Genetic characterization of honey bee (*Apis mellifera cypria*) populations in northern Cyprus*

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Received 15 April 2005 – revised 16 December 2005 – accepted 22 December 2005

Abstract — The variability of the honey bees of northern Cyprus was investigated using morphometric, mitochondrial DNA (mtDNA) and microsatellite analyses. Morphometric analysis resulted in a clear classification of the Cyprus bees as *Apis mellifera cypria*, but showed the influence of imported *A. m. anatoliaca* in some areas. In eastern Cyprus, several samples showed a similarity to *A. m. meda*, possibly corroborating a published report of similarity between *A. m. cypria* and Mediterranean *A. m. meda*. However, the importation of *A. m. meda* into Cyprus could not be ruled out. MtDNA analysis showed that most Cyprian samples belonged to the mitochondrial C lineage, but a small proportion of samples displayed restriction patterns typical for the mitochondrial O lineage. Population differentiation between Cyprus and honey bees from adjacent mainland populations was low, but the northwestern Cyprus population appeared to be introgressed to a larger extent by alleles from the Turkish mainland.

*Apis mellifera cypria* / mtDNA / microsatellites / morphometry / Cyprus

1. INTRODUCTION

Traditionally, subspecific classification and phylogeographic inferences in *Apis mellifera* L. have been based on the variation of behavior and morphology within the endemic range of the species. Using morphometric analyses, Ruttner (1988, 1992) hypothesized the existence of four evolutionary lineages within the species: M in northern and western Europe, A in Africa, C in southeastern Europe, and O in western Asia. Subsequent studies, based on variation of mitochondrial DNA, confirmed Ruttner’s hypotheses about the phylogeographic structure of *Apis mellifera* to a large extent (Garnery et al., 1992, 1993; Arias and Sheppard, 1996; Franck et al., 2000a). The most widely used marker in these studies was variation in the intergenic region between the *COI* and *COII* gene in *Apis mel-

Recent studies, using these methods, the morphological C and O branches were undistinguishable and were subsumed into a single mitochondrial lineage (C).

Recently, Franck et al. (2000a) reported the existence of a previously unknown mtDNA restriction enzyme pattern in honey bees sampled from Lebanon and inferred the existence of a fourth mitochondrial lineage of honey bees (‘mitochondrial O ’). This lineage may be analogous to the mtDNA lineage hypothesized based on restriction enzyme data (Palmer et al., 2000) and mitochondrial ND2 gene sequences (Arias and Sheppard, 1996). The distribution of the mitochondrial O lineage remains unknown, but may extend from Syria to Egypt (Arias and Sheppard, 1996).

The island of Cyprus is situated at the eastern end of the Mediterranean Sea, south of Turkey (75 km), west of Syria and Lebanon.
(105 km) and north of Egypt (380 km). The honey bees of Cyprus were described as a separate subspecies, *A. m. cypria*, by Pollman (1879) and shown by Ruttner (1988) to belong to the morphological O lineage of *Apis mellifera*. While other island populations and subspecies of honey bees in the Mediterranean have received more scientific interest (Crete: Ruttner, 1980; Sicily: Badino et al., 1985; Sinacori et al., 1998; Franck et al., 2000b; Malta: Sheppard et al., 1997; Balearics: De la Rúa et al., 2001, 2003), very little is known about the honey bee of Cyprus. The geographic location of Cyprus positions *A. m. cypria* in close proximity to subspecies to both the mitochondrial C and O lineages and the geographic region of transition between them. In this paper we report the results of an extensive morphometric and genetic analysis of the honey bees of Cyprus and compare their morphometric and genetic variability to that of neighboring subspecies.

### 2. MATERIALS AND METHODS

#### 2.1. Collection of bee samples

A total of 101 colonies were sampled from 12 locations in northern Cyprus in the years 2000 (40), 2002 (55), and 2004 (6) (Fig. 1, Tab. I). Samples were stored in 90% ethanol (2000, 2004) or in dry ice (2002).

#### 2.2. Morphometric analysis

A total of 18 colonies (3 from each location of the 2000 collection) were subjected to morphometric analysis. Between 11–15 worker bees per sample were dissected and measured for 39 morphometric characters according to methods of Ruttner et al. 

