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## Genetic divergence and phylogenetic relationships of honey bee *Apis mellifera* (Hymenoptera: Apidae) populations from Greece and Cyprus using PCR – RFLP analysis of three mtDNA segments<sup>1</sup>

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**Abstract** – The genetic structure and phylogenetic relationships among six honey bee populations were studied using RFLP analysis on three PCR-amplified mtDNA gene segments (16s rDNA, CO I, and ND 5). The populations were sampled from various areas of Greece and Cyprus and correspond to *Apis mellifera adami*, *A. m. macedonica*, *A. m. cecropia*, and *A. m. cyprica* races, based on origin (Ruttner, 1988). Seven, eight and seven restriction enzymes were found to have at least one recognition site at the 16s rDNA, CO I, and ND 5 segments, respectively. Seven different haplotypes were detected and diagnostic patterns enabled us to discriminate *A. m. macedonica* from the rest of the populations (races). The estimated net nucleotide sequence divergence among the populations examined was found to range from 0.00 to 1.18 with the highest value observed to be between *A. m. macedonica* and non-*A. m. macedonica* populations. The trees obtained (by UPGMA and Dollo parsimony methods) revealed that the most distant population was that of *A. m. macedonica*.

*Apis mellifera* / honey bee / mtDNA / genetic variability / Greece

### 1. INTRODUCTION

Intraspecific taxonomy of the honey bee *Apis mellifera* L. has been based mainly on morphology. At present, 26 subspecies of *A. mellifera* are recognized on the basis of morphometric characters (Ruttner, 1988, 1992; Sheppard et al., 1997). On the same morphometric basis (Ruttner, 1988, 1992), these subspecies have been grouped in four lineages. The first one includes the subspecies from northern and western Europe and northern Africa; the second the subspecies of the northern Mediterranean region and Eastern Europe; the third the

subspecies of sub-Saharan Africa and the fourth lineage the subspecies of the eastern Mediterranean region and Iran.

More recently, genetic systems such as allozymes (Nunamaker and Wilson, 1982; Badino et al., 1988), nuclear DNA (Hall, 1990; Tarès et al., 1993), mitochondrial DNA (mtDNA) (Moritz et al., 1986; Smith et al., 1989, 1991; Hunt and Page, 1992; Garnery et al., 1993; Oldroyd et al., 1995; Arias and Sheppard, 1996; Pedersen, 1996; De la Rúa et al., 2000) and microsatellites (Estoup et al., 1993; Garnery et al., 1998) have been used to study honey bee diversification. Such analysis of population genetic

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**Figure 1.** Sampling sites: PHT, Phthiotida; MAC, Macedonia; KTH, Kythira; KAS, Kasos; IKA, Ikaria; CYP Cyprus.

differentiation at the molecular level contributes to better understanding of honey bee population structure by allowing the comparison of morphological, behavioural, geographical and molecular variation.

The maternal inheritance of mtDNA, a property which has been demonstrated for honey bees (Meusel and Moritz, 1993), denotes that all the workers and drones in a colony share the mtDNA haplotype of the queen. Variation in the mtDNA of honey bees has been used to provide insight into their biogeography and, like morphometric data, reveals several main evolutionary lineages of *Apis mellifera* (Smith and Brown, 1988, 1990; Cornuet and Garnery, 1991b). However, one has to realize that the importation of foreign queens presents a situation where hybridization can change the distribution of mtDNA variants found in the genetic pool of local bees.

Ruttner's (1988) morphometric analysis concluded that *A. m. adami*, *A. m. macedonica*, *A. m. cecropia*, and *A. m. carnica* subspecies of *A. mellifera* exist in Greece and *A. m. cypria* exists in Cyprus. Several morphometric analyses of Greek continental populations showed no hybridization (Ifantidis, 1979). Moreover allozyme analysis of Greek continental honey bee populations (Thrace, Macedonia, Central Greece and Peloponnesse) and those of Crete (Aegean Sea) supported the existence of a dis-

tinct race in Crete distinguishable from honey bees populations from northern and central Greece (Badino et al., 1988).

In the present investigation, honey bee populations from various areas of Greece (Ikaria, Kasos, Kythira, Phthiotida, Macedonia) and Cyprus, corresponding to different subspecies, were studied using RFLP analysis of three mtDNA gene segments. The main aim of this research was: (1) to study the genetic structure of the aforementioned populations (races), (2) to examine their phylogenetic relationships, (3) to investigate the possibility of the gene flow existence as a result of migratory beekeeping and commercial breeding, and (4) to compare our data with those presented by Ruttner's (1988) morphometric ones. Such results could be also useful in terms of migratory beekeeping, commercial breeding and Greek honey bee monitoring.

