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Original Article

GENETIC CHARACTERIZATION OF THE CYPRIAN HONEY BEE (*APIS MELLIFERA CYPRIA*) BASED ON MICROSATELLITES AND MITOCHONDRIAL DNA POLYMORPHISMS

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Abstract

Honey bee populations from the island of Cyprus were analyzed using microsatellite and mitochondrial DNA markers. A total of 268 colonies were sampled in Cyprus, at six different locations-Kyrenia, Katydata, Flassou, Alabra, Troulloi, and Alassa-covering a wide area of the island. Results showed that the Cyprian honey bee *Apis mellifera cypria* could be distinguished from other *Apis mellifera* subspecies based on a „double pattern" of mitochondrial DNA belonging to the C1 lineage and microsatellite DNA belonging to the O lineage. All populations were homogeneous, except the population from Kyrenia, probably due to the introduction of queens or colonies belonging to the C2, C6, and M7 lineages.

Keywords: *Apis mellifera cypria*, genetic characterization, mitochondrial DNA, microsatellite DNA.

INTRODUCTION

Within the honey bee species *Apis mellifera* L., 29 different subspecies have been identified based on morphometric characteristics and ecological data (Ruttner, 1988; 1992; Sheppard et al., 1997; Engel, 1999; Sheppard and Meixner, 2003; Meixner et al., 2011). As the subspecies distributions correspond to distinct geographic areas, these groups are also described as "geographic races" (Ruttner, 1992). *A. mellifera* L. spp. are classified into five evolutionary lineages. The four primary lineages are found in the Mediterranean Basin, including the African lineage (A), west and north European lineage (M), south-east European lineage (C), and Near and Middle Eastern lineage (O) (Garnery et al., 1993; Arias and Sheppard, 1996; Franck et al., 2000; 2001; Miguel et al., 2007; Cánovas et al., 2008). The fifth proposed lineage is north-east African (Y) (Franck et al., 2001).

Mitochondrial DNA (mtDNA) markers have been widely used to address population and evolutionary questions in the honey bee *A. mellifera.* The sequencing and characterization of the mitochondrial DNA genome has been very useful for analyzing the phylogeny and population genetic structure of the *Apis* species and of *A. mellifera* subspecies, as it contains regions with variable evolutionary rates. In principle,

the general pattern of subspecies distribution has been supported by the results of various genetic studies using molecular tools (Garnery et al., 1993; Franck et al., 2000; 2001; Whitfield et al., 2006; Cánovas et al., 2008). Mitochondrial introgressions can be assessed using the "DraI" test, a molecular test that highlights the sequence variability between subunits I and II of the cytochrome oxidase gene (Garnery et al., 1993). This test has been widely used to analyze the biogeography of *A. mellifera* L. subspecies and races (e.g. Garnery et al., 1993; 1995; 1998a, b; De la Rúa et al., 1998; Rortais et al., 2011), as well as for other *Apis* species (Smith and Hagen, 1997). Using this method, different haplotypes can be distinguished and grouped into one of the four primary lineages described by Ruttner (1988) and reviewed by De la Rúa et al. (2009).

Importantly, such a test does not enable discrimination between the C and O lineages, resulting in erroneous characterizations of O-lineage colonies into the C lineage. Franck et al. (2000) reported the existence of a previously unknown mtDNA restriction enzyme pattern in honey bees sampled from Lebanon, and inferred the existence of an additional mitochondrial lineage of honey bees ('mitochondrial O'). This lineage may be analogous to a mtDNA lineage previously 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 may extend from Syria to Egypt (Arias and Sheppard, 1996). A recent study of *Apis mellifera syriaca* demonstrated its distribution in an area of contact between the A and O lineages (Alburaki et al. 2011). Pollman (1879) and Ruttner (1988) each described the honey bee of Cyprus as a distinct subspecies, *Apis mellifera cypria*. The geographic location of the Cyprian honey bee is in close proximity to subspecies belonging to both the mitochondrial C and O lineages, raising an intriguing question concerning the possible classification of *A. m. cypria* as part of one of these linages.

Here, we present a combined analysis of mtDNA and microsatellite data from *A. m. cypria*, which provides information on the genetic structure of the Cyprian honey bee population. These data, as well as those obtained from analogous studies using different approaches, can contribute to the conservation of the local honey bee populations in Cyprus.

