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Turkish honey bees belong to the east Mediterranean mitochondrial lineage

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Summary — Honey bees were collected from 12 localities in Turkey. The mitochondrial genomes of these bees were surveyed for the presence or absence of four restrictions sites that differentiate three lineages of honey bee mitochondrial DNA (west European, east Mediterranean and African). Based on these diagnostic sites, all samples match the east Mediterranean lineage. Samples from Thrace also possessed an additional *Xba*I restriction site in cytochrome oxidase I previously known only from *Apis mellifera carnica.*

Turkey / mitochondrial DNA / restriction site / *Apis mellifera anatoliaca* / *Apis mellifera caucasica*

INTRODUCTION

Turkey is located at the crossroads of Europe, Asia and the Middle East and has received both human and biological influences from all three sources. In addition, a wide range of climates and habitats are found within Turkey's borders. Thus, it is not surprising that numerous honey bee subspecies and ecotypes have been described from this region.

According to Ruttner's 1988 presentation of morphometric, behavioral and ecological data, three honey bee subspecies are found in Turkey: *Apis mellifera anatoliaca, A m caucasica and A m meda* (fig 1). *A m caucasica* occurs in the extreme northeast of Anatolia and along the eastern Black Sea coast. *A m meda* is found in the southeast. *A m anatoliaca* occurs over the rest of Turkey, including European Turkey. The Anatolian bees in western Turkey, in a region bounded in the east by Istanbul, Bursa, Eskişehir and Isparta, form a distinct subgroup within *A m anatoliaca*, the 'western Anatolian bees' (Ruttner, 1988).

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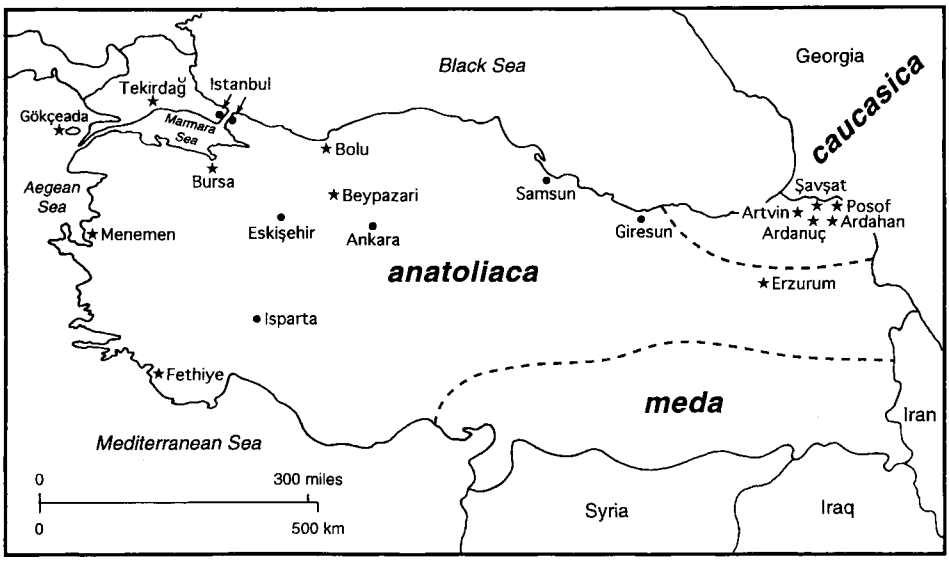

Fig 1. Approximate ranges of *A m anatoliaca, A m caucasica* and *A m meda* in Turkey as determined by the morphometric studies of Ruttner (1988). Samples for this study were collected from the starred localities.

