DO MOLECULAR MARKERS SUPPORT MORPHOMETRIC AND PHEROMONE ANALYSES? A PRELIMINARY CASE STUDY IN *APIS MELLIFERA* POPULATIONS OF MOROCCO

¿APOYAN LOS MARCADORES MOLECULARES LOS ANÁLISIS MORFOMÉTRICOS Y DE FEROMONAS? ESTUDIO PRELIMINAR EN POBLACIONES DE *APIS MELLIFERA* DE MARRUECOS

De la Rúa, P.[1], S. Radloff[2], R. Hepburn[3] and J. Serrano[1]

[1]Departamento de Zoología y Antropología Física. Facultad de Veterinaria. Universidad de Murcia. 30071 Murcia. España. E-mail: pdelarua@um.es (corresponding author); jserrano@um.es
[2]Department of Statistics. Rhodes University. Grahamstown, 6140. South Africa. E-mail: s.radloff@ru.ac.za
[3]Department of Zoology and Entomology. Rhodes University. Grahamstown, 6140. South Africa. E-mail: R.Hepburn@ru.ac.za

ADDITIONAL KEYWORDS

Honeybee. Mitochondrial DNA. Microsatellite. Morphometry. Biogeography.

PALABRAS CLAVE ADICIONALES

Abejas. ADN Mitocondrial. Microsatélite. Morfometría. Biogeografía.

SUMMARY

The intergenic tRNA^{leu}-COII mitochondrial region and four microsatellite *loci* were analyzed on worker honeybees sampled from the Atlantic coast of Morocco. Morphological and pheromonal analyses previously clustered them into the two subspecies *Apis mellifera intermissa* and *A. m. sahariensis*. Mitochondrial haplotypes and some microsatellite alleles were found to be restricted to each of the two subspecies. A hybrid zone between these subspecies could be inferred at the boundary of their geographic distribution, thus corroborating previous pheromonal analysis of the same samples.

RESUMEN

La variación de la región intergénica tARN^{leu}-COII del ADN mitocondrial y de cuatro *loci* de microsatélites ha sido analizada en muestras de abejas obreras de la costa atlántica de Marruecos. Análisis preliminares de estas mismas muestras a nivel morfológico y de feromonas agruparon estas muestras en dos subespecies (*Apis mellifera intermissa* y *A. m. sahariensis*). Los haplotipos mitocondriales y algunos alelos de microsatélites aparecieron restringidos a cada una de las subespecies. Según los resultados obtenidos se puede inferir la existencia de una zona de hibridación entre las dos subespecies en los límites de su distribución geográfica, corroborando de esta manera los resultados previos del análisis de feromonas realizado en las mismas muestras.

INTRODUCTION

The honeybee *Apis mellifera* L. has evolved into a high number of

geographic subspecies in the last 1-2 MY, when it possibly colonized the Ethiopian and West Palaearctic regions from the Middle East (Garnery *et al.*, 1992). Ruttner (1988) recognized 25 subspecies according to morphometric, geographic and behavioral traits, and parallel levels of variation have been found in molecular data (Franck *et al.*, 2000, 2001). Social organization, adaptation to local conditions and good dispersal power are among the main factors that explain the formation of discrete populations with varying degrees of intergradation between them.

Honeybee populations of North Africa are currently regarded as members of two subspecies, *Apis mellifera intermissa* Buttel-Reepen, 1906 and *A. m. sahariensis* Baldensperger, 1924. They were originally classified as members of the Western Mediterranean group by Ruttner (1988) according to morphometric, geographic and behavioral traits. The somewhat smaller *A. m. sahariensis* is found in several oases in Southern Morocco and is characterized by a reduced tendency to swarm, a restricted number of queen cells, an immediate elimination of virgins during swarming, a little use of propolis and a weak defensive response (Ruttner, 1988). In contrast, large populations of *A. m. intermissa* occur between the Atlas and the Mediterranean and Atlantic coasts. This subspecies is prone to swarming, shows aggressive behavior and an abundant use of propolis. An ecotype described as *A. m. major* was detected in a restricted area of the Rif Mountains. This large honeybee does not differ from *A. m. intermissa* in behavior and its taxonomic status remains undefined (Ruttner, 1988).