MATERIAL AND METHODS

Sampling

Between 2001 and 2005, one individual worker bee per colony was sampled from each of 268 managed colonies from six locations in Cyprus: Flassou (32°52'40.25"N, 32°52'40.25"E), Alabra (34°59'20.38"N, 33°23'39.68"E), Alassa (34°46'10.61"N, 32°16'56.73"E), Katydata (35°5'13.18"N, 32°52'53.40"E), Kyrenia (35°19'25.65"N, 38°18'30.94"E), and Troulloi (35°02'06.68"N, 33°37'17.69"E) (Fig. 1). The sampling in Flassou was performed in 2001, sampling in Alabra, Alassa, Katydata, and Kyrenia was conducted in 2003, and sampling in Troulloi was performed in 2005. Fifty colonies each were sampled from Flassou, Alabra, Alassa, Katydata, and Kyrenia, which is considered sufficient to characterize a „population" within an area (Nei, 1987). The apiary at Troulloi contained only 18 colonies; therefore, a maximum of only 18 colonies was sampled at this location. This apiary was included in the sampling because it has been maintained for decades by monks of a nearby monastery following traditional beekeeping practices - i.e., no selection by breeding, queen rearing, queen introduction or population exchanges.

All sampled bees were stored in absolute ethanol.

mtDNA analysis

Mitochondrial DNA (mtDNA) was extracted following the protocol described by Garnery et al. (1993), with minor modifications. A mitochondrial fragment containing the intergenic region between the $tRNA^{leu}$ gene and the second subunit of the cytochrome oxidase gene was amplified using the E2 and H2 primer pair, following the method described by Cornuet et al. (1991). The resulting amplified mtDNA fragments were electrophoretically separated on 1.4% agarose gels. For each bee, length poly-

morphisms were scored as Q (194-196 bp), PQ (1200 bp), PQQ (1400 bp), or PQQQ (1600 bp) according to Garnery et al. (1991). For each amplified sample, a 24-µL aliquot was digested with five units of the DraI restriction enzyme (Roche Applied Science) for 6 h at 37°C, and then visualized on a 10% acrylamide gel. The conventional honey bee haplotype names were used: A for African, C for north Mediterranean, and M for west Mediterranean.

Microsatellite analysis

Fourteen polymorphic microsatellite loci - A7, A8, A28, A88, A113, B124 (Estoup et al., 1995), A43, Ap33, Ap36, Ap43, Ap55, Ap66, Ap81, and B24 (Solignac et al., 2003) - were amplified in two multiplex reactions and sequenced. The resulting sequences were analyzed using GeneMapper Software (Applied Biosystems).

Data analysis

The data were statistically analyzed using the TREEMAKER package. Phylogenic analyses were performed based on the method described by Cavalli-Sforza and Edwards (1967). An exact test of sample differentiation based on haplotype frequencies was calculated according to the method of Raymond and Rousset (1995) using the GENEPOP v.3.1 package. Principal coordinate analysis (PCA) was performed using the NUEES v.0.8. package (Langella, 2001).

RESULTS

mtDNA analysis

The data from the mtDNA analysis showed that all honey bee populations belonged to the most common C lineage, the C1 haplotype (Garnery et al., 1992) - except for the population from Kyrenia, which exhibited four different haplotypes: C1, C2, C6, and M7 (Fig. 1).

Microsatellite analysis

The dendrogram topology (Fig. 2) indicated that all populations from Cyprus grouped together- except the population of Kyrenia, which was

