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Genetic variation in Turkish honeybees *Apis mellifera anatoliaca*, *A. m. caucasica*, *A. m. meda* (Hymenoptera: Apidae) inferred from RFLP analysis of three mtDNA regions (16S rDNA-COI-ND5)

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Abstract. In this study, the genetic structure of Turkish honey bee (*Apis mellifera* L.) populations, mainly obtained from the Central Anatolian region, were investigated at three different mitochondrial regions. A total of 165 worker bees were collected from 15 different populations in ten different locations. Portions of the mitochondrial 16S ribosomal RNA (16S rDNA), cytochrome C oxidase I (COI) and NADH dehydrogenase 5 (ND5) genes were amplified by PCR and then subjected to RFLP pattern analysis using 18 restriction enzymes (these having at least one recognition site in each region were used). Nucleotide polymorphisms were revealed using restriction enzymes *Bsp*143I, *Dra*I and *Ssp*I in 16S rDNA and *Taq*I in the COI gene segment. The polymorphisms were subsequently confirmed by direct DNA sequencing with sequences thereafter deposited in Genbank. In this study, six novel composite genotypes (haplotypes) were found in Turkish honey bee populations. The most common haplotype, type 1, was found in 12 of the sampled populations and overall accounted for 85.5% of the samples. TCS spanning network of haplotypes revealed that type 1 was the basal haplotype. Genetic distance (*D*) values were found to be low (0.0–0.0112) within Turkish honey bee populations. The average haplotype diversity (*h*) within populations was 0.082. Molecular phylogenetic analysis revealed that Konya/Sızma, Antalya/Elmalı and Konya/Selçuklu populations were the most distant from all the other Turkish honey bee populations surveyed.

INTRODUCTION

The western honey bee, *Apis mellifera* L., is native to Africa, the Near and Middle East, and Europe. Based on morphometric analysis, Ruttner (1988) grouped honey bee subspecies into four geographic branches: a south and central African branch (A), a north African and west European branch (M), an east European and north Mediterranean branch (C), and a Near and Middle Eastern branch (O). Subsequent studies using morphometry, along with mitochondrial DNA and microsatellite marker analysis, have confirmed or modified the species distributions within these groups and have added a new Middle Eastern branch from Yemen (Y) (Hall & Smith, 1991; Arias & Sheppard, 1996; Kauhausen-Keller et al., 1997; Smith et al., 1997; Palmer et al., 2000; 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, Adl et al., 2007); however, mtDNA analysis showed that they in fact belonged 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). From his morphometric analyses, Ruttner (1988) concluded that *A. m. anatoliaca*, *A. m. caucasica*, and *A. m. meda* all exist in Turkey. Nearly all of the geographical landmass of Turkey is seemingly occupied by *A. m. anatoliaca*, including European Turkey, except for the northeastern part where *A. m. caucasica* is found, whilst *A. m. meda* occurs in the southeastern part of the country. Recently, mitochondrial studies of Turkish honeybees have shown that *A. m. carnica* is also found in the Thrace region of European Turkey (Palmer et al., 2000), whilst *A. m. syriaca* is found in the southern part of the country near Hatay (Kandemir et al., 2006).

PCR-RFLP using mtDNA markers of honeybees have been particularly useful in differentiating evolutionary lineages and groups of subspecies (Hall & Smith, 1991; Garnery et al., 1992, 1993; Franck et al., 2000; Palmer et al., 2000). PCR-RFLP with *Dra*I restriction enzyme analysis of the cytochrome C oxidase COI and COII (COI–COII) intergenic region revealed more than 50 different haplotypes, mainly in lineages A and M and with less in lineage C (Garnery et al., 1992, 1993; Franck et al., 2000, 2001; Palmer et al., 2000; De la Rúa et al., 2006; Suppasat et al., 2007; Munoz et al., 2009; Nedic et al., 2009; Özdil et al., 2009a; Solorzano et al., 2009; Magnus & Szalanski, 2010). More recently, the other mitochondrial regions including 16S ribosomal DNA (rDNA) (Bouga et al., 2005; Collet et al., 2007; Kekeçoğlu et al., 2009), NADH dehydrogenase-2 (ND2) (Arias & Sheppard, 1996; Kandemir et al., 2006), and NADH dehydrogenase-5 (ND5) regions (Bouga et al., 2005; Martimianakis et al., 2011; Özdil et al., 2012), along with the COI region (Tanaka et al., 2001; Bouga et al., 2005; Kekeçoğlu et al., 2009; Sheffield et al., 2009; Martimianakis et al., 2011), were used to differentiate evolutionary lineages and groups of honey bee subspecies.