Both subspecies have been analyzed in some depth morphometrically and pheromonally (Hepburn and Radloff, 1996, 1998; Engel, 1999). Molecular analyses including samples from these subspecies have been carried out by Garnery *et al.* (1995) and Franck *et al.* (2001). These authors concluded that the relatedness of Moroccan honeybees to West European subspecies based on morphological traits was not fully supported by the molecular data. In this sense, the study of mitochondrial haplotypes showed that these bees are members of an evolutionary lineage named African (A) (Garnery *et al.*, 1995; Arias and Sheppard, 1996; Franck *et al.*, 1998). This lineage is defined by the presence of the sequence P_0 plus from one to three Q sequences in the intergenic $tRNA^{leu}$-COII mitochondrial region. In contrast, West European populations are characterized by a distinct P region (Garnery *et al.*, 1995). However, Arias and Sheppard (1996) noted that North African populations were slightly differentiated from Sub-Saharan ones, according to the sequence data of $tRNA^{Ile}$-ND2 mitochondrial region. Likewise, Moroccan bees show high levels of microsatellite polymorphism what characterize honeybee populations from Africa (Estoup *et al.*, 1995; Franck *et al.*, 1998).

This lack of a close correspondence between morphometric and molecular traits has been repeatedly found in most honeybee populations studied at some depth (Franck *et al.*, 2000, De la Rúa *et al.*, 2001a,b, Radloff *et al.*, 2001; Kandemir *et al.*, 2003). It is

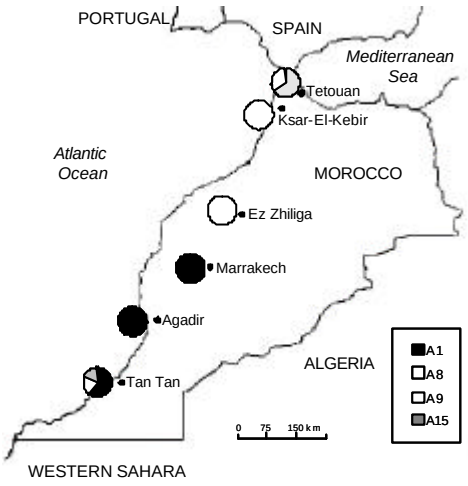

***Figure 1** . Locations in Morocco sampled in the present study and pie charts of the mtDNA haplotype frequency.* (Localidades de Marruecos muestreadas en este estudio y diagramas de distribución de frecuencias de haplotipos mitocondriales).

clear that both mechanisms of transmission of nuclear and mitochondrial characters, and selective processes acting on both genomes are involved in these differences. In this paper we investigate the mitochondrial and microsatellite variation of samples collected from traditional beekeepers along a transect in Morocco. These samples were previously analyzed morphometrically and pheromonally by Hepburn and Radloff (1996), who showed that the samples clustered into two geographic groups and reported the existence of a hybridization zone between Southern *A. m. sahariensis* and Northern *A. m. intermissa*. Likewise, they also noted a possible hybrid zone between this last subspecies and populations included in the ecotype *A. m. major*. We aim to test whether molecular markers corroborate the conclusions derived from the previous analyses.

MATERIAL AND METHODS

Three to five honeybee colonies were sampled at six localities in Morocco **(figure 1)**. These samples were assigned either to the subspecies *A. m. intermissa* or to *A. m. sahariensis* **(table I)** by Hepburn and Radloff (1996). DNA extraction was performed following the Chelex method (Walsh *et al.*, 1991) with slight modifications. One or two worker legs (one worker bee per colony) were dried for 30 min at 37 °C and then homogenized with 100 mL of 5 percent Chelex solution from Bio-Rad (South San Francisco, CA, USA) and protei-

***Table I.** Mitochondrial variation for the Moroccan honeybees by population and for each subspecies.* (Variación mitocondrial de las abejas de Marruecos por población y por cada subespecie).

	Frequency of haplotypes			
	A1 PoQ	A8 PoQ	A9 PoQQ	A15 PoQQQ
Subespecies				
intermissa	0.31	0.13	0.56	-
Tetouan	-	0.67	0.33	-
Ksar El Kebir	-	-	1	-
Ez Zhiliya	-	-	1	-
Marrakech	1	-	-	-
sahariensis	0.78	-	0.11	0.11
Agadir	1	-	-	-
Tan Tan	0.60	-	0.20	0.20
Total	0.48	0.08	0.4	0.04

nase K (10 mg/ml). This mixture was incubated for 1 h at 55 °C, 15 min at 99 °C, 1 min at 37 °C, and finally 15 min at 99 °C in a thermocycler. Two mL of this solution were used for the PCR amplification.