### Table I. Sampling locations, geographical positions, and number of colonies sampled for this study.

<table>
<thead>
<tr>
<th>Location</th>
<th>Geographical position</th>
<th># Colonies sampled</th>
<th>year</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Omorfo</td>
<td>35°12’N 32°59’E</td>
<td>5</td>
<td>2000</td>
</tr>
<tr>
<td>2-Lefke</td>
<td>35°06’N 32°51’E</td>
<td>4</td>
<td>2000</td>
</tr>
<tr>
<td>3-Gaziveren</td>
<td>35°11’N 33°01’E</td>
<td>6</td>
<td>2000</td>
</tr>
<tr>
<td>4-Kalecik</td>
<td>35°20’N 34°00’E</td>
<td>3</td>
<td>2000</td>
</tr>
<tr>
<td>5-Iskele</td>
<td>35°16’N 33°54’E</td>
<td>3</td>
<td>2000</td>
</tr>
<tr>
<td>6-Ardahan</td>
<td>35°21’N 33°52’E</td>
<td>47</td>
<td>2000, 2002</td>
</tr>
<tr>
<td>7-Yedikonuk</td>
<td>35°24’N 34°01’E</td>
<td>10</td>
<td>2002</td>
</tr>
<tr>
<td>8-Taslica</td>
<td>35°23’N 34°04’E</td>
<td>5</td>
<td>2002</td>
</tr>
<tr>
<td>9-Kantara</td>
<td>35°23’N 33°53’E</td>
<td>3</td>
<td>2002</td>
</tr>
<tr>
<td>10-Mersinlik</td>
<td>35°24’N 33°55’E</td>
<td>4</td>
<td>2002</td>
</tr>
<tr>
<td>11-Kaplica</td>
<td>35°23’N 33°54’E</td>
<td>5</td>
<td>2002</td>
</tr>
<tr>
<td>12-Girne</td>
<td>35°19’N 33°19’E</td>
<td>6</td>
<td>2004</td>
</tr>
</tbody>
</table>
Genetic characterization of *Apis mellifera cypria* (1978) and Ruttner (1988, 1992). Characters of pilosity and pigmentation were assessed with a stereomicroscope and an ocular micrometer. All other characters were measured with a CCD camera combined with a morphometric measurement program (Bee2, © Meixner, 2004). Reference data of honey bee subspecies of the eastern Mediterranean region were obtained from the database of the Institut für Bienenkunde, Oberursel. These included *A. m. carnica* (20 samples), *A. m. macedonica* (10), *A. m. cecropia* (10), *A. m. anatoliaca* (13), *A. m. syriaca* (9), *A. m. adami* (5), and *A. m. meda* (25). Reference data for *A. m. meda* came from samples of *A. m. meda* collected in Turkey and Syria (Ruttner, 1988; Ftayeh et al., 1994). Data were subjected to principal component analysis and discriminant analysis (Ftayeh et al., 1994). Data were subjected to principal component analysis and discriminant analysis (Arias and Sheppard, 1996) or a modified phenol-chloroform extraction (Arias and Sheppard, 1996) or a modified CTAB extraction protocol (Doyle and Doyle, 1987). A mitochondrial fragment containing the intergenic region between the tRNAleu gene and the second subunit of the cytochrome oxidase gene was amplified using the primer pair E2-H2 (Garnery et al., 1993): E2: 5′-GGC AGA ATA AGT GCA TTG-3′, H2: 5′-CAA TAT CAT TGA TGA CC-3′. The 25 µL reaction mix consisted of 0.8 µM of each primer, 0.2 mM of PCR Nucleotide mix (Boehringer Mannheim), 1.5 mM MgCl2, 1X Reaction Buffer (Promega), 1X Reaction Buffer (Promega), 1 U Taq Polymerase (Promega) and 1 µL of template. The amplification cycle consisted of an initial denaturation step of 2 min at 92 ºC, followed by 35 cycles of 30 s at 92 ºC, 30 s at 47 ºC and 2 min at 63 ºC, followed by a final extension step of 10 min at 63 ºC. Five µL of the PCR products were run on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV illumination. A 20 µL aliquot of each positive reaction was digested with the restriction enzyme *DraI* at 37 ºC overnight. Restriction fragments were separated on 10% polyacrylamide gels, stained with ethidium bromide and photographed under UV illumination.

