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GENETIC DIVERGENCE AND PHYLOGENETIC RELATIONSHIPS OF HONEY BEE POPULATIONS FROM TURKEY USING PCR-RFLP's ANALYSIS OF TWO mtDNA SEGMENTS

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Abstract

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The genetic structure and phylogenetic relationship among honey bee populations of Turkey were studied using RFLP analysis on two PCR-amplified mtDNA gene segments (COI, 16s rDNA). The honey bees were sampled from 54 mainland localities of Turkey and 2 Aegean islands. Two different mitotype were detected with SspI digestion of COI gene. One mitotype was seen in only central Anatolia.

The results of this research were compared with analogous studies on honey bee populations from Greece and it was found that the non-existence of 16s rDNA /DraI digestion is diagnostic only for Turkish honey bee populations. This result is very useful for the control of conservation of local honey bees, as the movement of colonies across the border line of these neighboring countries, may affect the genetic structure of honey bee populations.

Key words: Apis mellifera; genetic divergence; mtDNA; conservation; Turkey

Introduction

Evolutionary branches and lineages of Apis mellifera

The western honeybee originated in Asia and invaded Africa and Europe in three distinct evoluationary branches, branch (A), which included the subspecies from Africa (*A. m. lamarckii*, *A. m. yemenitica*, *A. m. scutellata*, *A. m. litorea*, *A. m. adansonii*, *A. m. capensis*), branch (M) which included the subspecies of North African and West European (*A. m. mellifera*, *A. m. iberica*, *A. m. intermissa* and

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branch (C) which included the subspecies from Eastern Europe, Northern Mediterranean and Middle East. Later on, subspecies in branch C were divided into two groups, branch C included *A. m. carnica*, *A. m. ligustica*, *A. m. macedonica*, *A. m. cecropia* and *A. m. sicula*, branch O included the Near and Middle Eastern subspecies (*A. m. caucasica*, *A. m. armeniaca*, *A. m. meda*, *A. m. anatoliaca*, *A. m. syriaca*, *A. m.cypria*, *A. m. adami*) (Ruttner, 1988).

The phylogenetic relationships based on molecular data (Cornuet and Garnery, 1991; Garnery et al., 1992; Arias and Sheppard, 1996) agree in general with those obtained with morphometrical data (Ruttner, 1988), except the fourth branch (O) which has recently been confirmed using mitochondrial and microsatellite variability (Franck et al., 2000; Palmer et al., 2000; Kandemir et al,. 2006a).

A. mellifera subspecies in Turkey

Southwest Asia included Anatolia is a zone of high morphological diversification and evolution for honeybees. Many clearly distinct races have been evolved in this region, which include a diversity of habitats. Honeybee races in this region include the subspecies, *A. m. anatoliaca*, *A. m. caucasica*, *A. m. meda* and *A. m. syriaca* (Ruttner, 1988).

Honeybee subspecies from Anatolia were studied extensively using morphometric and izoenzymic analysis (Asal et al., 1995; Guler and Kaftanoglu 1999a, 1999b; Guler 2001; Guler et al., 2002; Kandemir and Kence, 1995; Kandemir et al., 2000, 2005; Kekecoglu, 2007, Kekecoglu unpubl. data); molecular markers, mitochondrial DNA (mtDNA) analysis (Smith et al., 1997; Palmer et al., 2000; Kandemir et al., 2006a,b); microsatellite and RAPD analysis (Kence et al. 2003; Ivgin et al., 2004; Ivonava et al., 2004; Kandemir et al., 2006a; Bodur et al., 2004, 2007; Цzdil et al., 2009) and *A. m. carnica* have been recorded in Thrace (Smith et al., 1997; Kence et al., 2003; Kandemir et. al., 2000, 2005; Bodur et al., 2004, 2007).

The mtDNA is a favourite tool in systematic and population biology. It is generally maternally inherited without recombination. Only maternal inheritance of mtDNA has been demonstrated for honeybees (Smith 1991; Meusel and Moritz, 1993; Arias and Sheppard, 1996; Francisco et al., 2001; Pinto et al., 2003).