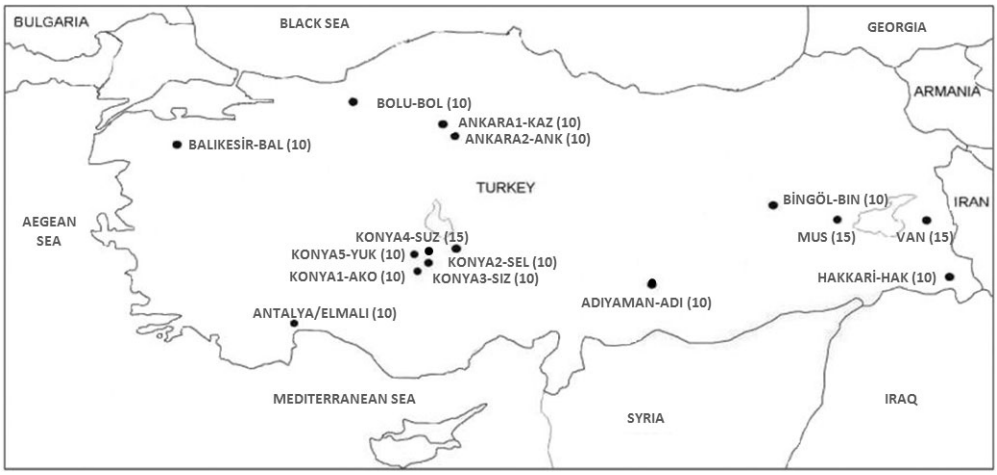

Fig. 1. Sampling locations of honey bees in Turkey. The number bracketed in parenthesis shows the number of colonies analyzed at each site.

The objective of the present research was to determine the level of genetic differentiation among and between Turkish honeybee populations as discriminated using PCR-RFLP pattern analysis of the 16S rDNA, COI and ND5 gene regions. mtDNA length variation and levels of genetic diversity as found in this study were compared with the results of other earlier mitochondrial studies of honey bees, such comparison thereby allowing much more complete assessment of the genetic structure of Turkish honey bee populations than hitherto previously possible using morphometrics alone.

MATERIAL AND METHODS

Sampling of the honeybees

Worker honey bees were collected between May 2008 and September 2009 from a total of 165 colonies from 15 different populations in ten different locations in Turkey (Fig. 1, Table 1). One sample was taken from each colony, these deriving from established colonies maintained by local (i.e. "none migratory") beekeepers. These samples were used for PCR-RFLP analysis of the three mtDNA regions studied.

DNA isolation

Worker bees were individually placed in 1.5 mL Eppendorf tubes containing 1.0 mL of 95% ethanol for transportation to the laboratory. Before DNA extraction, bees were rinsed in distilled water and air dried for about half an hour. Total genomic DNA was extracted from single bee thoraces according to Hall (1990). The concentration and purification of genomic DNA was quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific, Inc. Wilmington, Delaware, USA) spectrophotometer, and 20 ng of genomic DNA was used for the PCR.

RFLP Analysis

Mitochondrial regions were amplified according to Bouga et al. (2005). Three sets of primers were used for amplifying 16S rDNA, COI and ND5 gene regions, respectively. These were 5'-CAACATCGAGGTCGCAAACATC-3' and 5'-GTACCTTTT GTATCAGGGTTGA-3' for 16S rDNA, 5'-GATTACTTCCTC CCTCATTA-3' and 5'-AATCTGGATAGTCTGAATAA-3' for COI and 5'-TCGAAATGAATAGGATACAG-3' and 5'-GGTTGAGATGGTTTAGGATT-3' for the ND5 segment. PCR was run in a total volume 25 µl of the following reaction mixture: 2.5 µl of 10× reaction buffer with KCl as provided by the manufacturer (Fermentas Life Sciences, Vilnius, Lithuania), 2

TABLE 1. Sampling localities, geographical positions and number of colonies used for RFLP analyses.