MITOCHONDRIAL ANALYSIS

The intergenic $tRNA^{leu}$-COII region was amplified in a 12.5 mL reaction following Garnery *et al.* (1993), with the primers E2 located at the 5'-end of the $tRNA^{leu}$ gene, and H2 located at the 5'-end of the COII gene. The PCR reactions were conducted on a PTC-100 thermocycler (Bio-Rad, South San Francisco, CA, USA) and the conditions were as followed: 95 ºC for 5 min., 35 cycles of 95 ºC for 30 seg., 47 ºC for 30 seg. and 72 ºC for 90 seg.; a final extension at 72 ºC for 10 min. was added. Aliquots of 2.5 mL were electrophoretically separated on 1.5 percent agarose, stained with ethidium bromide and visualized under UV light to determine the size of the amplified products. Ten mL aliquots of the PCR products were digested with five units of the *Dra*I enzyme from Fermentas (Ontario, Canada) at 37 ºC for 4-12 h. The resulting fragments were visualized in 5 percent NuSieve agarose from Cambrex (Walkersville, MD, USA). Fisher's exact test and Monte Carlo 99 percent confidence intervals for the level of significance based on 10,000 samples were used to test for significant differences in haplotype distributions between the subspecies.

MICROSATELLITE ANALYSIS

Four polymorphic microsatellite *loci* were analyzed: A113, A7, A24 and A28. Multiplex PCR reactions were performed when the annealing temperature was compatible: A113 and A7 were amplified together at 58 ºC and A24 and A28 together at 55 ºC, both reactions with 1.5 mM MgCl2. The PCR conditions were as followed: 95 ºC for 5 min., 30 cycles of 95 ºC for 30 seg., 58 or 55 ºC for 30 seg. and 72 ºC for 30 seg.; ending with a final extension at 72 ºC for 30 min. The reactions were done in an Eppendorf thermocycler (Eppendorf AG, Hamburg, Germany) with fluorescent labeled primers and separated on a DNA automated sequencer (ABI 377) from Applied Biosystems (Foster City, CA, USA). Microsatellite allele sizes were scored by comparing the length of the PCR fragments to the standard 100 bp ROX from the same manufacturer. Population parameters (expected and observed heterozygosity and number of alleles) were calculated following Nei (1987) with The Excel Microsatellite Toolkit (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/). The exact tests for genic and genotypic differentiation were computed with GENEPOP web version 3.1c (http://wbiomed.curtin.edu.au/genepop). For each *locus*, an unbiased estimate of the P-value of the probability test (or Fisher's exact test) was performed, as described by Raymond and Rousset (1995).

RESULTS

1. MITOCHONDRIAL DNA

Four different haplotypes were found, all belonging to the African evolutionary lineage as they have the P_0 sequence in the mitochondrial intergenic region. These haplotypes

have one (A1 and A8), two (A9) or three (A15) repeats of the Q sequence (Garnery *et al.*, 1993; De la Rúa *et al.*, 1998). A1 and A9 have been observed in both subspecies, whereas A8 was found only in colonies of *A. m.*

Table II. *Microsatellite allele frequencies for the Moroccan honeybees by* locus. (Frecuencias de alelos de microsatélites de las abejas de Marruecos por *locus*).

Locus/Allele	Tetouan	Ksar El Kebir	Ez Zhiliya	Marrakech	Agadir	Tan Tan
A113						
202	-	-	0.33	-	-	-
204	0.25	0.50	0.17	0.70		0.30
206	-	-	-	-	-	0.10
210	-	-	-	-	-	0.10
212	0.25	-	-	-	0.25	-
214	0.25	0.33	0.50	0.10	0.50	0.30
216	-	-	-	-	0.12	-
220	0.25	0.17	-	0.20	-	0.20
224	-	-	-	-	0.12	-
A7						
96	0.50	0.17	-	-	-	-
97	-	-	-	-	-	0.20
98	-	0.17	-	-	-	-
103	-	0.17	-	-	-	0.20
104	-	0.17	-	-	0.12	-
106	-	0.17	-	0.67	0.25	0.10
107	0.50	-	-	-	0.12	-
109	-	0.17	0.50	0.33	0.50	0.30
114	-	-	0.50	-	-	0.20
A24						
94	-	-	0.37	0.25	-	0.60
98	-	0.25	-	-	-	-
99	-	-	0.25	-	0.50	-
101	0.50	-	0.25	-	-	-
102	-	0.25	0.12	-	-	0.4
103	0.50	0.50	-	0.75	-	-
105	-	-	-	-	0.50	-
A28						
127	-	0.17	0.37	0.12	0.25	0.20
128	-	-	0.12	0.12	-	0.10
129	-	0.33	0.12	0.12	-	-
130	0.25	-	-	-	0.12	0.30
131	-	-	-	0.12	-	0.20
132	0.75	0.50	0.12	-	0.50	-
133	-	-	0.25	0.50	0.12	0.10
136	-	-	-	-	-	0.10

