New mitochondrial lineages within the *Pipistrellus pipistrellus* complex from Mediterranean Europe

Pavel HULVA[1], Petr BENDA[2, 1], Vladimír HANÁK[1], Allowen EVIN[3] and Ivan HORÁČEK[1]

[1] *Department of Zoology, Faculty of Science, Charles University in Prague, Viničná 7, 128 44 Praha 2, Czech Republic; e-mail: hulva@natur.cuni.cz, horacek@natur.cuni.cz*

[2] *Department of Zoology, National Museum (Natural History), Václavské nám. 68, 115 79 Praha 1, Czech Republic; e-mail: petr.benda@nm.cz*

[3] *Origine, Structure et Evolution de la Biodiversité, Muséum National d'Histoire Naturelle, C.P. 50, 45 rue Buffon, 75005 Paris, France; e-mail: evin@mnhn.fr*

A b s t r a c t . In this study we are adding further information to phylogeography of *Pipistrellus pipistrellus* complex by discovery of isolated mitochondrial lineages in Europe, from the island of Crete (Greece) and central Mediterranean (Sicily, Corsica) and by adding new data about geographic distribution of clades within the complex. We performed phylogeographic study with aid of partial sequence of cytochrome *b* and with focus to the radiation centre of this group in the Mediterranean Basin. Within the clade *P. pygmaeus* s.l., we have discovered isolated lineage from Crete, which is sister taxon to *P. hanaki* from Libya. We have detected the occurrence of *P. pygmaeus* s.str. in northern Iran. In the clade *P. pipistrellus* s.l. we have discovered isolated cluster represented by populations from Sicily and Corsica, with phylogenetic relation to Moroccan lineage. This is a first evidence of separated phylogroup within the complex from central Mediterranean. We refer the occurrence of *P. pipistrellus* s.str. from the island of Cyprus. These data represent further arguments for importance of the Mediterranean region in phylogeny of the *P. pipistrellus* species complex. Proposed allopatric speciation scenario considers the role of environmental fragmentation during the Messinian Salinity Crisis, strengthen by preference of mountain habitats in Mediterranean populations. The species status of Cretan and central Meditraenan forms is also discussed.

Key words: *Pipistrellus pipistrellus*, *Pipistrellus pygmaeus*, Crete, Sicily, Corsica, Mediterranean, molecular phylogenetics, cytochrome b, cryptic species

Introduction

Unique characters of molecular markers compared to traditionally studied phenotypes enabled many new insights into biology of many widespread and well known species. In a model bat species, the common pipistrelle *Pipistrellus pipistrellus* (Schreber, 1774), the species status of two phonic types (A h l é n 1981, M i l l e r & D e g n 1981, W e i d & von H e l v e r s e n 1987, Z i n g g 1990) was confirmed by an application of molecular markers (B a r r a t et al. 1995, 1997, M a y e r & von H e l v e r s e n 2001a,b, B e n d a et al. 2003, H u l v a et al. 2004, R a c e y et al. 2007, M a y e r et al. 2007): *P. pipistrellus* s.str. and *P. pygmaeus* (Leach, 1825). This division was supported by behavioural (J o n e s & van P a r i j s 1993, B a r l o w & J o n e s 1997, P a r k et al. 1996) data and despite the fact that pronounced morphological differences were not discovered (B a r l o w et al. 1997, S e n d o r et al. 2002), other analyses reported minor or nonmetric discrimination traits (H ä u s s l e r et al. 2000, von H e l v e r s e n & H o l d e r i e d 2003).

Many recent studies have shown that sampling representing whole range is necessary to reconstruct phylogeography properly (see e.g. E g g e r t et al. 2002). The data from a large

part of pipistrelles range (H u l v a et al. 2004) revealed that *P. pipistrellus* and *P. pygmaeus* are two most widespread forms of the species complex, which has highest variation in the Mediterranean region. Two genetically distant allopatric populations were discovered in North Africa; in the Maghreb and in Cyrenaica (Libya). The latter one was described as separate species *P. hanaki* Hulva et Benda, 2004 (B e n d a et al. 2004). For the Maghrebian clade, H u l v a et al. (2004) suggested species status, based on genetic data suggesting reproductive isolation of this population. B e n d a et al. (2004), based on combined data with morphology analysis, concluded to classify it rather on subspecific level.