Locations	Abbreviation of the locations	Geographical position		# Colonies analyzed for RFLP analysis
Adıyaman	ADI	37°46′N	38°16′E	10
Ankara1 / Kazan	KAZ	39°58′N	32°52′E	10
Ankara2	ANK	40°12′N	32°41′E	10
Antalya / Elmalı	ELM	36°44′N	29°56′E	10
Balıkesir	BAL	39°39′N	27°53′E	10
Bingöl	BIN	39°00′N	40°41′E	10
Bolu / Yığılca	BOL	40°58′N	31°27′E	10
Hakkari	HAK	37°35′N	43°34′E	10
Konya1 / Akören	AKO	37°27′N	32°22′E	10
Konya2 / Selçuklu	SEL	37°57′N	32°26′E	10
Konya3 / Sızma	SIZ	38°05′N	32°24′E	10
Konya4	SUZ*	38°02′N	32°30′E	15
Konya5 / Yükselen	YUK	38°04′N	32°20′E	10
Muş / Varto	MUS	39°17′N	41°12′E	15
Van / Gevaş	VAN	38°18′N	43°06′E	15
Total				165

*SUZ – The Apiary of the Selçuk University.

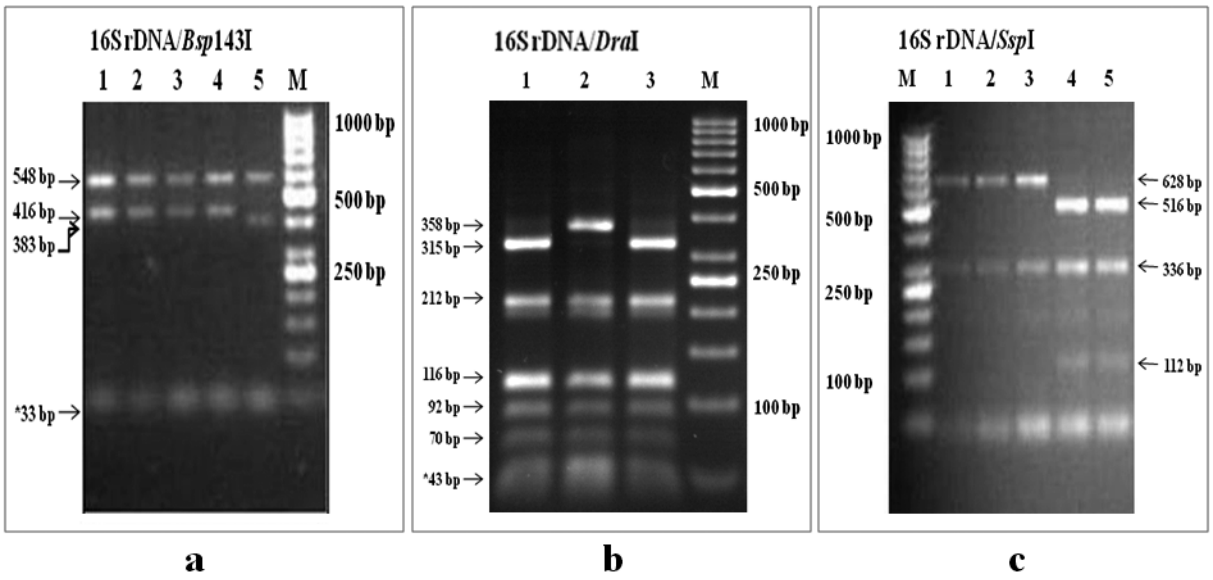

Fig. 2. *Bsp*143I, *Dra*I and *Ssp*I restriction patterns found in 16S rDNA gene regions of Turkish honey bee samples. a – *Bsp*143I digestion of 16S rDNA gene segment, lane 1-4: B type (548, 416 bp), lane 5: C type (548, 383, 33 bp); b – *Dra*I digestion of 16S rDNA gene segment, lane 1-3: C type (315, 212, 116, 116, 92, 70, 43 bp), lane 2: D type (358, 212, 116, 116, 92, 70 bp); c – *Ssp*I digestion of 16S rDNA gene segment, lane 1-3: A type (628, 336 bp), lane 4-5: B type (516, 336, 112 bp). M – DNA size marker.