intermissa and A15 in colonies of *A. m. sahariensis*. Overall, the most frequent haplotypes were A1 (48 percent) and A9 (40 percent), the other two haplotypes A8 and A15 showing a frequency of 8 percent and 4 percent, respectively. The distribution of haplotypes between subspecies is shown in **table I**. The haplotype A1 was only found south to Marrakech, whereas the haplotype A9 was mostly found in the north; except for a few individuals from Tan Tan **(figure 1)**. These differences in haplotype distribution are statistically significant (Fisher's exact test: 7.72, P= 0.024; 99 percent Monte Carlo confidence intervals for p-value: 0.022-0.030).

2. Microsatellite data

The allele frequencies of the colonies by *locus* are shown in **table II**. Nine alleles were observed at the *loci* A113 and A7, eight at the *locus* A28 and seven at the *locus* A24. The overall population parameters per locality and subspecies are shown in **table III**. There was a significant difference in mean gene diversity across all *loci* and populations within subspecies (0.79 ± 0.03 for *A. m. intermissa* and 0.82 ± 0.02 for *A. m. sahariensis*; Z test, p= 0.0154). The total number of alleles and the number of subspecies-specific alleles were both equal for the two subspecies across all *loci*, although the variation was different when considering each *locus* separately **(table IV)**.

Because of the reduced number of samples per locality (from 2 to 5) we have pooled the samples and performed differentiation tests at the subspecies level. The Fisher's method as implemented in the GENEPOP program was used for this purpose. The genic differentiation (concerning the distribution of alleles in the subspecies) and the genotypic differentiation tests (taking into account the distribution of genotypes between the subspecies) were not statistically significant, except

Table III. *Estimates of the overall number of alleles (n) and expected (He) and observed (Ho) heterozygosity values for the four* loci *per honeybee subspecies and locality.* (Estimaciones del número total de alelos y de los valores de heterocigosidad esperada y observada para los cuatro *loci* por subespecie de abeja y por localidad).

	N	n	He	Ho
A. m. intermissa	14	6.50±1.29	0.79±0.03	0.46±0.07
Tetouan	2	2.50±1.00	0.70±0.10	0.63±0.17
Ksar El Kebir	3	3.75±1.50	0.80±0.06	0.62±0.15
Ez Zhiliya	4	3.50±1.29	0.85±0.06	0.52±0.14
Marrakech	5	3.00±1.41	0.56±0.08	0.29±0.11
A. m. sahariensis	9	6.50±1.73	0.82±0.02	0.48±0.08
Agadir	4	3.50±1.00	0.73±0.02	0.31±0.12
Tan Tan	5	4.50±1.73	0.78±0.08	0.55±0.11

Table IV*. Number of alleles (Na) and number of subspecies-specific alleles per microsatellite* loci *(No).* (Número de alelos (Na) y de alelos específicos (No) de cada subespecie por *locus* de microsatélite).

Locus	Subspecies	Na	No
A113	*A. m. intermissa*	5	1
	A. m. sahariensis	8	4
A7	*A. m. intermissa*	8	2
	A. m. sahariensis	7	1
A24	*A. m. intermissa*	6	3
	A. m. sahariensis	4	1
A28	*A. m. intermissa*	7	1
	A. m. sahariensis	7	1
Total	*A. m. intermissa*	26	7
	A. m. sahariensis	26	7
	overall	33	14

for the *locus* A24 ($p<0.002$ and $p<0.03$, respectively).

DISCUSSION

Data on the mitochondrial variation within the subspecies *A. m. sahariensis* and *A. m. intermissa* suggest that the distribution of the mtDNA haplotypes is not random, as haplotype A15 is restricted to the former in the analyzed samples. Haplotype A1 is the most frequent in *A. m. sahariensis*, but is also unique to the population from Marrakech that corresponds morphometrically with *A. m. intermissa.* This introgression at the mitochondrial level is a common feature of the relationships between contiguous honeybee subspecies,and suggests that evolutionary trends of mitochondrial characters may be quite different from those concerning morphometric ones. Therefore, characters of the external morphology propose a relatively sharp discontinuous distribution between *A. m. sahariensis* and *A. m. intermissa,* whereas the molecular data seem to indicate the occurrence of appreciable flow between them. Pheromone distribution also supported this flow (Hepburn and Radloff, 1996). These authors concluded from the overall evidence that there must be at least two hybrid zones in Morocco, one close to Marrakech and another in the transition from the Atlantic plain to the Rif Mountains.