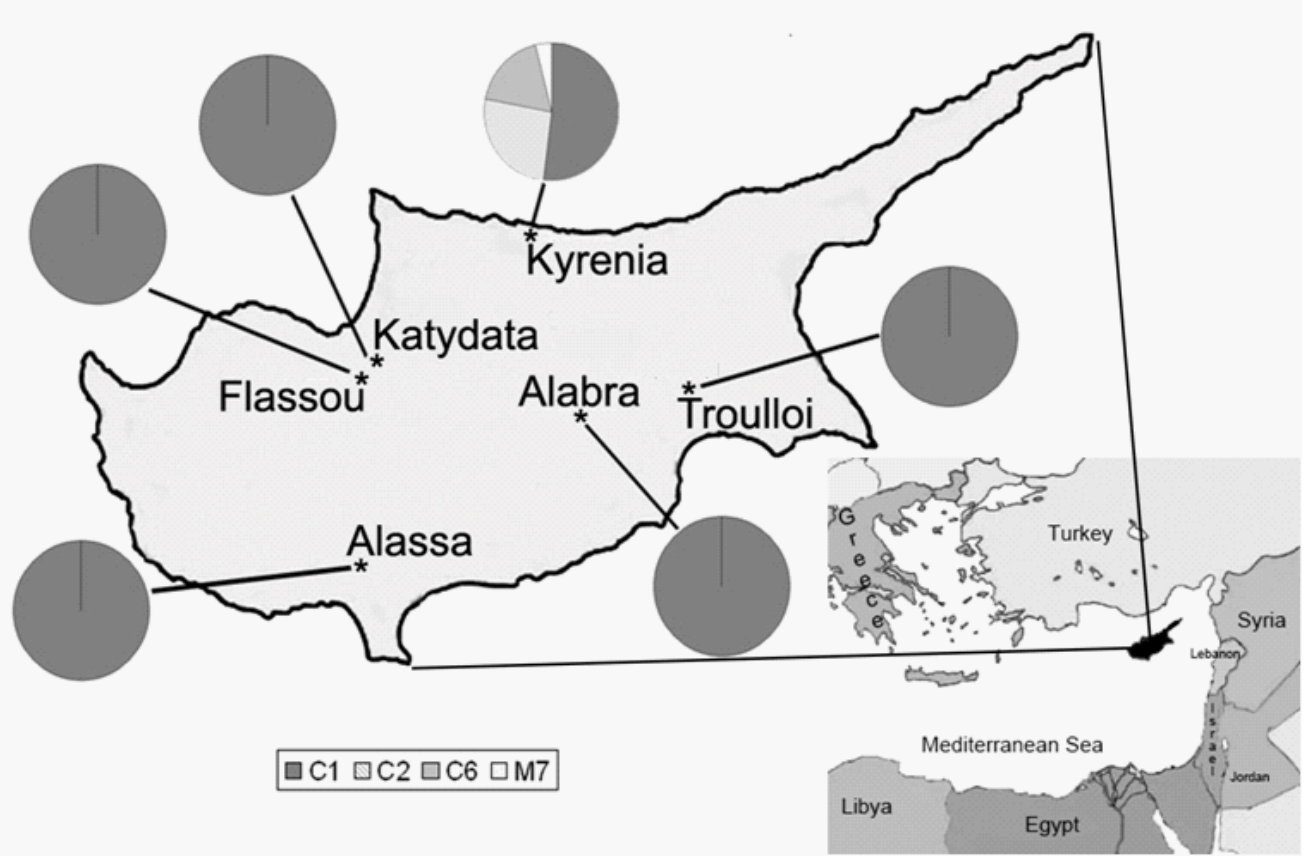

Fig. 1. Bee colony sampling sites in Cyprus, and population genetic structure (proportions of haplotypes) according to mitochondrial DNA analysis.