In this study we surveyed samples of Turkish honey bees for four diagnostic restriction sites in the mitochondrial genome. These sites were selected because earlier studies (eg, Smith and Brown, 1990; Crozier et al, 1991; Hall and Smith, 1991; Smith, 1991; Garnery et al, 1992; Meixner et al, 1993) showed them to be useful in distinguishing three mitochondrial lineages of honey bees: a west European lineage (mainly *A m mellifera*), an eastern Mediterranean lineage (eg, *A m ligustica* and *A m carnica*) and an African lineage (eg, *A m scutellata*), each of which is characterized by a particular pattern of restriction sites.

We chose to examine all four restriction sites because we had no a priori reason to believe that the Turkish bees would fall neatly into one of the three previously characterized mitochondrial lineages. They could equally well constitute a group of their own with a novel pattern of restriction sites. Indeed, morphometric data led Ruttner (1988) to propose a fourth 'Oriental' lineage that includes *A m caucasica, A m anatoliaca* and *A m meda*. Table I lists the diagnostic restriction sites and the portion of the mitochondrial genome in which they are found.

The three lineages of honey bee mitochondrial DNA (mtDNA) have more or less allopatric distributions, which match well the geographic distributions of three groups of honey bee subspecies recognized on the basis of morphometric characters (Ruttner, 1988 and references therein; Smith, 1991; Garnery et al, 1992). It seems likely that these three mtDNA lineages mark three pop-

ulations of honey bees that differentiated in isolation some time in the past, most likely during the Pleistocene, and subsequently came into contact again. Thus, study of the geographic distribution of mtDNA haplotypes can provide information about the history and biogeography of honey bee populations.

MATERIALS AND METHODS

Samples were collected in June 1995 from the collection sites listed below (see fig 1): (1) Thrace, seven colonies from villages near Tekirdağ; (2) Gökçeada, ten colonies (five surveyed for four restriction sites, an additional five surveyed for *Xba*I site); (3) Menemen, Agean Agricultural Research Institute, nine colonies (six surveyed for four restriction sites, an additional three surveyed for *Xba*I site); (4) Fethiye, nine colonies (six surveyed for four restriction sites, an additional three surveyed for *Xba*I site); (5) Beypazari, six colonies; (6) Erzurum, six colonies; (7) Ardahan, six colonies; (8) Artvin (near Ardanuç), six colonies from Hamurlu village; (9) Artvin, five colonies from Kaşikçi village; (10) Posof, Süngüllü and Şavşat villages, nine colonies; (11) Bolu, two colonies; (12) Bursa, one colony. The bees from Bolu, Şavşat and Beypazari were collected from colonies maintained at a honey bee breeding station in Kazan, Ankara. Adult workers were collected from the comb (or in one case from a swarm), and frozen in liquid nitrogen or preserved in 70% ethanol.

Total DNA was extracted from single thoraces of ethanol-preserved or frozen bees. Each thorax was crushed in 500 μL of sterile STE buffer (0.1 M NaCl, 0.05 M Tris pH 7.5, 0.001 M EDTA), 25 μL 20% sodium lauryl sulfate, and 25 μL of 10 mg/mL proteinase-K in STE. The mixture was incubated from 2 h to overnight in a 55–56 °C degree water bath. After digestion the mixture was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). DNA was precipitated with 1:10 volume of 3 M sodium acetate and two volumes of ice-cold 95% ethanol. DNA was pelleted by centrifuging for 15 min at 12 000 rpm in a bench-top microfuge. The pellet was rinsed once in 70% ethanol, air dried and resuspended in 100 μL of 1/10 TE (TE = 10 mM Tris, 1 mM EDTA pH 7.8).

Four regions of the mitochondrial genome were amplified by means of the polymerase chain reaction (PCR; Saiki et al, 1985) using the following thermal profile: 94 °C for 20 s, 40 °C for 1 min 30 s, 2 min ramp to 72 °C, 72 °C for 1 min, 35 cycles. Each PCR reaction contained 5 μL Promega 10 X PCR buffer, 4 mM $MgCl_2$, 0.1 μM of each primer, 0.5 μL Pharmacia DNA polymerization mix, 0.5 μL Promega *Taq* polymerase, and sufficient sterile distilled water to make 50 μL. The primers, regions of mitochondrial genome amplified, restriction enzymes used, and the pattern of restriction sites in each *A mellifera* mitochondrial lineage are shown in table I.