The aim

In the present investigation honey bee populations from 56 localities from all over Turkey were studied using RFLP's analysis of two mtDNA gene segments. The main aim of this research was to determine the extent of mtDNA variation of honey bees distributed in Turkey and to compare the results with these of analogous studies on honey bees from Greece, as the movement of colonies across the border line of these neighboring countries, may affect the genetic structure of honey bee populations.

Materials and Methods

Bees from 182 colonies were collected from 56 localities in Turkey and transported to the laboratory

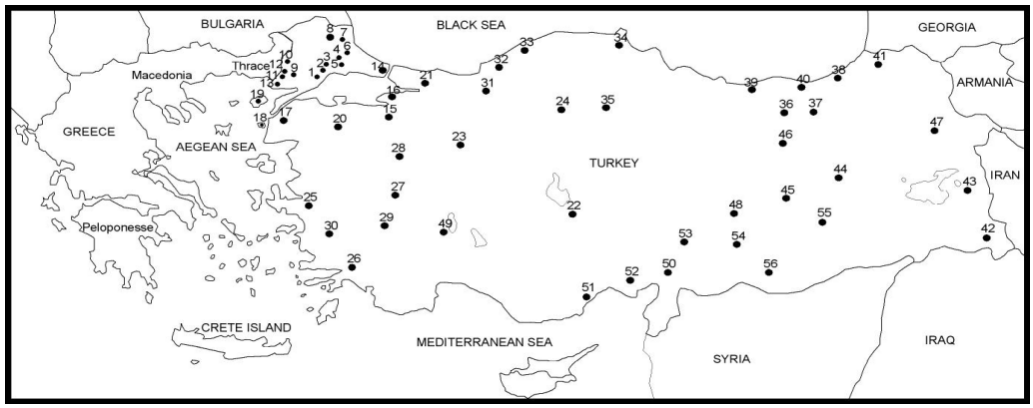

Fig. 1. Sampling site on the map of Turkey

Table 1
The 56 sampling sites grouped in 7 geographic regions of Turkey

Region		Localities
	1	Malkara
	2	Hayranbolu
	3	Muratlı
	4	Cerkezköy
	5	Corlu
	6	Saray
	7	Lüleburgaz
	8	Kırklareli
MARMAR	9	Kesan
	10	Meric
	11	Kocahıdır
	12	Ipsala
	13	Enez
	14	Istanbul
	15	Bursa
	16	Yalova
	17	Canakkale
	18	Bozcaada
	19	Gökceada
	20	Balıkesir
	21	Izmit
	22	Aksaray
CANATO	23	Eskisehir
	24	Cankırı
	25	İzmir
	26	Mugla
	27	Usak
AEGEAN	28	Kütahya
	29	Denizli
	30	Aydın
	31	Düzce
	32	Zonguldak
	33	Bartın
	34	Sinop
	35	Corum
BLKSEA	36	Gümüshane
	37	Bayburt
	38	Rize
	39	Giresun
	40	Trabzon
	41	Artvin
	42	Hakkari
	43	Van
	44	Bingol
EANAT	45	Elazıg
	46	Erzincan
	47	Agrı
	48	Malatya
	49	Isparta
	50	Osmaniye
MEDITE	51	Mersin
	52	Adana
	53	Maras
	54	Adıyaman
SEANAT	55	Diyarbakır
	56	Urfa

into small plastic vials with 96 % alcohol and stored at 4°C until used. According to morphometrical data, the honey bee populations were endemic to these areas (Kekecoglu 2007) (Figure 1, Table 1).

Total genomic DNA was extracted (two individuals/colony) according to the protocol of Hunt and Page (1992), after minor modifications (Bouga et al., 2005).

The mt DNA variation was analyzed by RFLP's, performed on PCR amplified products. The polymerase chain reaction (PCR) (Saiki et al., 1988) was performed as in Bouga et al. (2005).

The 16s rDNA gene segment was digested with *Sau3A I*, *Ssp I*, *Dra I*, *Hinc II*, *EcoR I*, *Pst I* and *Alu I* restricted enzymes and CO I gene segment with *Nco I*, *Sau3A I*, *Fok I*, *Bcl I*, *Ssp I*, *Sty I* and *Xho I* (Bouga et al., 2005).