Among the samples expressing restriction profiles of the C and O mitochondrial lineages, we sequenced the **COI-COII** region of one sample and the NADH dehydrogenase subunit 2 gene of two samples each, using a cycle sequencing protocol (Craxton, 1991) and an ABI 377 automated sequencer. The ND2 sequences were aligned with corresponding published sequence data from other *Apis mellifera* subspecies (Arias and Sheppard, 1996) using Clustal X (Thompson et al., 1997). Phylogenetic analyses using both neighbor-joining and parsimony methods were performed with MEGA 3.1 (Kumar et al., 2004). Sequences were deposited in GenBank under the accession numbers AY618919–AY618921.

2.4. Microsatellite analysis

The samples were analyzed for nine microsatellite loci: A7, A24, A28, A88, A113, B124 (Estoup et al., 1995), Ap55, Ap66, and Ap81 (Garnery et al., unpubl. data). Amplifications were performed in 10 µL reactions containing 1 µL extracted DNA, 1X reaction buffer, 3 mM dNTPs, 0.001 mg BSA, 1–4 mM of respective primers and 1.5 units Taq polymerase. Microsatellite primers were combined into two multiplex reactions with optimized concentrations of MgCl2: 1.2 mM for A7, A113, Ap55 and Ap81; and 1.5 mM for loci A24, A28, A88, Ap66 and B124. The PCR reaction conditions were identical for all loci and consisted of 7 min at 95 ºC, followed by 30 cycles of 95 ºC (30 s), 54 ºC (30 s), 72 ºC (30 s), and a final 60 min cycle at 72 ºC. Forward primers were fluorescent labeled and amplification products were separated on an ABI 3730 automatic sequencer. The resulting electropherograms were analyzed using GeneMapper Software (Applied Biosystems).

For analysis, the microsatellite data were combined with unpublished reference data from populations in Turkey (n = 47), Syria (n = 22) and Iran (n = 43). Exact tests for genetic structure and genetic differentiation between populations using unbiased estimates of Fst were calculated using the Genepop package version 3.4 (Raymond and Rousset, 1995). A neighbor-joining tree based on the microsatellite data and the chord distance of Cavalli-Sforza and Edwards was constructed using the Phylip program package (Felsenstein, 2005) with bootstrap values computed over 2000 replications.

3. RESULTS

3.1. Morphometry

In a principal component analysis based on three factors describing 38.8%, 11.9% and
7.9% of the morphological variation, respectively, the Cyprus samples mainly fell within the range previously published for *A. m. cypria* within the morphological O lineage (Ruttner, 1988). Two samples occupied positions away from the *A. m. cypria* cluster and appeared to be associated with *A. m. anatoliaca*. No relationship with *A. m. carnica* or other subspecies of the morphological C lineage was observed (plot not shown). The allocation of our samples to reference data of *A. m. cypria*, *A. m. anatoliaca*, *A. m. meda*, or *A. m. syriaca* was examined further using discriminant analysis. In this analysis (Fig. 2), 11 of the samples were clearly identified as *A. m. cypria* with probability scores of $P > 0.99$, while four samples were assigned to *A. m. cypria* with scores of $0.85 \geq P \geq 0.97$. Two samples (both from the same location) were identified as *A. m. anatoliaca*, and one sample (from a collection site in the east of Cyprus) was assigned to *A. m. meda*.

**3.2. Mitochondrial DNA**

Restriction enzyme digestion of the mitochondrial fragment containing the intergenic region with *Dra*I resulted in two different patterns assignable to the C and O mitochondrial lineages as described by Garnery et al. (1993) and Franck et al. (2000a). The majority (99 of 101) of our samples displayed the C2 mitochondrial haplotype previously reported from Italy, Greece and Iran (Garnery et al., 1993), and Turkey (Kandemir et al., 2006). Two samples from the eastern part of Cyprus displayed the O1b haplotype known to occur in honey bees of Lebanon (Franck et al., 2000a) and the western part of Syria (Meixner et al., unpubl. data).