## 2. MATERIALS AND METHODS

Bees from 72 colonies (12 colonies per population) were collected from Aegean Sea Islands Ikaria (IKA), Kasos (KAS), Kythira (KTH); Central Greece Phthiotida (PHT); Northern Greece Macedonia (MAC); and Cyprus (CYP). The sampling sites are shown in Figure 1. The honey bee populations endemic to these areas correspond to the

subspecies *A. m. adami* (IKA, KAS, and KTH), *A. m. cecropia* (PHT), *A. m. macedonica* (MAC) and *A. m. cyprina* (CYP) (Ruttner, 1988). The samples were transferred to the laboratory alive, and stored at  $-80^{\circ}\text{C}$  until used. Total DNA was extracted from each individual (one individual per colony), according to the protocol of Hunt and Page (1992), with minor modifications. Each individual (head and thorax) was homogenised in 200  $\mu\text{L}$  lysis solution (1% CTAB, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 75 M NaCl), and 1  $\mu\text{L}$  Proteinase K, (20 mg/mL). After incubation at  $60^{\circ}\text{C}$  for 1 hour, each sample was diluted with 50  $\mu\text{L}$  of a high salt buffer identical to that above except that the NaCl concentration was 1.5 M and Proteinase K was omitted, and then incubated again at  $55^{\circ}\text{C}$  for 10 min. DNA was purified with standard phenol: chloroform: isoamyl alcohol extractions, precipitated with 1/10 volume 3 M sodium acetate (pH 5.2) and two volumes of cold absolute ethanol, and resuspended in 75  $\mu\text{L}$  TE (Tris – EDTA, pH 7.6) buffer.

Variation of mtDNA was analysed by RFLP analysis performed on PCR amplified products. Three sets of primers were used for the amplification of 16s rDNA, CO I and ND 5 gene segments. The primers used for the 16s rDNA segment were 5-CAACATCGAGGTCGCAAACATC-3 and 3-AGTTGGGACTATGTTTTCCATG-5 (Nielsen et al., 1994), for the CO I segment were 5-GATTACTCTCCCTCATTA-3 and 3-AATAAGTCTGAT-AGGTCTAA-5 (Nielsen et al., 1999). For the ND 5 segment we designed primers based on the known mitochondrial genome of *A. m. ligustica*: 5-TCGAAATGAATAGGATACAG-3 and 3-TTAGGATTTGGTAGAGTTGG-5. The polymerase chain reaction (PCR) (Saiki et al., 1988) consisted of 2.4 units of *Taq* polymerase, 5  $\mu\text{L}$  of  $10\times$  reaction buffer provided by the manufacturer (GibcoBRL), 5  $\mu\text{L}$  dNTPs mix (2 mM), 3  $\mu\text{L}$   $\text{MgCl}_2$  (2 mM), approximately 100 ng DNA, 0.68  $\mu\text{M}$  of each primer and sterile water. PCR amplification conditions consisted of a 4 min denaturation step at  $94^{\circ}\text{C}$  followed by 35 cycles of  $94^{\circ}\text{C}$  for 1 min, annealing at  $55^{\circ}\text{C}$  (16s rDNA, CO I), or at  $48^{\circ}\text{C}$  (ND 5) for 1 min and extension at  $72^{\circ}\text{C}$  for 2 min. The reaction was concluded with an additional 6 min extension at  $72^{\circ}\text{C}$  following the final amplification cycle.

Amplified mtDNA segments from three individuals of each population were digested with 28 restriction enzymes to check the presence of recognition sites. The informative restriction enzymes were then applied to 12 individuals from each population (one individual per colony). The informative restriction enzymes used for the 16s rDNA gene fragment were *Sau3A I*, *Ssp I*, *Dra I*, *Hinc II*, *EcoR I*, *Pst I*, and *Alu I*; for the CO I gene fragment were, *NCO I*, *Sau3A I*, *Fok I*, *Bcl I*, *Ssp I*, *Sty I*,

*BstU I*, and *Xho I*; and for the ND 5 fragment were *Dra I*, *Taq I*, *Nla III*, *Alu I*, *Hinc II*, *Fok I*, and *Ssp I*.

The digested segments were separated electrophoretically on 2% agarose gels in  $0.5\times$  TBE buffer, stained with ethidium bromide and visualized under UV light. The sizes of DNA fragments were compared to a PCR marker (Promega) run on the same gel and were calculated using DNAfrag 3.03 (Nash, 1991) program. A letter in order of appearance identified single restriction patterns. Composite genotypes for each individual were then defined from all the restriction patterns of the three mtDNA segments. The restriction fragment data were converted to restriction site data (gain or loss of restriction site) (Swofford and Olsen, 1990).