mM $MgCl_2$, 2 mM of dNTP mix, 0.5 µM of each primer, 2 U of *Taq* polymerase and 20 ng of total purified honey bee DNA. For each primer pair, the following reaction profile was used: initial denaturation 94°C for 4 min, 35 cycles of 94°C for 1 min, annealing at 55°C (16S rDNA and COI) or 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% agarose gel to verify the size of the fragment. Amplified mtDNA regions from two individuals of each population were digested with 18 restriction enzymes to check for the presence of recognition sites. The informative restriction enzymes were then analyzed using ten to fifteen individuals from each population. The informative restriction enzymes used for the 16S rDNA gene fragment were: *Alu*I, *Bsp*143I (*Sau*3AI), *Dra*I, *Eco*RI, *Hin*cII, *Hph*I, *Pst*I, *Ssp*I, *Taq*I, *Vsp*I (*Ase*I); for the COI gene fragment *Alu*I, *Bcl*I, *Bse*GI (*Fok*I), *Bsp*143I (*Sau*3AI), *Hph*I, *Ssp*I, *Taq*I, *Xho*I, and for the ND5 gene fragment *Alu*I, *Dra*I, *Rsa*I, *Ssp*I, *Taq*I and *Vsp*I (*Ase*I).

The digested fragments were separated electrophoretically on 2% or 3% agarose gels in 1× TBE buffer, stained with ethidium bromide and photographed using a Vilber Lourmat gel imaging system. DNA fragment sizes were determined using Digital Image analysis software (Vilber Lourmat Deutschland GmbH Eberhardzell, Germany). The different restriction fragments detected were sequenced on an ABI Prism 310 automated sequencer (Applied Biosystems, Foster City, CA, USA) using standard protocols in order to verify the sequence variations. Sequences were aligned with the computer program Clustal X (Thompson et al., 1997).

Data analysis

Composite genotypes (haplotypes) for each individual were grouped from all the restriction patterns of the three mtDNA

TABLE 2. Restriction fragment patterns generated from analysis of 16S rDNA gene segment of Turkish honey bees.

16S rDNA		COI		ND5	
Restriction enzyme	Patterns observed (bp)	Restriction enzyme	Patterns observed (bp)	Restriction enzyme	Patterns observed (bp)
*Alu*I	B: 507, 392, 65	*Alu*I	A: 728, 300	*Alu*I	C: 554, 211, 57
*Bsp*143I (*Sau*3AI)	B: 548, 416 C: 548, 383, 33	*Bcl*I	C: 462, 317, 249	*Dra*I	B: 440, 270, 112
		*Bse*GI (*Fok*I)	B: 631, 318, 79	*Rsa*I	A: 792, 30
*Dra*I	C: 315,212,116,116,92,70,43 D: 358, 212, 116, 116, 92, 70	*Bsp*143I (*Sau*3AI)	B: 340, 317, 171, 78, 77, 45	*Ssp*I	B: 401, 192, 93, 82, 54
		*Hph*I	A: 925, 103	*Taq*I	B: 279, 240, 205, 98
*Eco*RI	B: 483, 481	*Ssp*I	C: 523, 213, 175, 85, 32	*Vsp*I (*Ase*I)	A: 228, 217, 198, 114, 65
*Hin*cII	B: 584, 380	*Taq*I	A: 432, 352, 244 B: 352, 262, 244, 170		
*Hph*I	A: 490, 474				
*Pst*I	B: 573, 391	*Xho*I	A: 616,412		
*Ssp*I	A: 628, 336 B: 516, 336, 112				
*Taq*I	A: 545,419				
*Vsp*I (*Ase*I)	A: 320, 243, 143, 129, 76, 53				

