis mellifera* from 15 different populations in Turkey. Five novel haplotypes were revealed for the ND5 gene segment of Turkish honeybees. The number of variable sites found was 6 for this region while 2 were parsimony informative sites. The average pairwise genetic distances were 0.3% for ND5 gene. In this study, the NJ tree of ND5 gene segment were constructed with the published sequences of *Apis mellifera* haplotypes. This study expands the knowledge about the mitochondrial ND5 region in *Apis mellifera* and it is also the first comprehensive sequencing analysis of ND5 region in Turkish honeybees.

Key words: *Apis mellifera* L., DNA sequence diversity, ND5, Turkey

INTRODUCTION

The honey bee, *Apis mellifera* L., occurs naturally in Europe, the Middle East and Africa (Ruttner 1988). From morphometric and molecular studies, the 29 subspecies of the honey bee, *Apis mellifera* L., are grouped into five evolutionary lineages: M from northern and western Europe and northern Africa, A from southern and central Africa, C from the northern Mediterranean region and eastern Europe, O from the eastern Mediterranean and the Near and Middle East region, and Y from the east African country of Ethiopia (Ruttner 1988; Hall and Smith 1991; Garnery *et al.* 1992; Arias and Sheppard 1996; Kauhausen-Keller *et al.* 1997; Franck *et al.* 2000, 2001).

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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 mtDNA analysis showed that they belonged to the C lineage (Smith *et al.* 1997; Palmer *et al.* 2000; Franck *et al.* 2000, 2001; Kandemir *et al.* 2006; Özdil *et al.* 2009a, 2009b; Bouga *et al.* 2011). Ruttner's (1988) morphometric analyses concluded that *A. m. anatoliaca*, *A. m. caucasica* and *A. m. meda* exist in Turkey. Nearly all of Turkey is occupied by *A. m. anatoliaca*. *A. m. caucasica* is found in the northeastern part of Turkey and *A. m. meda* is found in the southeastern part of Turkey. Recently, the mitochondrial studies of Turkish honeybees had also shown that *A. m. carnica* is found in the European part of Turkey called Thrace (Palmer *et al.* 2000) and *A. m. syriaca* is found in the south part of the country near the Hatay region (Kandemir *et al.* 2006).

The mitochondrial genome has been a useful molecule for population genetic studies of *Apis mellifera* L. Length and sequence variations within the mitochondrial genome of honeybees have been particularly useful in differentiating evolutionary lineages and groups of subspecies (Hall and Smith 1991; Garnery *et al.* 1992, 1993; Franck *et al.* 2000; Palmer *et al.* 2000). *Apis mellifera ligustica* was the first Hymenopteran subspecies for which the whole mitochondrial genome has been sequenced (Crozier and Crozier 1993). To date, many mitochondrial regions, of which the $tRNA^{leu}$-COII region (formerly COI-COII intergenic region) (Garnery *et al.* 1992, 1993; Franck *et al.* 2000; De la Rua *et al.*2001, 2004; Palmer *et al.* 2000, Sušnik *et al.* 2004; Kozmus *et al.* 2007; Muňoz *et al.* 2009; Nedić *et al.* 2009; Özdil *et al.* 2009a; Magnus and Szalanski 2010; Szalanski and Magnus 2010) was the first, have been sequenced and phylogenetic relationships among *Apis mellfera* subspecies has been described. But we still have little knowledge about the other mitochondrial regions. The ND5 gene segment was first sequenced in Greek honeybees and this is the only study of the phylogenetic relationships among East European subspecies (Martimianakis *et al.* 2011).

The objective of this research was to determine the genetic diversity and phylogenetic relationships of *Apis mellifera* subspecies of Turkey as determined by sequencing of ND5 gene segment. Length variations and nucleotide substitutions found in this gene segment were compared with the other mitochondrial surveys and such results could be also useful in determining the genetic structure of honey bees.

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

MATERIALS AND METHODS

Sampling and DNA extraction

A total of 93 honey bees each representing a different colony was collected from 15 widespread locations in Turkey (Fig. 1, Table 1). Workers were collected in 95% ethanol and subsequently air-dried. Total DNA was extracted from each bee's thorax according to Hall (1990). The concentration and purification of genomic DNA was quantified with a NanoDrop ND-1000 spectrophotometer, and 20 ng of genomic DNA was used for the PCR.

Sequence Analysis

The ND5 mitochondrial gene segment was amplified according to 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 with the computer program Clustal X (Thompson *et al.* 1997). For estimates of the similarity index and evolutionary divergence between DNA sequences MEGA5 Software was used. Resulting in a consensus of the phylogenetic tree, the methods of Maximum Parsimony (MP) and Neighbor-Joining (NJ) analysis were performed using the same software (Tamura *et al.* 2011).