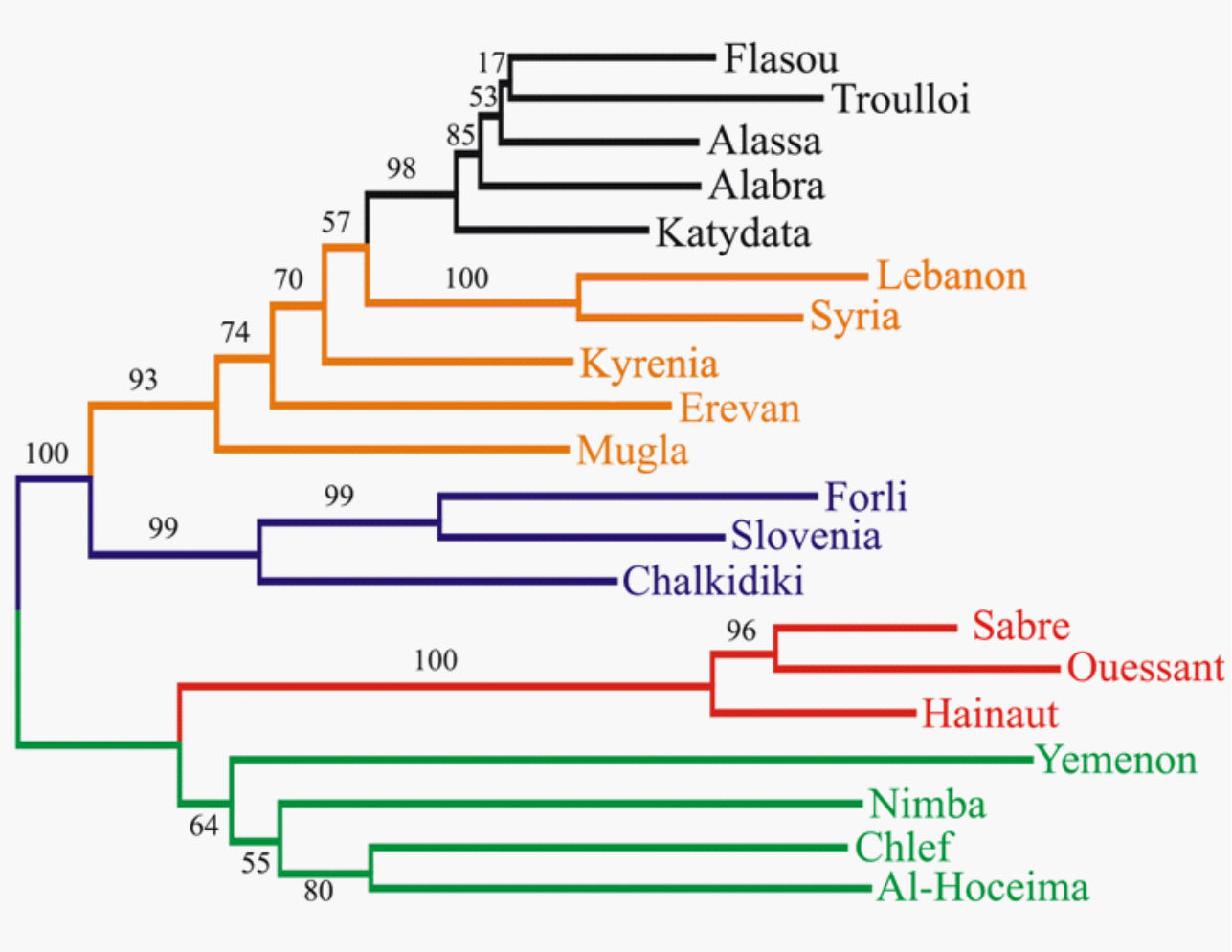

Fig. 2. A neighbor-joining tree based on mitochondrial DNA analysis of six populations from Cyprus (black), three populations belonging to the M linage (red), three belonging to the C linage (blue), four populations to the O linage (orange), and four belonging to the A linage (green). Bootstrap values are indicated for the main nodes.

located at the base of the other Cyprian populations and separated by a long branch with a high bootstrap value (94%). The Cyprian populations were located up on the branch of the O lineages with populations from Syria and Lebanon, but were separated from these groups with high bootstrap values (94 and 100%, respectively). This situation was confirmed by the PCA results (Fig. 3 and 4). The first axis represented 39.07% of the total variance, and opposed populations from the M, C, and O lineages. Projections of the Cyprian and Middle-Eastern populations indicated that these populations exist in close proximity with the C and O lineages. The second axis expressed 54.38% of the total variance, and opposed populations from the C lineage and from the Middle-East. The Cyprian populations appeared to be more closely related to Middle-Eastern populations than to the C or O lineages. These results were also confirmed by the PCA performed on the distance matrix between individuals. Compared to the other analyzed bee populations, that from Kyrenia existed as a distinct clade belonging to the O lineage. The third axis expressed 14.3% of the total variance. The projection of Cyprian populations on this axis showed their differentiation from the populations belonging to the O lineage, and their distance from the populations belonging to the C linage. The only exception was, once again, the population from Kyrenia which was close to the O lineage populations.