It is noticeable that haplotype A15 has been detected in Morocco for the first time, whilst is common in the Canary Islands and Madeira (De la Rúa *et al.*, 1998, 2001a, in press). This result supports a mainland origin of this haplotype or, alternatively, a recent recolonization event from the Canaries, instead of being a particular haplotype restricted to some Macaronesian archipelagos.

Mitochondrial data corroborate the idea that the sea between North Africa and the Iberian Peninsula must be an effective barrier to the flow between honeybee populations. The A9 haplotype that predominates in Northern Morocco (and perhaps A8 in the Rif Mountains) is rare in the Iberian Peninsula, where A2 predominates (De la Rúa *et al.*, 2004 and references therein). As mitochondrial markers are good indicators of historical events, these results corroborate the hypothesis of an ancient colonization of Mediterranean islands and the Iberian Peninsula by African populations characterized by the A2 haplotype (De

la Rúa *et al.*, 2001b) that has been also detected in Morocco (Franck *et al.*, 2001).

The distribution of the alleles of the microsatellite *loci* also shows the genetic differentiation of the two subspecies, as 42 percent of the total number of alleles is subspecies-specific (26 percent for each subspecies). This result is mirrored in the genetic differentiation tests, particularly at the *locus* A24. The high genetic variability of African honeybee populations has been explained as a result of the pronounced migratory behavior and tendency to swarm (Franck *et al.*, 1998), and as a consequence of a large effective population size (Franck *et al*., 2001). This migratory behavior is different in the two subspecies of Moroccan honeybees, and could explain the significant differences observed in the expected heterozygosity values obtained in this study. However, this explanation should be further assessed by increasing both the number of samples of both subspecies and microsatellite *loci.*

Microsatellite data are not conclusive on the role of the Strait of Gibraltar as a geographic barrier to flow. Whereas southeast Iberian populations show the lower levels of variability of European races (De la Rúa *et al.*, 2002) in comparison to African populations (Franck *et al.*, 1998, 2001), others located in the southern tip of the Andalusia (Iberian Peninsula) facing Morocco (De la Rúa *et al.*, 2004) show a microsatellite variation close to that found in North African populations (Franck *et al.*, 2001) as described also in this paper. These results merit further studies to reconstruct the complex sequence of evolutionary events that led to the present geographic patterns of the honeybee in the western Palaearctic region.

We can conclude that a correlation between morphometric and molecular characters exits in this case. Some morphometric traits are apparently conservative over time, inert to environmental influences and therefore suitable to characterize *A. mellifera* subspecies (De la Rúa *et al.*, 2005). Also some parts of the mtDNA molecule are helpful to reconstruct events that may have happened hundreds of thousands of years ago. The use of both data sets is the trend in the characterization of new subspecies as *A. m. ruttneri* (Sheppard *et al.*, 1997) and *A. m. pomonella* (Sheppard and Meixner, 2003) recently described on the basis of morphometric and molecular characters. In any case, further analyses with more samples and including more populations may confirm these results.

ACKNOWLEDGEMENTS

This work has been supported by the COBICE program and Fundación Séneca (Agencia Regional de Ciencia y Tecnología, Spain). We thank B. V. Pedersen for laboratory facilities and A.B. Jensen for her help in the handling of the ABI 377 sequencer. P. De la Rúa is supported by the Program Ramón y Cajal of the Spanish Ministry of Education and Science. We appreciate the corrections and suggestions made by two anonymous reviewers.

REFERENCES

Arias, M.C. and W.S. Sheppard. 1996. Molecular phylogenetics of honeybee subspecies (*Apis mellifera* L.) inferred from mitochondrial DNA sequence. *Mol. Phyl. Evol*., 5: 557-566.

De la Rúa, P, J. Galián and J. Serrano. 1998. Mitochondrial variability of honeybee populations from the Canary Islands. *Mol. Ecol.,* 7: 1543-1547.

De la Rúa, P, J. Galián, J. Serrano and R.F.A. Moritz. 2001a. Genetic structure and distinctness of *Apis mellifera* L. populations from the Canary Islands. *Mol. Ecol.,* 10: 1733-1742.