The degree of nucleotide diversity and the degree of nucleotide divergence between and within populations was estimated using the REAP computer package programme (McElroy et al., 1991). Phylogenetic trees were constructed by the UPGMA (Sneath and Sokal, 1973) method, based on net nucleotide divergence between the populations and Dollo parsimony analysis (Farris, 1977), based on the presence or absence of restriction sites. The confidence of the branches was evaluated by the application of the bootstrap method (Felsenstein, 1985). Phylogenetic comparisons were made using PHYLIP (V.3.4) (Felsenstein, 1993) and trees were drawn using the TREEVIEW program (Page, 1996). The degree of geographical heterogeneity of mtDNA haplotype distribution was assessed using a  $\chi^2$  statistic analysis described by Rhoif and Bentzen (1989). The significance level was obtained by 1000 Monte-Carlo randomization using the MONTE program from the REAP package (McElroy et al., 1991).  $N_{ST}$  (Lynch and Crease, 1990) was used to estimate the degree of population subdivision at the nucleotide level. Values of  $N_{ST}$  range from 0 (no population subdivision) to 1 (complete population subdivision).

### 3. RESULTS

The PCR-amplified 16s rDNA, CO I and ND 5 mtDNA gene segments were found to exhibit lengths of 964 bp, 1028 bp and 822 bp, respectively, for all populations examined. Seven, eight and seven restriction enzymes were found to have at least one recognition site in the amplified 16s rDNA, CO I and ND 5 segments, respectively. The restriction enzymes generated a total of 34 restriction sites corresponding to an estimated 178 bp analyzed. Fragment patterns produced by each restriction enzyme for the three mtDNA analysed segments are presented in Tables I, II and III.

**Table I.** Fragment size estimates (in base pairs) of all fragment patterns observed on mtDNA 16s rDNA gene segment among the populations studied.

16s rDNA														
	Sau3A I		Ssp I		Dra I	Hinc II	EcoR I	Pst I	Alu I					
	A	B	A	B	A	A	A	A	A	A				
964	—		628	—	557	—	598	—	492	—	621	—	572	—
548		—	516		407	—	366	—	472	—	343	—	392	—
416		—	336	—	—									
			112	—										

**Table II.** Fragment size estimates (in base pairs) of all fragment patterns observed on mtDNA CO I gene segment among the populations studied. (Fragment marked with asterisk was not observed but assumed under the criterion of minimum mutational steps).

CO I															
	NCO I		Sau3A I	Fok I	Bcl I		Ssp I	Sty I		BstU I		Xho I			
	A	B	A	A	A	B	A	A	B	A	B	A	B		
1028	—	371	—	476	—	765	—	487	—	1028	—	1028	—		
595	—	349	—	425	—	465	—	277	—	626	—	658	—	616	—
433	—	280	—	127	—	326	—	264	—	402	—	370	—	412	—
		28*	—			263	—								
						237	—								

**Table III.** Fragment size estimates (in base pairs) of all fragment patterns observed on mtDNA ND 5 gene segment among the populations studied.

ND 5														
	Dra I	Taq I		Nla III		Alu I		Hinc II	Fok I		Ssp I			
	A	A	A	A	A	B	A	A	A	A				
429	—	375	—	585	—	554	—	—	418	—	430	—	385	—
285	—	258	—	237	—	268	—		404	—	392	—	206	—
108	—	189	—			171	—						124	—
						97	—						107	—

Diagnostic patterns were revealed in the Macedonian population after the digestion of CO I gene segment with the restriction enzymes NCO I (pattern type A) and Sty I (pattern type B). Moreover, digestion of ND 5 gene segment with the restriction enzyme Alu I produced an additional diagnostic pattern (named Type A) characteristic of the Macedonian population. All other populations studied (IKA, KTH, KAS, PHT, CYP) were found to exhibit patterns B, A and B for the above enzymes,

rather than the A, B and A found in the Macedonian population. These patterns were also found in four colonies collected from Phthiotida. However, according to information from the beekeepers, these colonies originated from Macedonia. Intrapopulation variation was revealed in Phthiotida and Kythira for the 16s rDNA gene segment digested with Sau3A I and Ssp I, respectively. Intrapopulation variation was also found in Phthiotida populations for the CO I gene segment digested with

BstU I, Xho I, Sty I, NCO I and in the Kasos population for the same gene segment digested with the restriction enzyme Bcl I.

The seven different haplotypes (composite genotypes) detected, their frequencies and the haplotype and nucleotide diversity values are presented in Table IV. The highest value of haplotype and nucleotide diversity was observed in the Phthiotida population. The net nucleotide divergence estimated among the six populations was found to range from 0.00 to 1.18 with the highest values being observed between the Macedonian and non-Macedonian populations (Tab. V).