The digested segments were then separated electrophoretically on 2% agarose gels in 0.5 x TBE buffer, stained with ethidium bromide and visualized under UV light. The sizes of DNA fragments were compared to the PCR marker (Promega G316A, Promega Corp.) run on the same gel and were calculated using DNA frag 3.03 (Nash, 1991) program. Composite genotypes for each individual were then defined from all the restriction patterns of the two mtDNA segments. The restriction fragment data were converted to restriction data (gain or loss of restriction site).

The evolutionary distance from restriction site data (Nei and Tajima, 1981; Nei and Miller, 1990) was estimated using the REAP computer package (McElroy et al., 1991). Phylogenetic tree was constructed by the UPGMA (Sneath and Sokal, 1973) method, based on evolutionary distance from restriction site data, using the PHYLIP (version 3.4) (Felsenstein, 1993) software package. The tree was drawn using TREEVIEW program (Page, 1996). Results from analogous study on honey bees from different areas from Greece: Central Greece, Phthiotida (PHT), Northern Greece, Macedonia (MAC) and the Aegean islands, Kythira (KTH), Kasos (KAS), Ikaria (IKA) (Bouga et al., 2005) were included in the above mentioned statistical process.

Results

The sizes of PCR-amplified mtDNA segments for all populations examined were found to be approximately lengths about 964bp and 1028bp for 16s rDNA and CO I gene segments respectively. Seven restriction enzymes had at least one recognition site on the amplified 16s rDNA and CO I segments. The restriction enzymes used generated a total of 16 restriction sites corresponding to an estimated average number of 78 bases surveyed in Turkish honey bees. Fragment patterns produced by each restriction enzyme for the two mtDNA segments are presented in Tables 2 and 3. Diagnostic patterns were revealed discriminating Turkish honey bee populations (pattern type B) from the Greek ones (pattern type A) after the digestion of 16s rDNA gene segment with the restriction enzyme *Dra I* which recognizes sites in Greek honey bee populations but not in Turkish honey

Table 2

Fragment size estimates (in base pairs) of all fragment patterns observed on mtDNA 16s rDNA gene segment among the populations studied

16s rDNA								
Sau3AI		Ssp I	Dra I		Hinc II	EcoR I	Pst I	Alu I
A	B	A	A	B	A	A	A	A
964	548	628	557	964	598	492	621	572
	416	336	407		366	472	343	392

Table 3

Fragment size estimates (in base pairs) of all fragment patterns observed on mtDNA CO I gene segment among the populations studied

CO I									
Nco I		Sau3AI	Fok I	Bcl I	Ssp I		Sty I		Xho I
A	B	A	A	A	A	B	A	B	A
	1028	371	476	465	487	530	1028		616
595		349	425	326	277	498		626	412
433		280	127	237	264			402	
		28							

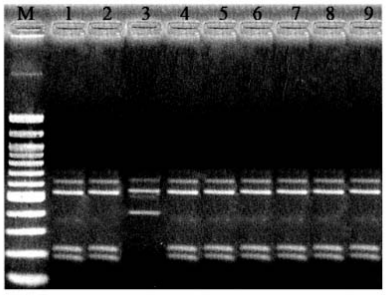

Fig. 2. *SspI* Resitriction banding pattern of amplified COI subunite gene lane 1,2: Marmara (MARMAR); lane 3: Central Anatolia (CANATO); lane 4: Aegean (AEGEAN); lane 5,6: Black Sea (BLKSEA); lane 7: East Anatolia (EANAT); lane 8: Mediterrian (MEDITE); lane 9: Southeast Anatolia (SEANAT)

bees. Macedonian honey bees from Greece are also discriminating from Turkish honey bees after the digestion of CO I gene segment with the restriction enzymes *NCO I* and *Sty I* (patterns A and B respectively).

Comparing the results from previous analogous investigation (Bouga et al., 2005) as concerning the main haplotype that has been found in honey bee populations from Greece and Turkish honey bees, in 16s rDNA gene segment digested with *Sau3A I* exhibit different patterns (A for Greece and B for Turkey).

It is very interesting that honey bees from Central Anatolia are discriminating from all Greek as well as Turkish honey bee populations as concerning the digestion of CO I gene segment with the restriction enzyme *Ssp I* (pattern B) (Figure 2) .