De la Rúa, P, J. Galián, J. Serrano and R.F.A. Moritz. 2001b. Molecular characterization and population structure of the honeybees from the Balearic Islands (Spain). *Apidologie,* 32: 417-427.

De la Rúa, P, J. Galián, J. Serrano and R.F.A. Moritz. 2002. Microsatellite analysis of non-migratory colonies of *Apis mellifera iberica* from south-eastern Spain. *J. Zool. Syst. Evol. Res*., 40: 1-5.

De la Rúa, P., R. Hernández-García, B.V. Pedersen, J. Galián and J. Serrano. 2004. Molecular diversity of *Apis mellifera iberica* L. (Hymenoptera: Apidae) from western Andalusia. *Arch. Zootec.,* 53: 195-203.

De la Rúa, P., S. Fuchs and J. Serrano. 2005. Biogeography of European honeybees. *In* Lodesani, M; Costa, C (ed) Beekeeping and conserving biodiversity of honeybees. Northern Bee Books, Hebden Bridge, UK; pp. 15-52.

Engel, M.S. 1999. The taxonomy of recent and fossil honeybees (Hymenoptera: Apidae: *Apis*). *J. Hym. Res.,* 8: 165-196.

Estoup, A., L. Garnery, M. Solignac and J.M. Cornuet. 1995. Microsatellite variation in honeybee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. *Genetics,* 140: 679-695.

Franck, P., L. Garnery, M. Solignac and J.M. Cornuet. 1998. The origin of West European subspecies of honeybees (*Apis mellifera*): new insights from microsatellite and mitochondrial data. *Evolution,* 52: 1119-1134.

Franck, P., L. Garnery, M. Solignac and J.M. Cornuet. 2000. Molecular confirmation of a fourth lineage in honeybees from the Middle East. *Apidologie,* 31: 167-180.

Franck, P., L. Garnery, A. Loiseau, H.R. Hepburn, M. Solignac and J.M. Cornuet. 2001. Genetic diversity of the honeybee in Africa: microsatellite and mitochondrial data. *Heredity,* 86: 420-430.

Garnery, L., J.M. Cornuet and M. Solignac. 1992. Evolutionary history of the honey bee *Apis mellifera* inferred from mitochondrial DNA analysis. *Mol. Ecol.,* 1: 145-154.

Garnery, L., M. Solignac, G. Celebrano and J.M. Cornuet. 1993. A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L. *Experientia,* 49: 1016-1021.

Garnery, L., E.H. Mosshine and J.M. Cornuet. 1995. Mitochondrial DNA variation in Moroccan and Spanish honey bee populations. *Mol. Ecol.,* 4: 465-471.

Hepburn, H.R. and S.E. Radloff. 1996. Morphometric and pheromonal analyses of *Apis mellifera* L. along a transect from the Sahara to the Pyrenees. *Apidologie,* 27: 35-45.

Hepburn, H.R. and S.E. Radloff. 1998. Honeybees of Africa. Springer-Verlag, Berlin, Germany, 367 pp.

Kandemir, I., M.D. Meixner, A. Ozcan and S.W. Sheppard. 2003. Morphometric, allozymic and mtDNA variation in honeybee populations in Northern Cyprus. 38-th International Apicultural Congress of Apimondia, Ljubljana, Slovenia.

Nei, M. 1987. Molecular Evolutionary Genetics, Columbia University Press, New York, NY, USA.

Radloff, S.E., H.R. Hepburn, C. Hepburn and P. de la Rúa. 2001. Morphometric affinities and

population structure of honeybees of the Balearic Islands (Spain). *J. Apicultural Res.,* 40: 97-104.

Raymond, M. and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Heredity,* 86: 248-249

Ruttner, F. 1988. Biogeography and taxonomy of honeybees. Springer Verlag, Berlin, Germany; 284 pp.

Sheppard, W.S., M.C. Arias, M.D. Meixner and A. Grech. 1997. *Apis mellifera ruttneri*, a new honey bee subspecies from Malta. *Apidologie,* 28: 287-293.

Sheppard, W.S. and M.D. Meixner. 2003. *Apis mellifera pomonella*, a new honey bee subspecies from Central Asia. *Apidologie,* 34: 367-375

Walsh, P.S., D.A. Metzqer and R. Higuchi. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques,* 10: 506–512.

Recibido: 26-4-06. Aceptado: 22-6-06.