Inclusion of mitochondrial ND2 sequence data from C2 or O1b haplotypes in the phylogenetic analyses of subspecies consistently clustered the C2 sample with subspecies from the C lineage branch. The O1b sample clustered with the bees sampled from Egypt and Syria, previously hypothesized to form a fourth mitochondrial lineage (Arias and Sheppard, 1996) (tree not shown).

**3.3. Microsatellite analysis**

Heterozygosity estimates of microsatellite loci in the Cyprus populations ranged from 0.286 (Ap81) to 0.857 (A113) with a mean across loci of $0.553 \pm 0.26$ for northwestern Cyprus and $0.554 \pm 0.22$ for northeastern Cyprus. All loci were in Hardy-Weinberg
equilibrium with respect to the populations studied. The number of alleles, the allele size range in bp and the expected and observed heterozygosities (H. exp. and H. obs.) and the allele frequencies for each individual locus are presented in Table II. The results of pairwise population comparisons using multilocus F-statistics between the northwestern and northeastern Cyprus populations and the reference populations were low and ranged between 0.003 (northwestern Cyprus, Turkey) and 0.081 (northeastern Cyprus, Syria) (Tab. III).

The populations of northwestern and northeastern Cyprus showed significant differences in their microsatellite variability (P < 0.001, Fisher exact test). When compared to surrounding mainland populations, the allelic distribution of the northwestern Cyprus population was not significantly different from the population of Turkey, but different from Syria and Iran (P < 0.001). The bees of eastern Cyprus differed significantly from all adjacent mainland honey bee populations (Turkey, Syria, Iran) (P < 0.001).

A neighbor-joining tree based on the Cavalli-Sforza and Edwards chord distance resulted in low resolution between the populations from Iran and the branch combining the other groups from the Near East. Within this branch, the honey bee populations from Cyprus were incorporated into a subcluster with Syria (Fig. 3).

4. DISCUSSION

Several different subspecies of honey bees belonging to two different evolutionary lineages (C and O) come together in the eastern Mediterranean and the Near East. Although these two evolutionary lineages are distinguishable by morphological methods, the delineation based on restriction analysis of mitochondrial DNA is incongruent and seemingly confusing. The honey bee subspecies of the entire Near East, including Turkey, morphologically belong to the O evolutionary lineage sensu Ruttner (1988). However, in the southern portion of this range a division between mitochondrial lineages C (sensu Garnery et al., 1993) and O (sensu Franck et al., 2000a) occurs further south and east. Thus, C mitochondrial haplotypes occur in many subspecies belonging to the O morphological lineage sensu Ruttner, including most of the honey bees of Turkey (Kandemir et al., 2006) and those that occur east into Iran and Central Asia at the eastern edge of the Apis mellifera range (unpublished data; Sheppard and Meixner, 2003). Further south, extending from southern Turkey (Kandemir et al., 2006) through Lebanon (Franck et al., 2000a), Syria and Egypt (Arias and Sheppard, 1996; unpubl. data), honey bee populations are characterized by haplotypes belonging to the (perhaps unfortunately named) mitochondrial lineage O (as described and named by Franck et al., 2000a).

Our results show that the contemporary honey bee population of (northern) Cyprus retain A. m. cypria characteristics as described by Ruttner (1988), although in some areas the influence of other subspecies, especially A. m. anatoliaca, can be detected. Beekeepers in Cyprus predominantly use primitive hives, but the use of modern equipment, migratory beekeeping and commercial pollination practices are increasing (Kandemir, 2003). The two Cyprian samples that were morphometrically classified as A. m. anatoliaca and the one with an intermediate score between A. m. anatoliaca and A. m. cypria all came from modern beekeeping operations involved in citrus pollination (located in northwestern Cyprus) and may reflect past or recent importation of A. m. anatoliaca queens. In contrast, three other samples with intermediate scores showed an affinity to A. m. meda and, together with the one sample classified as A. m. meda, originated from the eastern part of the island where traditional beekeeping in trunk hives is still predominant.