The existence of peripheral populations within the range of the *Pipistrellus pipistrellus* complex and their allopatric occurrence brought support for a hypothesis of allopatric speciation driven by fragmentation of the range, rather than for sympatric speciation promoted by assortative mating and disruptive selection (H u l v a et al. 2004). Such scenario was further supported by molecular clock analysis. According to the molecular dating, the split between two major lineages happened at about 3.1–4.1 Ma in the southern Mediterranean, supposedly North Africa, in connection with environmental changes accompanying the Messinian Salinity Crisis. It was followed by a split between Cyrenaican phylogroup and *P. pygmaeus* s. str. in the eastern Mediterranean at 2.1–1.8 Ma and between the Maghrebian lineages and *P. pipistrellus* s. str. in the western Mediterranean at 1.6–0.9 Ma. Europe seems to be colonized by *P. pipistrellus* s. str. via the Iberian route and by *P. pygmaeus* s. str. via Balkan route during the current glacial cycle with subsequent secondary overlap of ranges and character displacement in echolocation frequencies (H u l v a et al. 2004). This scenario was also supported by fossil record (S e v i l l a 1988, 1989, H o r á č e k & J a h e l k o v á 2006).

Although the pre-Pleistocene age of the main divergence was shown to be similar to other European sibling species in mammals (see e.g. S e d d o n et al. 2001), the recolonization of Europe did not result in formation of the "hedgehog pattern" with a relatively narrow contact zone, but in broad area of sympatry. Evidently, the time of separation was enough to enable a formation of reproductive isolating barriers. This interesting pattern with two widespread sympatric lineages and a mosaic of marginal populations in the Meditarranean region represents promising model for studying of speciation. In this paper, we add further information to this field by discovering of a new lineages within the *Pipistrellus pipistrellus* complex in Europe, which are represented by a specimens collected on Crete, Sicily and Corsica. Although the existence of the population of pipistrelles on Crete was mentioned several times (K a h m a n n 1959, H a n á k et al. 2001, M a y e r & von H e l v e r s e n 2001a), its representatives were never morphologically and/or genetically examined. Only M a y e r & von H e l v e r s e n (2001a) published a record of echolocation call, which suggested presence of *P. pipistrellus* s.str. on this island.

Materials and Methods

In this study, we used the sequences from 34 specimens of *Pipistrellus pipistrellus* / *P. pygmaeus* complex covering different parts of eastern and central Mediterranean (Table 1) plus the data representing whole range of this group from our previous studies (H u l v a et al. 2004: 1025–1026, Table 1; B e n d a et al. 2004: 216–217, Appendix 2). Special attention in this study was given to specimens from Crete and Sicily, as representatives of new isolated lineages within the complex (see genetics part of Results) and detailed morphometric

Table 1. Specimens sequenced for this study.