A UPGMA tree based on net nucleotide divergence is shown in Figure 2. In this tree, the most distant population was that of Macedonian, with all other populations grouped together in another main clade. The majority-rule consensus tree based on Dollo parsimony analysis of the presence or absence of restriction sites is shown in Figure 3 and shows an analogous topology, taking into consideration haplotypes 2 and 4. These haplotypes are grouped together and strongly supported by high bootstrap values. In this tree, haplotypes 1, 3, 5 and 6 are grouped in another clade, with haplotype 7 placed in a distantly separate clade.

Statistically significant differences in haplotype frequencies were detected among all populations, ( $\chi^2 = 124.74$ ,  $P < 0.001$ ). The estimated  $N_{ST}$  value of 0.71 showed that 29% of the overall genetic diversity observed was within populations, as opposed to 71% that was attributable among populations.

#### 4. DISCUSSION

The honey bee (*Apis mellifera* L.) exhibits patterns of genetic variation rarely found in other animal groups, because this single species has an unusually large geographical distribution encompassing very distinct environmental conditions (Ruttner, 1988). Thus, many local types and subspecies have been recognized, with the differentiation of population structure at distinct geographical scales attributed both to the adaptive and stochastic processes (Wagner, 1990). However, morphometric data are not very well suited for inferring population structure (especially introgression) within this species, since selective factors may

result in convergence. We consider mtDNA to be an ideal marker for such population studies, as all individuals of each colony typically share the same haplotype. It is noteworthy that several technical improvements introduced in beekeeping management may have interfered with the natural distribution of populations. The importation of foreign queens and the practice of moving hives several times per year are factors that can affect the genetic structure of a local honey bee population through genetic introgression (Garnery et al., 1998).

Overall, two main haplotypes were detected among the seven revealed from the Greek and Cyprus populations. The first (Type 1) characterized all populations tested except that of Macedonia, which was characterized by the second (Type 2). In terms of subspecies, *A. m. adami*, *A. m. cecropia* and *A. m. cyprica* (Type 1 haplotype) were quite different from *A. m. macedonica* (Type 2 haplotype). These differences have not been reported from previous studies involving these subspecies (Cornuet and Garnery, 1991a; Smith, 1991).

While no significant differences were found among *A. m. adami* (Ikaria, Kasos and Kythira), *A. m. cecropia* (Phthiotida) and *A. m. cyprica* (Cyprus), the existence of these honey bee races in Greece and Cyprus was reported by Ruttner (1988) based on morphometric analysis. While our results are not in strong congruence with those of Ruttner's (1988), it is important to note that subspecies classification is based on discriminant analysis of morphometric characters. Mitochondrial variation, especially in the C and O morphological lineages, has not been found to be highly discriminatory of subspecies (Garnery et al., 1993). However, the fact that our study reveals markers that can discriminate *A. m. macedonica* from neighboring subspecies, means the method should be useful to study patterns of introgression.

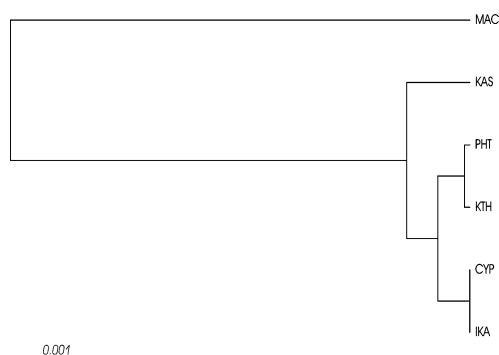
As regards the intrapopulation polymorphisms which characterized honey bee populations from Kasos, Kythira and Phthiotida, we suggest that the existence of unique haplotypes in Kasos (Type 7) and Kythira (Type 5) in relatively high frequencies could be attributed to the maintenance of a pure local race, since Kasos beekeepers restrict the importation of foreign queens (unpublished data). However further investigation is needed to adequately characterize mitochondrial variation in these

**Table IV.** Composite genotypes (haplotypes), haplotype frequencies, haplotype diversity including standard error, nucleotide diversity (%) and sample size of all the populations studied.