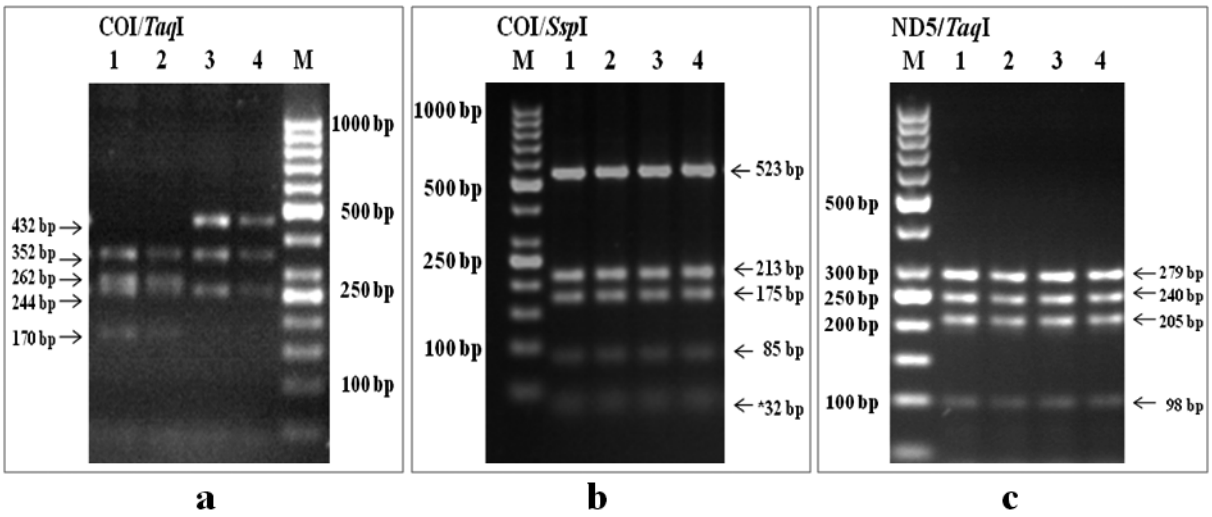

Fig. 3. *Taq*I restriction patterns in COI and ND5 genes and *Ssp*I restriction pattern in COI gene region of Turkish honey bee samples. a – *Taq*I digestion of COI gene segment, lane 1-2: B type (352, 262, 244, 170 bp), lane3-4: A type (432, 352, 244 bp); b – *Ssp*I digestion of COI gene segment, lane 1-4: C type (523, 213, 175, 85, 32); c – *Taq*I digestion of ND5 gene segment, lane 1-4: B type (279, 240, 205, 98). M – DNA size marker.

amplicons tested. Haplotype diversity between populations was estimated according to Nei & Tajima (1981) and Nei (1987). Genetic distances (Nei, 1978) among haplotypes and frequency distribution among and between populations were calculated using the Restriction Enzyme Analysis Package REAP (McElroy et al., 1991).

While Neighbor-Joining, Maximum Parsimony and Maximum Likelihood methods all have difficulties in resolving relationships among closely related haplotypes, statistical parsimony allows display of genealogical relationships among sequences with a limited number of mutations. The results of statistical parsimony among six haplotypes were represented using a network calculated with TCS version 1.21 software (Clement et al., 2000).

RESULTS

The sizes of the PCR-amplified mtDNA regions for all populations studied were found to average 964 bp for 16S rDNA, 1028 bp for COI and 822 bp for ND5 mtDNA gene regions. Ten, eight and six restriction enzymes were found to have at least one restriction site in the amplified 16S rDNA, COI and ND5 regions, respectively. Observed fragment patterns generated by each restriction enzyme for the three mtDNA regions are summarized in Table 2.

*Bsp*143I, *Dra*I and *Ssp*I restrictions in 16S rDNA (Fig. 2) and *Taq*I restriction in the COI gene each generated two different restriction profiles in Turkish honeybees (Fig. 3).

Diagnostic and novel patterns were revealed in the Konya/Sızma (SIZ) and Antalya/Elmalı (ELM) populations after the digestion of 16S rDNA segment with the restriction enzyme *Bsp*143I (pattern type C). The nucleotide sequences of the pattern type B and type C of 16S rDNA gene segment (Fig. 2) were deposited in Genbank with accession numbers JF825878 and JF825879, respectively. In the pattern type B, a C→T transition was found at position 13513 that creates the additional *Bsp*143I restriction site (Fig. 4).

Moreover digestion of 16S rDNA with the restriction enzyme *Dra*I produced two novel restriction patterns (pattern type C and D) which have not been reported previously (Fig. 2). Thus five or six *Dra*I restriction sites were found in 16S rDNA gene segment. In pattern type C, six diagnostic restriction sites were found, and this pattern was seen to be the most common type in Turkish honey bees, whereas pattern type D lacked a *Dra*I restriction site and was only found in some of the

TABLE 3. Composite genotypes (haplotypes), haplotype diversity and sample size of all the populations studied.

