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

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Original article

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

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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 the *COII* gene in *Apis mellifera* mtDNA, as determined by sequencing or restriction analysis (Garnery et al., 1992, 1993, 1998; Franck et al., 1998). However, 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

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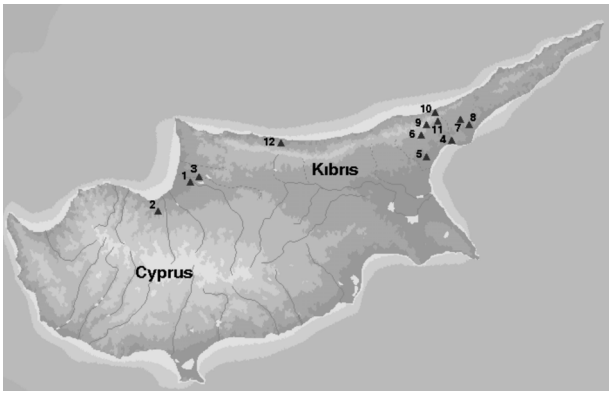

Figure 1. Map indicating sampling locations in Cyprus.

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

Location	Geographical position		# Colonies sampled	year
1-Omorfo	35°12'N	32°59'E	5	2000
2-Lefke	35°06'N	32°51'E	4	2000
3-Gaziveren	35°11'N	33°01'E	6	2000
4-Kalecik	35°20'N	34°00'E	3	2000
5-Iskele	35°16'N	33°54'E	3	2000
6-Ardahan	35°21'N	33°52'E	47	2000, 2002
7-Yedikonuk	35°24'N	34°01'E	10	2002
8-Taslica	35°23'N	34°04'E	5	2002
9-Kantara	35°23'N	33°53'E	3	2002
10-Mersinlik	35°24'N	33°55'E	4	2002
11-Kaplica	35°23'N	33°54'E	5	2002
12-Girne	35°19'N	33°19'E	6	2004

(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.

(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 using the SPSS 12.0.1 statistical software.

2.3. Restriction and sequence analysis of mitochondrial DNA

Total nucleic acids of one bee per sample were isolated with 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 $MgCl_2$ (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 °C, followed by 35 cycles of 30 s at 92 °C, 30 s at 47 °C and 2 min at 63 °C, followed by a final extension step of 10 min at 63 °C. 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 *Dra*I at 37 °C 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 *ND*2 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 $MgCl_2$: 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 °C, followed by 30 cycles of 95 °C (30 s), 54 °C (30 s), 72 °C (30 s), and a final 60 min cycle at 72 °C. 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

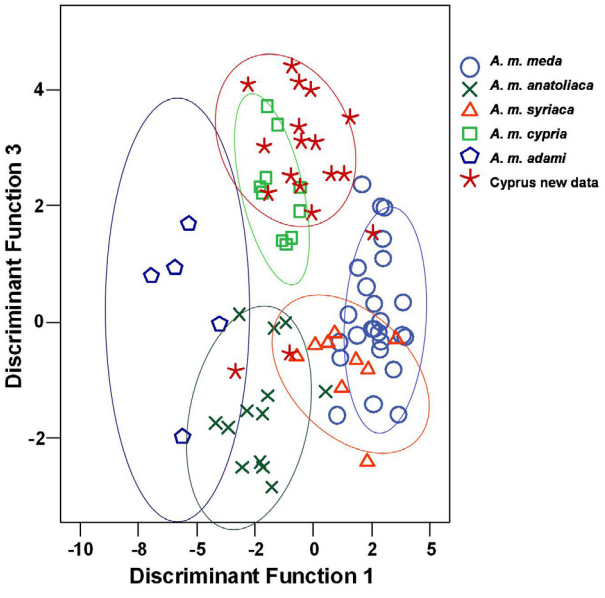

Figure 2. Discriminant analysis containing the samples from Cyprus and reference samples of subspecies belonging to the morphological O-branch. X-axis: discriminant function 1, Y-axis: discriminant function 3. The ellipses of confidence (75%) for each group are included. The confidence ellipse of the new Cyprus samples was constructed excluding the three samples not classified as *A. m. cypria*.

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 ± 0.26 for northwestern Cyprus and 0.554 ± 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.

Regions in northern Cyprus	Genetic Parameters	Locus								
		A7	**A24**	**A28**	**A88**	**Ap66**	**B124**	**A113**	**Ap55**	**Ap81**
Northwest	Sample size	14	20	17	17	16	17	14	14	14
	Allele size range (bp)	87–155	94–106	127–139	130–148	85–135	212–230	216–237	153–177	135–139
	Number of alleles	14	5	4	7	8	7	9	6	3
	H exp.	0.9158	0.6592	0.3460	0.6107	0.7	0.7284	0.8393	0.6224	0.2526
	H n.b.	0.9497	0.6791	0.3565	0.6292	0.722	0.7504	0.8704	0.6455	0.2619
	H obs.	0.7143	0.6471	0.4118	0.6471	0.563	0.7647	0.8571	0.6429	0.2857
Northeast	Sample size	69	74	72	74	71	75	58	54	64
	Allele size range (bp)	78–159	94–106	101–139	133–145	93–137	218–238	210–237	171–185	125–145
	Number of alleles	29	5	9	6	7	8	13	7	9
	H exp.	0.8951	0.7218	0.3440	0.6179	0.566	0.5189	0.8353	0.5485	0.2834
	H n.b.	0.9016	0.7267	0.3464	0.6221	0.570	0.5224	0.8426	0.5537	0.2857
	H obs.	0.7826	0.7432	0.3750	0.7838	0.549	0.5733	0.7414	0.5185	0.1406

Table III. Fst results from Genepop.