Mitochondrial analysis predominantly placed our Cyprus collection into the mitochondrial C lineage, but also showed a small proportion of restriction profiles characteristic for the mitochondrial O lineage. Whether this observation reflects a mixed ancestry of the Cyprus population or a more recent introduction of honey bees from the eastern shore of the Mediterranean is unknown. While O mitochondrial lineage haplotypes might be a remnant of the Pleistocene fauna of Cyprus
Table II. Genetic parameters: sample size, allele size range, number of alleles, expected heterozygosity (H. exp.), expected heterozygosity unbiased estimate (H. n.b.), and observed heterozygosity (H. obs.) (Nei, 1978) of nine microsatellite loci for northeast and northwest Cyprus. The allele frequencies for each locus are available in Appendix of the online version.

<table>
<thead>
<tr>
<th>Regions in northern Cyprus</th>
<th>Genetic Parameters</th>
<th>Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Number of alleles</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>H exp.</td>
<td>0.9158</td>
</tr>
<tr>
<td></td>
<td>H n.b.</td>
<td>0.9497</td>
</tr>
<tr>
<td></td>
<td>H obs.</td>
<td>0.7143</td>
</tr>
<tr>
<td></td>
<td></td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Number of alleles</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>H exp.</td>
<td>0.8951</td>
</tr>
<tr>
<td></td>
<td>H n.b.</td>
<td>0.9016</td>
</tr>
<tr>
<td></td>
<td>H obs.</td>
<td>0.7826</td>
</tr>
</tbody>
</table>
Table III. Fst results from Genepop.

<table>
<thead>
<tr>
<th></th>
<th>Northwest</th>
<th>Northeast</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyprus</td>
<td></td>
<td></td>
<td>0.018</td>
</tr>
<tr>
<td>Iran</td>
<td>0.021</td>
<td>0.048</td>
<td></td>
</tr>
<tr>
<td>Turkey</td>
<td>0.003</td>
<td>0.050</td>
<td></td>
</tr>
<tr>
<td>Syria</td>
<td>0.069</td>
<td>0.081</td>
<td></td>
</tr>
</tbody>
</table>

In contrast to the high differentiation observed using mitochondrial markers, microsatellite analysis indicated a relatively low level of differentiation among the Near Eastern populations studied, irrespective of their assignment to mitochondrial lineages C or O. While overall Fst values between Cyprus and all reference populations were low, the honey bee population of northwestern Cyprus was introgressed to a larger extent by microsatellite alleles from Turkey, suggesting the role of queen importation from the Turkish mainland. Thus, while our results confirm the distinctness of *A. m. cypria* as island subspecies of Cyprus, they also show that importation of bees from adjacent mainland areas may become a threat to its conservation in the future.

ACKNOWLEDGEMENTS

This project was partially supported by USDA-IFAFS grant 2001-52103-11417 to WSS, and ZKU-2003-13-06-04, TUBITAK-TBAG 2403 grants and TUBITAK-BAYG 2219 scholarship to IK. We gratefully acknowledge the Institut für Bienenkunde in Oberursel, Germany, for use of morphometric reference data. We thank Stefan Fuchs, Lionel Garnery and three anonymous reviewers for comments on earlier versions of the manuscript.


L’analyse morphométrique a nettement classé les abeilles de Chypre comme étant *A. m. cypria*, mais a montré également l’influence dans certaines régions des importations d’*A. m. macedonica*. Dans la partie orientale de Chypre plusieurs échantillons présentaient des similitudes avec *A. m. meda* et là non plus l’importation d’*A. m. meda* à Chypre n’a pu être écartée. Les analyses de restriction comme celles de la séquence de l’ADNmt ont montré que la plupart des échantillons chypriotes appartenaient à la lignée mitochondriale C, mais une petite proportion d’échantillons présentait des profils de restriction typiques de la lignée mitochondriale O. D’après la fréquence allélique des microsatellites
la différenciation entre les échantillons chypriotes et les populations voisines du continent était faible. La population du nord-ouest de Chypre semble avoir subi une large introgression par des allèles venant du continent turque. Ainsi, alors que nos résultats confirment la particulière d’A. m. cypria comme sous-espèce de l’île de Chypre, ils montrent aussi que l’importation d’abeilles du continent voisin peut devenir une menace pour sa conservation à l’avenir.

Apis mellifera cypria / ADNmt / microsatellite / morphométrie / Chypre


Apis mellifera cypria / mtDNA / Morphometrie / Zypern / Mikrosatelliten

REFERENCES
Genetic characterization of *Apis mellifera cypria* 555