Haplotype	Composite genotypes																								Populations															
	16S rDNA										COI								ND5																					
	AluI	*Bsp*1431	*DraI*	*EcoRI*	*HincII*	*HphI*	*PstI*	*SspI*	*TaqI*	*VspI*	*AluI*	*BclI*	*BseGI*	*Bsp*1431	*HphI*	*SspI*	*TaqI*	*XhoI*	*AluI*	*DraI*	*RsaI*	*SspI*	*TaqI*	*VspI*	ADI	KAZ	ANK	ELM	BAL	BIN	BOL	HAK	AKO	SEL	SIZ	SUZ	YUK	MUS	VAN	
Type1	B	B	C	B	B	A	B	A	A	A	A	C	B	B	A	C	A	A	C	B	A	B	B	A	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00	0.60		1.00	1.00	1.00	1.00	
Type 2	B	C	C	B	B	A	B	A	A	A	A	C	B	B	A	C	B	A	C	B	A	B	B	A											0.20					
Type 3	B	C	D	B	B	A	B	B	A	A	A	C	B	B	A	C	A	A	C	B	A	B	B	A				0.30												
Type 4	B	C	C	B	B	A	B	A	A	A	A	C	B	B	A	C	A	A	C	B	A	B	B	A				0.70												
Type 5	B	B	C	B	B	A	B	B	A	A	A	C	B	B	A	C	A	A	C	B	A	B	B	A										0.40						
Type 6	B	B	C	B	B	A	B	A	A	A	A	C	B	B	A	C	B	A	C	B	A	B	B	A											0.80					
	Haplotype diversity (*h*)																								0.00	0.00	0.00	0.47	0.00	0.00	0.00	0.00	0.00	0.53	0.36	0.00	0.00	0.00	0.00	0.082
	N																								10	10	10	10	10	10	10	10	10	10	10	15	10	15	15	

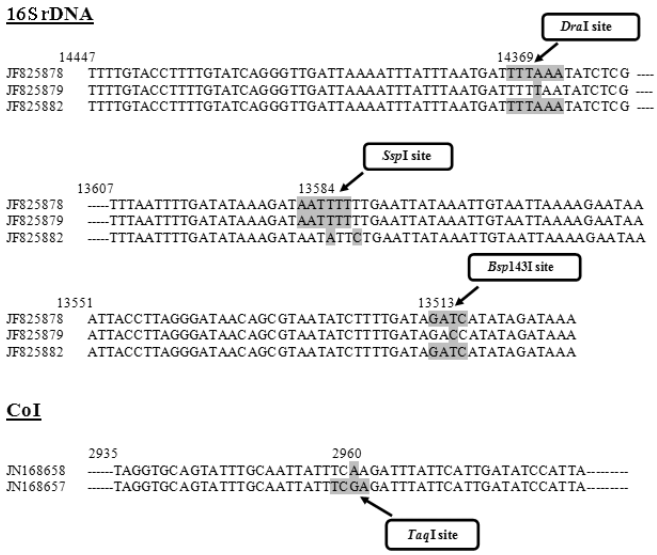

Fig. 4. Nucleotide variations of different restriction sites found to be polymorphic in this study.

Antalya/Elmalı (ELM) populations tested. An A→T transversion deleted one of the *Dra*I restriction site at position 14369, the nucleotide sequences of the pattern type D of the 16S rDNA gene region being deposited in Genbank with accession number JF825879 (Fig. 4).

Diagnostic patterns were also revealed in the Konya/Selçuklu (SEL) and Antalya/Elmalı (ELM) populations after the digestion of 16S rDNA region with the restriction enzyme *Ssp*I (pattern type B) (Fig. 2), a result compatible with Bouga et al. (2005). T→A transversion at position 13584 deleted an *Ssp*I restriction site; this sequence was deposited in Genbank with accession number JF825882 (Fig. 4). A T→C transition at position 13587 was also found in JF825882.

Digestion of COI segment with the restriction enzyme *Taq*I produced different restriction pattern (pattern type B) from the Konya/Sızma (SIZ) population, a pattern that have not been observed before (Fig. 3). An A→G transition at position 2960 produced an additional *Taq*I restriction site; this sequence was deposited in Genbank with accession number JN168657 (Fig. 4).