Haplotype	Composite genotype										Sample locality									
	16s rDNA					COI					ND 5									
Type 1	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	0.50	0.50	0.67	1.00	1.00
Type 2	A	A	A	A	A	A	A	A	B	B	A	A	A	A	A	0.08	1.00			
Type 3	A	A	A	A	A	A	A	A	A	A	A	B	A	A	A	0.08				
Type 4	A	A	A	A	A	A	A	A	A	B	A	A	A	A	A	0.08				
Type 5	B	B	A	A	A	A	A	A	A	B	A	A	A	A	A	0.25	0.25			
Type 6	B	A	A	A	A	A	A	A	A	B	A	A	A	A	A	0.25				
Type 7	A	A	A	A	A	A	A	A	B	A	A	B	A	A	A		0.33			
Haplotype diversity																				
Standard error																				
Nucleotide diversity (%)																				
N																				

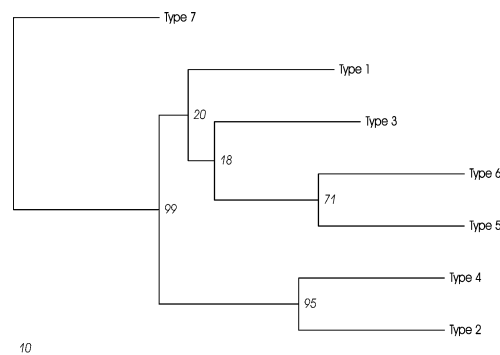
**Table V.** Net Nucleotide divergence ( $\times 10^2$ ) for mtDNA analysis among the populations studied.

	PHT	MAC	KTH	KAS	CYP	IKA
PHT	–					
MAC	0.74	–				
KTH	0.01	1.14	–			
KAS	0.16	1.18	0.20	–		
CYP	0.05	1.05	0.10	0.10	–	
IKA	0.05	1.05	0.10	0.10	0.00	–

**Figure 2.** UPGMA (Sneath and Sokal, 1973) dendrogram, showing the relationships between the populations studied.

populations. We conclude that Phthiotida is an area of hybridization, as five out of the seven haplotypes were found in this honey bee population, as were high haplotype and nucleotide diversity values. These findings may result from the widespread migratory beekeeping and queen importation that occurs in the area. The unique haplotype found in low frequency in Phthiotida may be either a relic native variant or be attributable to the importation of foreign queens. Again, further investigation of the populations in this area is needed.

The net nucleotide divergence detected among the studied populations are in the same range as those estimated in analogous studies (Cornuet and Garnery, 1991; Smith, 1991). The sharp genetic differentiation between *A. m. macedonica* and the other honey populations was supported by the phylogenetic dendrograms, the diagnostic patterns of some haplotypes and the  $N_{ST}$  and  $\chi^2$  values (for haplotype distribution). Thus, while it was difficult to discriminate among the honey bee populations

**Figure 3.** Dollo parsimony (Farris, 1977) dendrogram, showing the relationships between the 7 mtDNA haplotypes detected. Numbers indicate the percentage out of 1000 bootstrap replicates that each node occurred in this majority – rule consensus tree.

studied, except from Macedonia, their genetic structure is a prominent project for further investigation. Migratory beekeeping and commercial breeding during past decades seems to be an important factor which contributed to the genetic differentiation of the populations studied. The fact that the Macedonian population can be discriminated from the others will be of great economic importance in breeding because beekeepers in Greece typically prefer honey bees from the Macedonia area.

**Résumé – Divergence génétique et relations phylogénétiques des populations d'abeilles domestiques (*Apis mellifera*) de Grèce et de Chypre d'après l'analyse par PCR-RFLP de 3 segments d'ADNmt.** La taxonomie infraspécifique de l'Abeille domestique (*Apis mellifera* L.) est traditionnellement basée sur la morphologie. Plus récemment ont été utilisés des marqueurs génétiques tels que les allozymes ou l'ADN mitochondrial (ADNmt). L'ADNmt possède certaines propriétés



dont l'hérédité maternelle, qui en fait un outil favori pour la systématique et la biologie des populations. L'hérédité maternelle de l'ADNmt a été montrée par Meusel et Moritz (1993) : toutes les ouvrières et tous les mâles d'une colonie partagent le même ADNmt que la reine. Les populations d'abeilles étudiées ont été prélevées dans six régions de Grèce et de Chypre (Fig. 1 ; îles de la mer Egée : Ikaria, Kasos et Cythère ; Grèce centrale : Phthiotida ; Grèce du Nord : Macédoine ; Chypre). En fonction de leur origine ces populations correspondaient aux sous-espèces *A. m. adami* (Ikaria, Kasos, Cythère), *A. m. cecropia* (Phthiotida), *A. m. macedonica* (Macédoine) et *A. m. cypria* (Chypre) (Ruttner, 1988).