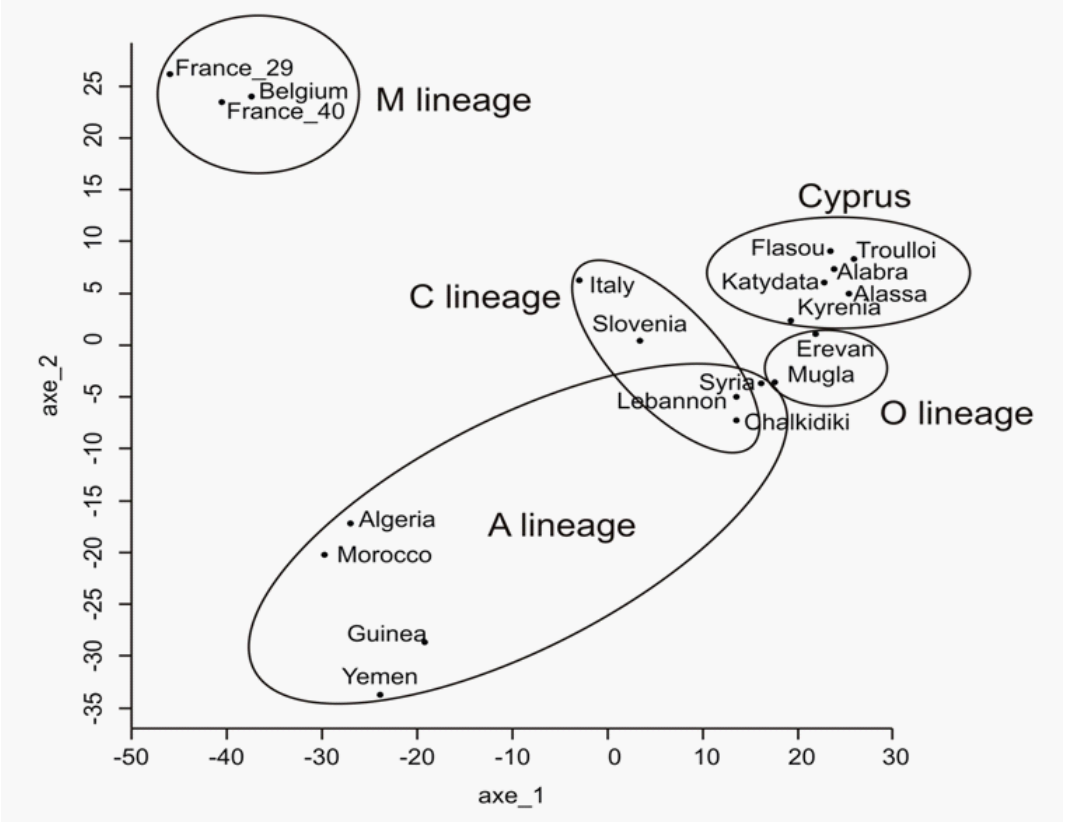

Fig. 3. Comparison between honey bee populations from Cyprus and 14 control populations using principal coordinate analysis. Axis 1 represents 39.07% of the total variance, and opposes populations from the M, C, and O lineages. Axis 2 expresses 54.38% of the total variance, and opposes populations from the C lineage and from the Middle East.