The six different and novel haplotypes (composite genotypes) which were detected in the fifteen populations studied and the haplotype frequencies and haplotype diversity values are presented in Table 3. Type 1 haplotype was the most common haplotype, found in 85.5% of samples. Rarer haplotypes, type 2–6, type 3–4 and type 5, were found in Konya/Sızma (SIZ), Antalya/Elmalı (ELM) and Konya/Selçuklu (SEL) samples, respectively.

TCS spanning network of haplotypes revealed that type 1 was the basal haplotype and type 3 was the most distantly related to type 1, with three base pair differences, and only occurred in the Antalya/Elmalı population (Fig. 5).

The genetic distance (*D*) values were found to be low (0.0–0.0112) within Turkish honey bee populations. The average haplotype diversity (*h*) within populations was 0.082.

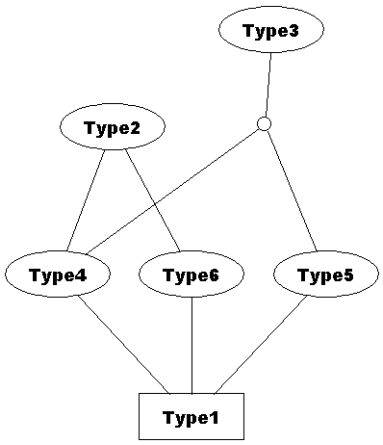

Fig. 5. Statistical parsimony network (TCS software, version 1.21) based on concatenated data sets of 16S rDNA and COI sequences (1992 bp, 5 parsimony informative sites).

DISCUSSION

In a recent study of Turkish honey bee samples, we earlier showed that all insects then tested belonged to the East Mediterranean (C) lineage as found using several restriction enzymes (Özdil et al., 2009a). In the present study, we have used different regions of the mitochondrial genome to verify the nature and distribution of genetic variation within and between Turkish honey bee populations.

When we compare our results with Bouga et al. (2005) and Kekeçoğlu et al. (2009), we find similar restriction profiles with generally small base differences in the fragments, except for *Dra*I, *Ssp*I and *Taq*I digestions in 16S rDNA, COI and ND5 gene regions, respectively (Table 2). In these regions, different restriction profiles were detected in our samples (Figs 2 and 3). *Dra*I restriction in 16S rDNA revealed five or six restriction sites in this study whereas, restriction analyses were previously reported to have revealed one or null restriction sites (Bouga et al., 2005, Kekeçoğlu et al., 2009). Following *Ssp*I digestion, four restriction sites were also found in the COI region (Fig. 3), a result contrasting with the one or two sites previously reported in earlier studies (Bouga et al., 2005; Kekeçoğlu et al., 2009). Similarly, the restriction of the ND5 gene region with *Taq*I revealed three sites (Fig. 3), compared with the two as reported in Greek honeybees (Bouga et al., 2005). The results of the present study were confirmed with complementary sequencing studies performed in order to check for the presence or absence of the restriction sites. The nucleotide polymorphisms responsible for the different restriction profiles were subsequently submitted to Genbank (JF825878, JF825879, JF825882 and JN168657, JN168658)

Overall, two main groups of haplotypes were detected among the six haplotypes from Turkish honey bee populations. The first haplotype (Type 1) was characteristic of nearly all Turkish honey bee populations analyzed (85.5%) except for the Southern part of Turkey, characterized by the remaining haplotypes (Type 2–6). In terms of subspecies, *A. m. caucasica* (KAZ, ANK) and *A. m.*

meda (ADI, BIN, HAK, MUS, VAN) ecotypes were found to show Type 1 haplotype, whereas *A. m. anatoliaca* ecotypes from southern and central region displayed the remaining haplotypes (Type 2 to 6). Type 2–6, type 3–4 and type 5, were found in Konya/Sızma (SIZ), Antalya/Elmali (ELM) and Konya/Selçuklu (SEL) samples, respectively. The provinces of Konya and Antalya are found in the central and southern part of Turkey and are close to each other. These two regions are dominated by *A. m. anatoliaca* and *A. m. anatoliaca* ecotypes, so the existence of unique haplotypes in Konya/Sızma-SIZ (Type 2 and 6) and Antalya/Elmalı-ELM (Type 3 and 4) populations at high frequencies could be attributed to different founder queens since beekeepers from these two regions do not use commercial *A. m. caucasica* queens. However, further follow up studies are needed to characterize mitochondrial DNA variation in the honeybees of these regions along with morphometric analyses in order to accurately characterize the particular regional honeybee populations.

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