L'analyse par RFLP de trois régions d'ADNmt avait pour but d'étudier la structure génétique des populations et d'évaluer l'influence de l'apiculture de transhumance et de l'élevage commercial sur la différenciation génétique des populations. Les segments de gènes mitochondriaux étudiés [16s-ADNr (965 bp), CO-I (1028 bp) and ND-5 (822 bp)] ont été amplifiés par PCR. On a trouvé sept enzymes de restriction spécifique du gène 16s-ADNr, du gène CO-I et du gène ND-5. Le degré de diversité nucléotidique entre populations et au sein des populations a été estimé par le progiciel REAP (McElroy et al., 1991). Les arbres phylogénétiques ont été construits selon la méthode UPGMA (Sneath et Sokal, 1973), basée sur la divergence nucléotidique, et la méthode de parcimonie de Dollo (Farris, 1977), basée sur la présence ou l'absence de sites de restriction, à l'aide du progiciel PHYIP (V3.4) (Felsenstein, 1993).

Des profils de digestion diagnostics ont été obtenus pour la population macédonienne à partir de la restriction par NCOI et StyI du segment du gène COI et par AluI du segment du gène ND5. On a trouvé une variation intrapopulation chez les populations de Phthiotida et de Cythère pour le segment de gène 16s-ADNr, digéré respectivement par les enzymes Sau3A-I et Ssp-I, chez la population de Phthiotida pour le segment de gène CO-I digéré par BstUI, Xho I, Sty-I, NCO-I, et chez la population de Kasos pour CO-I digéré par Bcl I.

Parmi les sept haplotypes détectés, la valeur la plus élevée de l'haplotype et de la diversité nucléotidique a été enregistrée chez la population de Phthiotida. Le maximum de divergence nucléotidique parmi les populations testées a été trouvée entre les populations macédoniennes et les autres (Tab. V). Les arbres phylogénétiques montrent que la population la plus éloignée est la macédonienne, les autres sont regroupées.

#### *Apis mellifera* / variabilité génétique / ADNmt / Grèce

#### Zusammenfassung – Bestimmung der genetischen Divergenz und phylogenetischen Verwandtschaft von Populationen der Honigbienen *Apis*

***mellifera* (Hymenoptera: Apidae) in Griechenland und Zypern mit der PCR – RFLP Analyse von drei mtDNA Segmenten.** Die intraspezifische Taxonomie der Honigbiene *Apis mellifera* beruhte traditionell auf Morphologie. Neuerdings werden genetische Marker wie Allozyme und Mitochondrien DNA (mtDNA) benutzt. Auf Grund besonderer Eigenschaften der mtDNA, wie z.B. ihre mütterliche Vererbung, ist sie zu einem beliebten Bezugssystem in der Systematik und der Populationsbiologie geworden. Die mütterliche Vererbung der mtDNA wurde für Honigbienen (Meusel and Moritz, 1993) bewiesen, und deshalb haben alle Arbeiterinnen und Drohnen in einem Volk die gleiche mtDNA wie die Königin. Von den zu untersuchenden Populationen der Honigbienen wurden in 6 verschiedenen Gebieten in Griechenland und Zypern Proben gesammelt (auf den Mittelmeerinseln: Ikaria, Kasos und Kythira; in Zentralgriechenland: Phthiotida; in Nordgriechenland: Macedonia und auf Zypern). Auf Grund ihrer Herkunft sollten die Proben den Unterarten *A.m. adami* (Ikaria, Kasos und Kythira), *A.m. cecropia* (Phthiotida), *A.m. macedonica* (Mazedonien); und *A.m. cypria* (Zypern) entsprechen (Ruttner, 1988).

Die RFLP Analyse von 3 mtDNA Segmenten wurde durchgeführt. Sie waren zur Bestimmung der genetischen Struktur der Population und des Einflusses der Wanderimkerei und der kommerziellen Zucht auf ihre genetische Differenzierung geeignet. Die Gensegmente [16srDNA (965 bp), COI (1028 bp) und ND5 (822 bp)] wurden durch PCR vervielfacht. Es wurden spezifische Restriktionsenzyme gefunden: 7 für das 16srDNA Gen, 8 für COI und 7 für ND5. Der Grad der nuklearen Diversität zwischen und innerhalb der Populationen wurden mit dem REAP Computer Paket (McElroy et al., 1991) bestimmt. Mit der UPGMA Methode (Sneath and Sokal, 1973), die auf einem Netz nukleotider Divergenz zwischen den Populationen beruht und mit der Dollo parsimony Methode (Farris, 1977), die auf An- oder Abwesenheit von Restriktionsstellen beruht, wurden mit Hilfe des PHYLIP Software Pakets (V.3.4) (Felsenstein, 1993) phylogenetische Stammbäume berechnet.