The haplotypes (composite genotypes) that were detected are presented in Table 4. The evolutionary distance from restriction site data is shown in Table 5; the most distant honey bee population is this of Central Anatolia. The phylogenetic tree is shown in Figure 3, produced by the UPGMA method based on evolutionary distance from restriction site data. As it is shown, Greek and Turkish honey bees are grouped in two different clades and the honey bees from Central Anatolia (CANOTO) are discriminating from the rest ones, as well as Macedonian honey bees (MAC) from Greece.

Discussion

The honey bees of Turkey, a country which encompasses a wide range of climates and habitats within its borders, belong to two different evolutionary lineages (C and O) as it is mentioned in the Introduction Section. It is noteworthy that several technical improvements introduced in beekeeping management may have interfered with the natural distribution of populations. The importation of foreign queens and the practice of moving colonies several times per year are factors that can affect the genetic structure of a local honey bee population through genetic introgression (Garnery et al., 1998).

Based on mtDNA analysis, Smith et al. (1997)

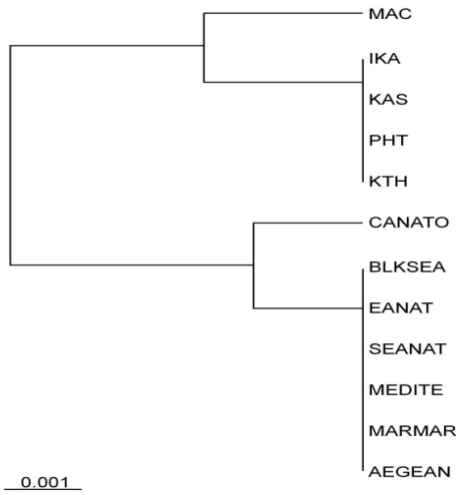

Fig. 3. UPGMA (Sneath and Sokal, 1973) dendrogram, showing the relationships between the populations studied. Northern Greece, Macedonia (MAC), Ikaria (IKA), Kasos (KAS), Central Greece, Phthiotida (PHT) and the Aegean islands, Kythira (KTH); Central Anatolia (CANOTO), Black Sea (BLKSEA), East Anatolia (EANAT), Southeast Anatolia (SEANAT), Mediterranean (MEDITE), Marmara (MARMAR), Aegean (AEGEAN)

Table 4
Composite genotypes (haplotypes). The different patterns are in bold

Haplotype	Composite genotype													
	16s rDNA							CO I						
	Sau3AI	*Ssp I*	*Dra I*	*Hinc II*	*EcoR I*	*Pst I*	*Aiu I*	*Nco I*	*Sau3A I*	*Fok I*	*Bcl I*	*Ssp I*	*Sty I*	*Xho I*
PHT	A	A	A	A	A	A	A	B	A	A	A	A	A	A
MAC	A	A	A	A	A	A	A	A	A	A	A	A	B	A
KTH	A	A	A	A	A	A	A	B	A	A	A	A	A	A
KAS	A	A	A	A	A	A	A	B	A	A	A	A	A	A
IKA	A	A	A	A	A	A	A	B	A	A	A	A	A	A
MARMAR	B	A	B	A	A	A	A	B	A	A	A	A	A	A
CANATO	B	A	B	A	A	A	A	B	A	A	A	B	A	A
AEGEAN	B	A	B	A	A	A	A	B	A	A	A	A	A	A
BLKSEA	B	A	B	A	A	A	A	B	A	A	A	A	A	A
EANAT	B	A	B	A	A	A	A	B	A	A	A	A	A	A
MEDITE	B	A	B	A	A	A	A	B	A	A	A	A	A	A
SEANAT	B	A	B	A	A	A	A	B	A	A	A	A	A	A

claimed that honey bee populations from Thrace belong to *A. m. carnica* and are included in C lineage. Later on, Palmer et al. (2000) and Kandemir et al. (2006a) concluded that Thrace populations are not different from Anatolian honey bee populations. Our results are consistent with those of Palmer et al. (2000) and Kandemir et al. (2006a).