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	6
ANKARA / KAZAN	KAZ	39°58'N	32°52'E	6
ANKARA /AUZ*	AUZ	40°12'N	32°41'E	6
ANTALYA / ELMALI	ELM	36°44'N	29°56'E	8
BALIKESIR	BAL	39°39'N	27°53'E	5
BINGÖL	BIN	39°00'N	40°41'E	6
BOLU / YIĞILCA	BOL	40°58'N	31°27'E	6
HAKKARI	HAK	37°35'N	43°34'E	6
KONYA / AKÖREN	AKO	37°27'N	32°22'E	5
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	6
VAN / GEVAŞ	VAN	38°18'N	43°06'E	6

*AUZ: The Apiary of the Ankara University
**SUZ: The Apiary of the Selçuk University

For the construction of the phylogenetic trees, we used *Apis cerena* (accession number: NC_014295) sequences retrieved from Genbank, as outgroup in order to root the trees. The sequences obtained in this study have been deposited to Genbank with accession numbers JN410833 to JN410837. The resulting sequences were compared to published sequences available in Genbank.

RESULTS

The sizes of the PCR-amplified ND5 segment for all populations studied were found to be 782 bp (primers excluded). Five novel haplotypes were revealed for the ND5 gene segment in Turkish honeybees. The number of variable sites was found to be 6 for this region 2 of which were parsimony informative sites. The average pairwise genetic distances were 0.3% for ND5 gene (Kimura 1980).

Table 2 lists the different haplotypes of the ND5 gene found in this study and in Martimianakis *et al.* 2011. The sequence information, Genbank accession numbers and variable sites of these haplotypes and additional new

haplotypes that are found in this study are summarized in Table 2. The mtDNA nucleotide positions are taken from Crozier and Crozier (1993).

The trees drawn by Maximum Parsimony and Neighbor-Joining analysis exhibited nearly the same topology for ND5 mtDNA region so only the NJ tree is presented here. At population level, sequencing of ND5 mitochondrial region has been studied less than the other mitochondrial regions. Only Martimianakis *et al.*, (2011) gives information about sequencing of this region in samples from Greece and some East European Countries. The phylogenetic tree of Greek and Turkish honey bee haplotypes constructed by the NJ method is shown in Fig. 2.

DISCUSSION

Several mitochondrial studies of Turkish honey bee populations have been conducted (Smith *et al.* 1997; Palmer *et al.* 2000; Kandemir *et al.* 2006, Özdil *et al.* 2009a). With restriction digests, nearly all Turkish colonies analyzed previously by the other surveys were found to belong to the C Mediterranean

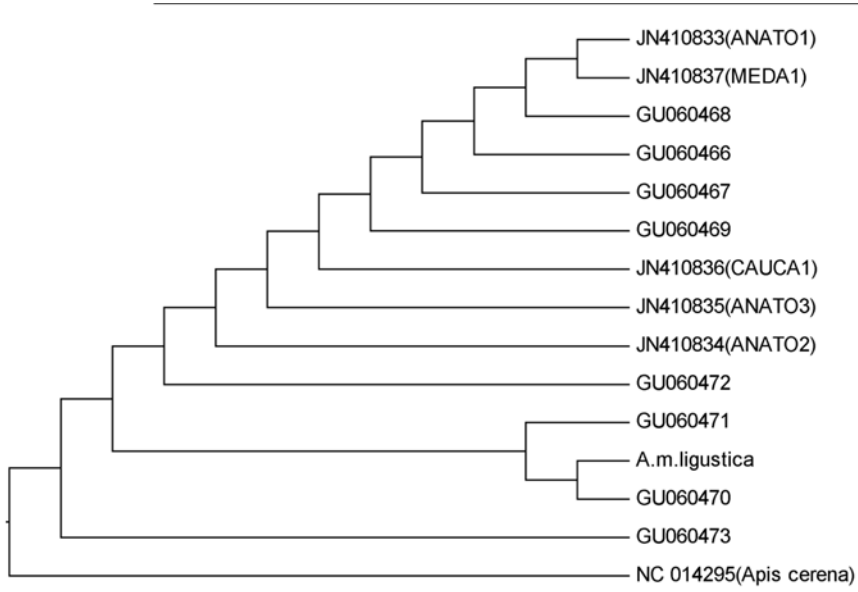

Fig. 2. Neighbour-Joining dendogram based on ND5 sequences of *Apis mellifera* haplotypes. Sequences obtained in this study are written in capital letters.

Table 2. Haplotypes, Genbank accession numbers and variable sites of the ND5 region of *Apis mellifera* L. The variable sites that are found in this study are indicated in bold numbers.