Nur in den mazedonischen Populationen zeigten sich diagnostische Muster, und zwar beim COI Gensegment, geschnitten mit den Restriktionsenzymen NCOI und StyI, und beim ND 5 Gensegment, geschnitten mit den Enzymen AluI. Populationsinterne Variationen wurden bei den Populationen von Phthiotida und Kythira gefunden, und zwar beim 16srDNA Gensegment, geschnitten mit den Sau3A I bzw. Ssp I Enzymen. Populationsinterne Variationen wurden außerdem bei der Phthiotida Population im COI Gensegment gefunden, geschnitten mit BstU I, Xho I, Sty I, NCO I und bei der Kasos Population geschnitten mit Bcl I Enzym.

Von 7 nachgewiesenen Haplotypen (zusammengesetzte Genotypen) wurden die meisten Haplotypen und die höchste nukleotidische Diversität bei der Phthiotida Population gefunden. Die geschätzte

nukleare Divergenz zwischen den untersuchten Populationen war am größten zwischen den mazedonischen und nicht-mazedonischen Populationen (Tab. V). Der phylogenetische Stammbaum zeigte, dass die Population in Mazedonien am weitesten entfernt liegen, während sich die anderen zusammen gruppieren. Insgesamt könnte diese Methode nützlich für zwei Sachen sein: für die Bestimmung des Einflusses der Wanderimkerei und für die kommerzielle Zucht der Honigbienen.

***Apis mellifera* / Honigbiene / mtDNA / genetische Variabilität / Griechenland**

**REFERENCES**

- Arias M.C., Sheppard W.S. (1996) Molecular Phylogenetics of Honey Bee Subspecies (*Apis mellifera* L.) Inferred from Mitochondrial DNA Sequence, *Mol. Phylogenet. Evol.* 5, 557–566.
- Badino G., Celebrano G., Manino A., Ifantidis M.D. (1988) Allozyme variability in Greek Honeybees (*Apis Mellifera* L.), *Apidologie* 19, 377–386.
- Cornuet J.M., Garnery L. (1991a). Genetic diversity in *Apis mellifera*, in: Smith D.R. (Ed.), *Diversity in the Genus Apis*, Westview, Boulder, Colorado, USA, pp. 103–115.
- Cornuet J.M., Garnery L. (1991b) Mitochondrial DNA variability in honeybees and its phylogeographic implications, *Apidologie* 22, 627–642.
- De La Rúa P., Simon U.E., Tide A.C., Moritz R.F.A., Fuchs S. (2000) MtDNA variation in *Apis cerana* populations from the Philippines, *Heredity* 84, 124–130.
- Estoup A.L., Solignac M., Harry M., Cornuet J.M. (1993) Characterization of (GT)<sub>n</sub> and (CT)<sub>n</sub> microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*, *Nucleic Acids Res.* 21, 1427–1431.
- Farris J.S. (1977) Phylogenetic analysis under Dollo's law, *Syst. Zool.* 26, 77–88.
- Felsenstein J. (1985) Confidence limits on phylogenies: An approach using the Bootstrap, *Evolution* 39, 783–791.
- Felsenstein J. (1993) PHYLIP (Phylogeny Inference Package), Version 3.5C, Distributed by the author, Department of Genetics, University of Washington, Seattle, W.A.
- Garnery L., Solignac M., Celebrano G., Cornuet J.M. (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., Franck P., Baudry E., Vautrin D., Cornuet J.M., Solignac M. (1998) Genetic diversity of the west European honey bee (*Apis mellifera mellifera* and *Apis m. iberica*), II Microsatellite loci, *Genet. Sel. Evol.* 30, 49–74.
- Hall H.G. (1990) Parental Analysis of Introgressive Hybridization Between African and European Honeybees Using Nuclear DNA RFLPs, *Genetics* 125, 611–621.
- Hunt J.G., Page Jr. E.R. (1992) Patterns of inheritance with RAPD molecular markers reveal novel types of polymorphism in the honey bee, *Theor. Appl. Genet.* 85, 15–20.
- Ifantidis M.D. (1979) Morphological characters of the Greek Bee *Apis Mellifica Cecropia*, XXVII Congr. Int. Apic. Athens, pp. 271–277.
- Lynch M., Crease T.J. (1990) The Analysis of Population Survey Data of DNA sequence variation, *Mol. Biol. Evol.* 7, 377–394.
- McElroy D., Moran P., Bermingham E., Komfield J. (1991) The Restriction Enzyme Analysis Package, version 4.0 University of Maine, Orono, M.E.
- Meusel M.S., Moritz R.F.A. (1993) Transfer of paternal mitochondrial DNA in fertilization of honeybees (*Apis mellifera* L.) eggs, *Curr. Genetics* 24, 539–543.
- Moritz R.F.A., Hawkins C.F., Crozier R.H., McKinley A.G. (1986) A mitochondrial DNA polymorphism in honey bees (*Apis mellifera* L.), *Experientia* 42, 322–324.
- Nash J.H.E. (1991) DNAfrag, program version 3.03, National Research Council of Canada, Ottawa, Ontario, Canada.
- Nielsen D., Page R.E. Jr., Crosland M.W.J. (1994) Clinal variation and selection of MDH allozymes in honey bee populations, *Experientia* 50, 867–871.
- Nielsen D., Ebert P.R., Hunt J.G., Guzmán-Novoa E., Kinnee S.A., Page R.E. Jr. (1999) Identification of Africanized Honey Bees (Hymenoptera: Apidae) Incorporating Morphometrics and an Improved Polymerase Chain Reaction Mitotyping Procedure, *Ann. Entomol. Soc. Am.* 92, 167–175.
- Nunamaker R.A., Wilson W.T. (1982) Isozyme changes in the honeybee *Apis mellifera* L. during larval morphogenesis, *Insect Biochem.* 12, 99–104.
- Oldroyd B.P., Cornuet J.M., Rowe D., Rinderer E.T., Crozier R.H. (1995) Racial admixture of *Apis mellifera* in Tasmania, Australia: similarities and differences with natural hybrid zones in Europe, *Heredity* 74, 315–325.
- Page R.D. (1996) TREEVIEW: an application to display phylogenetic trees on personal computers, *Comput. Appl. Biosci.* 12, 357–358.
- Pedersen B.V. (1996) On the phylogenetic position of the Danish strain of the black honeybee (the Laeso bee), *Apis mellifera mellifera* L. (Hymenoptera: Apidae) inferred from mitochondrial DNA sequences, *Entomol. Scand.* 27, 241–250.
- Rohlf J., Bentzen P. (1989) The statistical analysis of mitochondrial DNA polymorphisms:  $\chi^2$  and the problem of small samples, *Mol. Biol. Evol.* 6, 539–545.
- Ruttner F. (1988) Biogeography and Taxonomy of Honeybees, Springer-Verlag, Berlin.
- Ruttner F. (1992) Naturgeschichte der Honigbienen, Ehrenwirth Verlag, München, Germany.