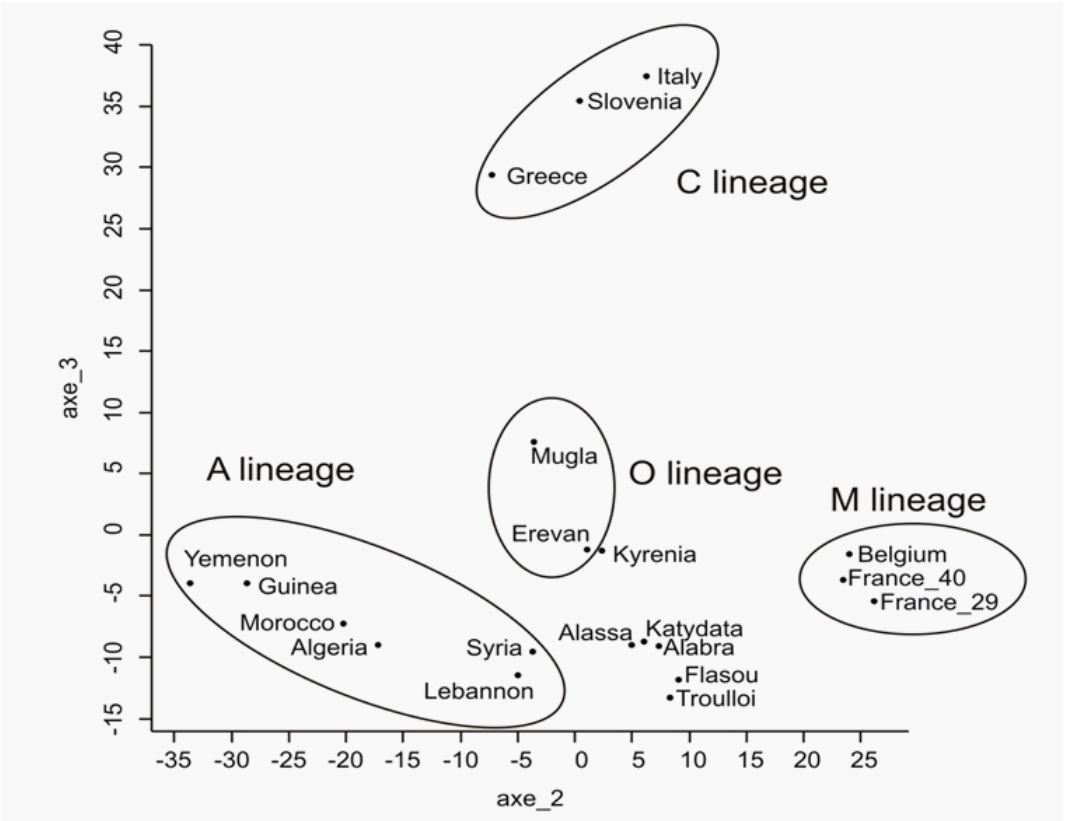

Fig. 4. Comparison between honey bee populations from Cyprus and 14 control populations using principal coordinate analysis. Axis 3 expresses 14.3% of the total variance. The projection of Cyprian populations on this axis shows their differentiation from the populations belonging to the O lineage and their distance from the populations belonging to the C lineage.

DISCUSSION

The results of the present study, confirm that the honey bee populations in Cyprus belong to the C lineage-except for the population of Kyrenia, which is of high interest because it belongs to four different haplotypes C1, C2, C6, and M7 (Fig. 1). Our data based on microsatellite analysis also showed that the population from Kyrenia was distinct from the other analyzed populations, seeming to be more similar to those from the Middle East.

Our current findings support the assumption that *A. m. ligustica* bees were imported to the northern part of Cyprus. Previously, Kandemir et al. (2006) found colonies of the mitochondrial C lineage in north Cyprus, along with a small proportion of colonies having restriction profiles characteristic of the mitochondrial O lineage. Microsatellite analysis of these same colonies indicated a relatively low level of differentiation among the Near-Eastern populations, irrespective of their assignment to the mitochondrial lineages C or O. While overall Fst values between the populations of Cyprus and the reference populations are low, the honey bee population of north-western Cyprus was introgressed to a larger extent by microsatellite alleles from Turkey, suggesting importation of queens from Turkey. Their morphometric analysis also showed that the contemporary honey bee populations 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*, could be detected.

Bouga et al. (2005b) performed isoenzymatic analysis of honey bees from Greece and the southern part of Cyprus, and their results showed that Cyprian honey bee populations are well discriminated from the Greek populations based on the ADH and LAP enzymatic systems. MtDNA analysis of the same samples (Bouga et al., 2005a; Martimianakis et al., 2011) demonstrated that honey bees from Cyprus belong to the East Mediterranean C lineage. Additionally, results based on geometric morphometry show that honey bee populations located near the northern part of Cyprus are different from those located in the southernmost part, suggesting a possible introgression within these populations from the north (Lykoudis et al., 2004).

The most important finding of the present study is the observed unique "double pattern"-i.e., the existence of mtDNA belonging to the C and O linage-which can be used for the discrimination and characterization of *A. m. cypria*. This finding proves that the domestic subspecies still exists on the island, despite the possible importations of other bee races in the northern part of Cyprus. These data can contribute to the conservation of the local bees, which are better adapted to the harsh climatic conditions of Cyprus (Ruttner, 1988) and therefore important to maintain.

CONCLUSIONS

The present combination of mtDNA and microsatellite analyses revealed a „double pattern" of both C and O linages in the *A. m. cypria* genome. This provides an accurate criterion to discriminate the domestic honey bee subspecies of the island, which remains homogenous in most of Cyprus.

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