MtDNA nucleotide positions		**7425****	7636	**7697****	7777	**7830**	7968	**7991****	8006	8011	8019	8026	8088	**8089**	**8095****
Haplotypes/Reference	GenBank Accession Numbers														
Crozier and Crozier 1993	NC_001566[1]	T	C	A	T	C	A	G	G	C	T	C	C	C	T
Martimianakis *et al.* 2011	GU060466[2]	*	.	.	.	A	.	.	.	.	.	T	.	T	.
	GU060467[2]	*	.	.	.	A	G	.	.	.	.	.	.	T	.
	GU060468[2]	*	.	.	.	A	.	.	.	.	.	.	.	T	.
	GU060469[3]	*	.	.	.	A	.	.	A	.	.	.	.	T	.
	GU060470[3]	*	.	.	.	.	.	.	.	.	.	.	.	.	.
	GU060471[3]	*	.	.	.	.	.	.	.	.	.	.	.	T	.
	GU060472[3]	*	.	.	A	A	.	.	.	.	.	.	.	T	.
	GU060473[3]	*	T	.	.	A	.	.	.	T	C	T	T	T	.
New – ANATO1	JN410833[4]	A	.	.	.	A	.	.	.	.	.	.	.	T	.
New – ANATO2	JN410834[4]	.	.	T	.	A	.	.	.	.	.	.	.	T	.
New – ANATO3	JN410835[4]	.	.	.	.	A		C	.	.	.	.	.	T	.
New – CAUCA1	JN410836[4]	.	.	.	.	A	.	.	.	.	.	.	.	T	G
New – MEDA1	JN410837[4]	.	.	.	.	A	.	.	.	.	.	.	.	T	.

[1] NC_001566: 16343 bp of *Apis mellifera ligustica* complete mitochondrial genome.[2] GU060466-GU060468: 553 bp of ND5 gene (bases from 7595 to 8147); [3] GU060469-GU060473: 687 bp of ND5 gene (bases from 7461 to 8147); [4] 782 bp of ND5 gene (bases from 7416 to 8197) of Turkish honey bees found in this study. . indicates identical nucleotides at that site. * means there is no sequence information at that site of the haplotype. ** Variable sites that are newly found in this study are indicated in bold numbers.

lineage. This finding was expected because the Turkish populations consist mainly of *A. m. anatoliaca*, *A. m. caucasica* and *A. m. meda*.

Previously, we had shown the phylogenetic relationships and PCR-RFLP profile of Turkish honey bees using several restriction enzymes in both the mitochondrial and nuclear genome of *Apis mellifera* (Özdil *et al*. 2009, 2011). In this study we presented a comprehensive sequencing analysis of the ND5 mitochondrial gene segment to verify genetic divergence in Turkish honey bee populations. We found five different haplotypes for the ND5 gene segment.

Sequencing of the ND5 mitochondrial region has been studied less than the other mitochondrial regions. Only eight different haplotypes of *Apis mellifera* were reported in this region. Here we added five new haplotypes in this study (Table 2). Three came from the Anatolian geographical locations, one from each Iranian and Caucasian geographical location. While ANATO1 (JN410833) haplotype was only obtained in some of the honeybees from SIZ population, ANATO2 (JN410834) was found in both SIZ and ELM populations. These two haplotypes were found to be the rarest Anatolian haplotypes whereas ANATO3 (JN410835) haplotype was found in a wide geographical area such as BAL, BOL, AKO, SEL and SUZ. Caucasian haplotype, CAUCA1 (JN410836), was obtained from ARD, KAZ and AUZ where ARD was the center of Caucasian honey bees. And MEDA1 (JN410837) haplotype was found in honey bees from the South-East part of Turkey where mostly *A. m. meda* is found.

The base substitutions at position 7830 (C→A) and 8089 (C→T) which were reported in Martimianakis *et al.* (2011), were observed in all of the Turkish honeybees in this study. The NJ dendogram (Fig. 2) based on ND5 sequences of the two surveys showed that that these races cannot be discriminated from each other since all of them belong to East European (C) lineage. But it is seen that Haplotype 1-4 (Genbank records: GU060466-GU060469) in Martimianakis *et al.* (2011) and the haplotypes that are found in Turkish honeybees are much closer than the other haplotypes.

In addition to previous findings of the ND5 gene, here we reported sequencing of this mitochondrial DNA gene segment in Turkish honey bees and compared these results with other East European races. Our data showed

that samples from SIZ (Konya/Sızma) and ARD (Ardahan), having unique haplotypes, maintain their native origin and they might be pure *A. m. Anatoliaca* and *A. m. caucasica,* respectively. High migratory beekeeping activity and the use of Caucasian queens for queen rearing in Turkey have resulted in the loss of genetic diversity. So it is highly important to identify the genetic structure of local honey bee races and improve strategies to conserve them in their areas.

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