Ten-microliter aliquots of each PCR amplification product were digested with the appropriate restriction enzyme (table I) following the manufacturer's (Promega) recommended temperature and buffer conditions. The resulting restriction fragments were then separated by electrophoresis through an 0.8% agarose/TBE gel, (90 V, room temperature, 30–60 min), stained with ethidium bromide, and viewed and photographed under ultraviolet illumination. Restriction sites were scored as present (PCR amplification product cut, resulting in two bands on the gel) or absent (PCR amplification product not cut, resulting in one band).

In some mtDNAs, *Xba*I digests of the COI to COII fragment (table I) revealed the presence of two *Xba*I restriction sites instead of the expected one or zero. In order to examine the geographical distribution of this site more closely additional samples (a total of ten from Gökçeada, nine from Menemen and nine from Fethiye) were examined. One sample with and one without the additional site were sequenced to identify the sequence difference between haplotypes. Sequencing was carried out with the Promega fMol cycle sequencing kit and a ^{32}P-end labeled sequencing primer (see table I).

RESULTS AND DISCUSSION

The particular restriction sites surveyed here were selected because previous studies had shown little or no variation within mitochondrial lineages, and reliable differences among lineages. All samples showed the set of restriction sites typical of the eastern

Table I. Summary of the procedure used to diagnose the mitochondrial genomes of Turkish honey bees. One of four pairs of primers ('primer pair') was used to amplify a portion of the mitochondrial genome ('location of amplified fragment'), which was then digested with the appropriate restriction enzyme. Each of three main lineages of honey bee mtDNA (west European, east Mediterranean and African) is characterized by a different set of restriction sites.

Primer pair	*Location of fragment amplified*	*Restriction enzyme*	*West European*	*East Mediterranean*	*African*
5′ TATGTACTACCATGAGGACAAATATC 3′[a] 3′ TAAGGATTATTTAATCCTCCACATTA 5′	cytochrome oxidase b (cytb)	*Bgl*II	+	+	–
5′ TTTTGTACCTTTTGTATCAGGGTTG 3′[b] 3′ CCCTGCTATTCTGGGATATC 5′	large ribosomal subunit (lsRNA)	*Eco*RI	–	+[d]	–
5′ TCTATACCACGACGTTATTC 3′[bc] 3′ CCAGTAGTTACTATAACTAG 5′	3′ end cytochrome oxidase I to 5′ end cytochrome oxidase II (COI-COII)	*Xba*I	–	+	–
5′ TTAAGATCCCCAGGATCATG 3′[b] 3′ GTTATCCACGTCATAAACGT 5′	5′ end cytochrome oxidase I (COI)	*Hin*CII	+	–	–

'+' Indicates restriction site present, '–' indicates restriction site absent.
[a] Crozier et al, 1991; [b] Hall and Smith, 1991; [c] also used as a sequencing primer; [d] may be absent in some *A m ligustica*; Garnery et al, 1992.

Mediterranean mitochondrial lineage: the *Bgl*II site in cytochrome b (cytb), *Xba*I site in COI and the *Eco*RI site in the large subunit ribosomal RNA gene (lsRNA) were present, and the *Hinc*II site in COI was absent. However, it should be noted that the mtDNA of Turkish bees may differ from previously studied east Mediterranean mtDNAs in possessing or lacking restriction sites that we did not survey in this study.

In six out of seven colonies from Thrace a second *Xba*I restriction site was present in COI. Sequencing showed that samples with and without the additional *Xba*I site differed by a single substitution in base pair 3204 in the honey bee mitochondrial genome (using the numbering system of Crozier and Crozier, 1993): ttaga in those without the restriction site, tctaga in the sample with the additional site.