Comparing our findings with these of Bouga et al. (2005), honey bees in Turkey and Greece were found to be well discriminated; it is noted that for the first time are reported diagnostic patterns that distinguish the honey bee populations of these neighboring countries. Honey bees from Turkey are also discriminating from Greek Macedonian honey bees.

Taking in consideration the research of Harizanis and Bouga (2003), the results of our investigation show that the main haplotype of Turkish honey bees, excluding non-existence of 16s rRNA/*DraI* restriction site, look alike Crete island's population (*A. m adami*), perhaps of a possible common genetic origin.

It is very interesting that a unique haplotype is found in honey bees from Eskisehir region in Central Anatolian. It is not clear whether this haplotype is as a result of importation of foreign queens or that pure race of *A. m. anatoliaca* occurs in this region.

The above mentioned results are very useful for the conservation of Turkish honey bees but further investigation is necessary.

Acknowledgments

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General Summary

The genetic structure and pylogenetic relationship among honey bee populations of Turkey were studied using RFLP's analysis on two PCR-amplified mtDNA gene segments (COI, 16s rDNA). The honey bees were sampled from 54 mainland localities of Turkey and 2 Aegean islands (Gokceada, Bozcaada). The 16s rDNA gene segment was digested with

Table 5
Evolutionary distance from restriction site data (×102)

	PHT	KTH	KAS	IKA	MAC	MARMAR	AEGEAN	MEDITE	SEANAT	EANAT	BLKSEA	CANATO
PTH												
KTH	0.00											
KAS	0.00	0.00										
IKA	0.00	0.00	0.00									
MAC	1.38	1.38	1.38	1.38								
MARMAR	1.48	1.48	1.48	1.48	2.87							
AEGEAN	1.48	1.48	1.48	1.48	2.87	0.00						
MEDITE	1.48	1.48	1.48	1.48	2.87	0.00	0.00					
SEANAT	1.48	1.48	1.48	1.48	2.87	0.00	0.00	0.00				
EANAT	1.48	1.48	1.48	1.48	2.87	0.00	0.00	0.00	0.00			
BLKSEA	1.48	1.48	1.48	1.48	2.87	0.00	0.00	0.00	0.00	0.00		
CANATO	2.40	2.40	2.40	2.40	3.92	0.79	0.79	0.79	0.79	0.79	0.79	

Sau3A I, Ssp I, Dra I, Hinc II, EcoR I, Pst I and Alu I restricted enzymes and CO I gene segment with Nco I, Sau3A I, Fok I, Bcl I, Ssp I, Sty I and Xho I.

The evolutionary distance from restriction site data was estimated using the REAP computer package. Phylogenetic tree was constructed by the UPGMA method, based on evolutionary distance from restriction site data, using the PHYLIP (version 3.4) software package. The tree was drawn using TREEVIEW program. Results from analogous study on honey bees from different areas from Greece: Central Greece, Phthiotida (PHT), Northern Greece, Macedonia (MAC) and the Aegean islands, Kythira (KTH), Kasos (KAS), Ikaria (IKA) were included in the above mentioned statistical process.

Fragment patterns produced by each restriction enzyme for the two mtDNA segments are presented in Tables 2 and 3. Diagnostic patterns were revealed discriminating Turkish honey bee populations (pattern type B) from the Greek ones (pattern type A) after the digestion of 16s rDNA gene segment with the restriction enzyme Dra I which recognizes sites in Greek honey bee populations but not in Turkish honey bees. Macedonian honey bees from Greece are also discriminating from Turkish honey bees after the digestion of CO I gene segment with the restriction enzymes NCO I and Sty I (patterns A and B respectively). It is very interesting that honey bees from Central Anatolia are discriminating from all Greek as well as Turkish honey bee populations as concerning the digestion of CO I gene segment with the restriction enzyme Ssp I (pattern B). The phylogenetic tree is shown in Figure 3, produced by the UPGMA method based on evolutionary distance from restriction site data. As it is shown, Greek and Turkish honey bees are grouped in two different clades and the honey bees from Central Anatolia (CANOTO) are discriminating from the rest ones, as well as Macedonian honey bees (MAC) from Greece. The above mentioned results are very useful for the conservation of Turkish honey bees but further investigation is necessary.

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