	Northwest Cyprus	Northeast Cyprus
Northeast Cyprus	0.018	
Iran	0.021	0.048
Turkey	0.003	0.050
Syria	0.069	0.081

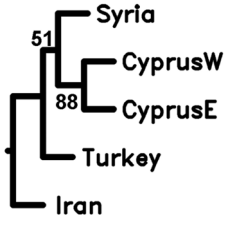

Figure 3. Neighbor-joining tree based on the Cavalli-Sforza and Edwards chord distance between populations (based on nine microsatellite loci). Bootstrap values are based on 2000 replications.

that contained African elements such as dwarf hippos and elephants (Schüle, 1993), it is more likely that the presence of these O lineage colonies in Cyprus resulted from more recent human-mediated introductions. For example, it is known that extensive importations of honey bee queens were made from present day Syria and Lebanon to Cyprus in the 19th century (Strange, 2001).

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.

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Résumé – Caractérisation génétique des populations d'abeilles domestiques (*Apis mellifera cypria*) à Chypre. L'île méditerranéenne de Chypre possède sa propre sous-espèce d'abeille domestique, *Apis mellifera cypria* Pollmann 1879, mais on connaît peu de choses concernant sa variabilité génétique et ses relations avec les sous-espèces voisines. De par la position géographique de Chypre, *A. m. cypria* avoisine directement les sous-espèces des lignées mitochondriales C et O. Nous avons étudié la variabilité des abeilles de Chypre par les méthodes morphométriques et par des analyses de l'ADN mitochondrial et des microsatellites. Au total 101 échantillons ont été prélevés dans 12 localités du nord de Chypre ; 18 d'entre eux ont fait l'objet d'une analyse morphométrique. Les mesures de 39 caractères ont été analysées par les méthodes de statistique multivariées. Des échantillons de référence provenant des régions continentales voisines ont également été analysés. Un fragment mitochondrial contenant la région intergénique entre le gène ARNtleu et la seconde sous-unité du gène de la cytochrome oxydase a été amplifié et digéré par l'enzyme de restriction *DraI*. Le fragment contenant la région intergénique et un fragment contenant l'ARNt pour l'isoleucine et une partie du Gène mitochondrial ND2 ont été séquencés pour deux échantillons représentant chacun des haplotypes observés dans l'analyse de restriction. Pour l'analyse de la variabilité des microsatellites neuf locus différents ont été amplifiés avec des amorces marquées par une substance fluorescente et analysés dans un séquenceur automatique.
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 particularité 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**

Zusammenfassung – Genetische Charakterisierung von Populationen der Honigbiene in Nordzypern (*Apis mellifera cypria*). Die Mittelmeerinsel Zypern besitzt ihre eigene Unterart der Honigbiene, *Apis mellifera cypria* Pollmann 1879, aber bisher ist noch wenig über ihre genetische Variabilität und ihre Beziehungen zu benachbarten Unterarten bekannt. Bedingt durch die geographische Lage Zyperns ist *A. m. cypria* sowohl zu Unterarten der mitochodrialen C als auch der O Linie direkt benachbart. Wir untersuchten die Variabilität von *A. m. cypria* sowohl mit morphometrischen Methoden als auch mit Analysen der mitochondrialen DNA und von neun Mikrosatellitenloci. Insgesamt wurden 101 Proben an 12 Orten in Nordzypern gesammelt, wovon 18 einer morphometrischen Analyse unterzogen wurden. Es wurden 39 morphometrische Merkmale gemessen und mit multivariaten statistischen Methoden analysiert, wobei Referenzproben von umliegenden Festlandspopulationen einbezogen wurden. Ein mitochondriales Fragment, das die nichtkodierende Region zwischen dem tRNAleu Gen und dem CytochromoxidaseII Gen enthält, wurde amplifiziert und mit dem Restriktionsenzym *Dra*I verdaut. Für jeweils zwei Proben mit den im Restriktionsversuch beobachteten verschiedenen Haplotypen wurde das Fragment mit der nichtkodierenden Region sowie ein Fragment das die tRNAileu sowie einen Teil des mitochondrialen ND2 Gens enthält, sequenziert. Für die Untersuchung der Mikrosatellitenvariabilität wurden neun verschiedenen Loci mit Fluoreszenzfarbstoff markierten Primern amplifiziert und in einem automatischen Sequenzierer analysiert.
Die morphomterische Analyse ergab eine eindeutige Zuordnung der Proben aus Zypern zu *A. m. cypria*, aber in einigen Gegenden war auch ein Einfluss von importierter *A. m. anatoliaca* deutlich. Im östlichen Zypern zeigten einige Proben Ähnlichkeit mit *A. m. meda*, aber auch hier kann ein Import von *A. m. meda* nach Zypern nicht ausgeschlossen werden. Sowohl Restriktions- als auch Sequenzanalyse der mtDNA ergaben, dass die meisten zyprischen Proben zur mitochondrialen C Linie gehören, jedoch wies ein kleiner Prozentsatz der Proben typische Haplotypen der O Linie auf. Auf der Basis der Allelfrequenzen der Mikrosatelliten war die Differenzierung zwischen den Proben aus Zypern und den benachbarten Festlandspopulationen gering, jedoch erschien die Population aus Nordwestzypern stärker von Allelen des türkischen Festlands beeinflusst. Damit bestätigen unsere Ergebnisse zwar die Besonderheit von *A. m. cypria* als Unterart der Insel Zypern, sie zeigen aber auch, dass Importe von Bienen vom benachbarten Festland eine potentielle Bedrohung für die Erhaltung dieser Biene darstellen.

***Apis mellifera cypria* / mtDNA / Morphometrie / Zypern / Mikrosatelliten**

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