- Saiki R., Gelfand D.H., Stoffel S., Scharf S.J., Higuchi R., Horn G.T., Mullis K.B., Erlich H.A. (1988) Primer – directed enzymatic amplification of DNA with thermostable DNA polymerase, *Science* 239, 487–491.
- Sheppard W.S., Arias M.C., Grech A., Meixner M.D. (1997) *Apis mellifera ruttneri*, a new honey bee subspecies from Malta, *Apidologie* 28, 287–293.
- Smith D.R. (1991) Mitochondrial DNA and Honey Bee Biogeography, in: Smith D.R. (Ed.), *Diversity in the Genus Apis*, Westview, Boulder, Colorado, USA, pp. 131–176.
- Smith D.R., Brown W.M. (1988) Polymorphisms in mitochondrial DNA of European and Africanized honeybees (*Apis mellifera*), *Experientia* 44, 257–260.
- Smith D.R., Brown W.M. (1990) Restriction Endonuclease Cleavage Site and Length Polymorphisms in Mitochondrial DNA of *Apis mellifera mellifera* and *A. m. carnica* (Hymenoptera: Apidae), *Ann. Entomol. Soc. Am.* 83, 81–88.
- Smith D.R., Taylor O.R., Brown W.M. (1989) Neotropical Africanized honeybees have African mitochondrial DNA, *Nature* 339, 213–215.
- Smith D.R., Palapoli M.F., Taylor B.R., Garnery L., Cornuet J.M., Solignac M., Brown W.M. (1991) Geographical Overlap of Two Mitochondrial Genomes in Spanish Honeybee (*Apis mellifera iberica*), *J. Hered.* 82, 96–100.
- Sneath P.H.A., Sokal R.R. (1973) *Numerical Taxonomy – The principle and practice of numerical classification*, W.H. Freeman and Co., San Francisco.
- Swofford D.L., Olsen G.J. (1990) Phylogeny reconstruction In *Molecular Systematics*, Hillis D.M. and Moritz C. (Eds.), Sinauer, Sunderland, M.A., pp. 411–501.
- Tarès S., Cornuet J.M., Abad P. (1993) Characterization of an Unusually Conserved Alu I Highly Reiterated DNA Sequence Family From the Honeybee *Apis mellifera*, *Genetics* 134, 1195–1204.
- Wagner G.P. (1990) A comparative study of morphological integration in *Apis mellifera* L., *Z. Zool. Syst. Evolutionsforsch.* 28, 48–61.