This additional *Xba*I site has thus far been found only in *A m carnica* from Austria and the Balkan peninsula (Smith and Brown, 1990; Meixner et al, 1993). We found this site only in Thrace, and not in samples from Gökçeada, Bursa, Menemen or Fethiye, indicating that the distribution of this character does not correspond to the distribution of the 'western Anatolian' subgroup of bees identified by morphometrics. Populations of bees in Thrace are near populations of *A m macedonica* (Ruttner, 1988) and further to the north in the Balkans and Austria, *A m carnica*. The presence of this restriction site suggests that the bees of Thrace are related to the bees farther north in the Balkan peninsula, specifically *A m carnica*. Sampling colonies along transects from Austria through the Balkan peninsula and Greece, and into northwestern Anatolia (eg, Istanbul, Bursa) would clarify the distribution of this character and perhaps shed additional light on the relationship among *A m carnica* and the bees of Greece, the Balkans and Turkey.

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Résumé — Les abeilles (*Apis mellifera* L) de Turquie appartiennent à la lignée mitochondriale de la Méditerranée orientale. Des abeilles ont été prélevées dans douze localités de Turquie (fig 1) : Thrace (sept colonies), Gökçeada (dix colonies), Bolu (deux colonies), Bursa (une colonie), Menemen (neuf colonies), Fethiye (neuf colonies), Beypazari (six colonies), Erzurum (six colonies), Ardahan (six colonies), Ardanuç (six colonies), Artvin (cinq colonies) et dans des villages près de la frontière géorgienne (neuf colonies). On a recherché dans le génôme mitochondrial de ces abeilles la présence ou l'absence de quatre sites de restriction qui différencient trois lignées d'ADNmt chez l'abeille mellifère : la lignée d'Europe occidentale, celle de la Méditerranée orientale et l'africaine. Les fragments diagnostiqués indiquent que tous les échantillons appartiennent à la lignée de la Méditerranée orientale. Des échantillons de Thrace possèdent en outre un site de restriction supplémentaire *Xba*I dans la cytochrome oxydase I, qui n'était connu à ce jour que chez *A m carnica ;* il n'a été trouvé dans aucune autre population de Turquie.

***Apis mellifera anatoliaca* / *Apis mellifera caucasica* / ADNmt / génétique population / site restriction / Turquie**

Zusammenfassung — Türkische Honigbienen gehören zur ostmediterranen mitochondrialen Abstammungslinie. Honigbienen wurden in 12 Gegenden in der Türkei gesammelt (Abb 1): Thrace (sieben Völker), Gökçeada (zehn Völker), Bolu (zwei Völker), Bursa (ein Volk), Menemen (neun Völker), Fethiye (neun Völker), Beypazari (sechs Völker), Ezurum (sechs Völker), Ardahan (sechs Völker), Ardanuç (sechs Völker), Artvin (fünf Völker) und von Dörfern nahe der Grenze zu Georgien (neun Völker). Das mitochondriale Genom von diesen Bienen wurde in Bezug auf das Vorhandensein bestimmter Restriktionsfragmente untersucht, welche zwischen den drei Abstammungslinien von Honigbienen mtDNA (westeuropäisch, ostmediterran und afrikanisch) unterscheiden. Diesen diagnostizierenden Fragmenten zufolge (siehe Tab I), entsprechen alle Proben der mediterranen Abstammungsline. Die Bienen aus Thrace zeigten ein zusätzliches Fragment bei der Restriktion von Cytochromoxidase I mit *Xba*I, welches zuvor nur von *Apis mellifera carnica* bekannt war; dieses Fragment wurde in keinem der anderen türkischen Bienenvölkern gefunden.

***Apis mellifera anatolica* / *Apis mellifera caucasica* / mitochondriale DNA / Restriktionsschnittstelle / Türkei**

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