Species	Haplotype	Locality	Provider	Accession No.
P. pygmaeus s.str.	EUR2	Korud Abad (Golestan), Iran	Benda P., Reiter A.	AY316321
P. pygmaeus s.str.	IRA4	Tutaki (Gilan), Iran	Benda P., Reiter A.	EU084882
P. pygmaeus	CYP1	Troodos, Troodos forest, Cyprus	Benda P., Hanák V., Horáček I.	AJ504442
P. pygmaeus	CYP1	Troodos, Kryos river, Cyprus	Benda P., Hanák V., Horáček I.	AJ504442
P. pygmaeus	CYP1	Troodos, Kryos river, Cyprus	Benda P., Hanák V., Horáček I.	AJ504442
P. pygmaeus	CYP1	Agios Nikolaos, Cyprus	Benda P., Hanák V., Horáček I.	AJ504442
P. pygmaeus	CYP1	Agios Nikolaos, Cyprus	Benda P., Hanák V., Horáček I.	AJ504442
P. pygmaeus	CYP1	Agios Nikolaos, Cyprus	Benda P., Hanák V., Horáček I.	AJ504442
P. pygmaeus	CYP1	Agios Nikolaos, Cyprus	Benda P., Hanák V., Horáček I.	AJ504442
P. pygmaeus	CYP2	Troodos, Kryos river, Cyprus	Benda P., Hanák V., Horáček I.	EU084883
P. pygmaeus	CYP2	Troodos, Kryos river, Cyprus	Benda P., Hanák V., Horáček I.	EU084883
P. pygmaeus	CYP2	Agios Nikolaos, Cyprus	Benda P., Hanák V., Horáček I.	EU084883
P. pygmaeus	CYP3	Troodos, Kryos river, Cyprus	Benda P., Hanák V., Horáček I.	EU084884
P. hanaki	CRE1	Omalos, Crete, Greece	Hulva P., Hanák V., Benda P.	EF370417
P. pipistrellus	SIC1	Ália, Sicily, Italy	Hulva P., Bolfíková B., Zahradníček O., Tocháčková P.	EU084885
P. pipistrellus	COR1	Ospedale, Corsica, France	Evin A.	EU420890
P. pipistrellus	COR2	Ospedale, Corsica, France	Evin A.	EU420891
P. pipistrellus	COR2	Ospedale, Corsica, France	Evin A.	EU420891
P. pipistrellus	COR3	Castifao, Corsica, France	Evin A.	EU420892
P. pipistrellus	COR3	Cuttoli-Corticchiato, Corsica, France	Evin A.	EU420892
P. pipistrellus	COR3	Vezzani, Corsica, France	Evin A.	EU420892
P. pipistrellus s.str.	IRA2	Karizbalagh (Khorasan Razni), Iran	Benda P., Reiter A.	EU084886
P. pipistrellus s.str.	IRA2	Shurlaq (Khorasan Razni), Iran	Benda P., Reiter A.	EU084886
P. pipistrellus s.str.	IRA3	Tunel-e-Golestan (Golestan), Iran	Benda P., Reiter A.	EU084887
P. pipistrellus s.str.	SYR1	Port al Khalars, Lebanon	Horáček I., Hulva P., Lučan R., Němec P.	AY316337
P. pipistrellus s.str.	SYR3	Afqa Cave, Lebanon	Horáček I., Hulva P., Lučan R., Němec P.	AY426096
P. pipistrellus s.str.	SYR3	Afqa Cave, Lebanon	Horáček I., Hulva P., Lučan R., Němec P.	AY426096
P. pipistrellus s.str.	SYR3	Qadisha Cave, Lebanon	Horáček I., Hulva P., Lučan R., Němec P.	AY426096
P. pipistrellus s.str.	LEB1	Nahr es Safa, Lebanon	Horáček I., Hulva P., Lučan R., Němec P.	EU084888
P. pipistrellus s.str.	LEB1	Port al Khalars, Lebanon	Horáček I., Hulva P., Lučan R., Němec P.	EU084888
P. pipistrellus s.str.	LEB2	Nahr es Safa, Lebanon	Horáček I., Hulva P., Lučan R., Němec P.	EU084889
P. pipistrellus s.str.	LEB2	Afqa Cave, Lebanon	Horáček I., Hulva P., Lučan R., Němec P.	EU084889
P. pipistrellus s.str.	LEB3	Adonis, Lebanon	Horáček I., Hulva P., Lučan R., Němec P.	EU084890
P. pipistrellus s.str.	CYP4	Kryos River, Troodos, Cyprus	Ruedi M.	EU084891

investigation was performed in order to obtain basal characteristics of these forms. In the island of Crete, an adult male bat, preliminarily identified as *Pipistrellus pipistrellus* s. str. according to its echolocation call with maximum energy at around 45 kHz (recorded with a heterodying detector Pettterson D200) was netted at an entrance of the Spilion Tsanis cave, 0.5 km NE of Omalos (35° 21' N, 23° 54' E), Lefka Ori Mts (ca. 1025 m a.s.l.) on October 1st, 2006. The bat has been deposited in the collection of the National Museum (Natural History), Prague, under the number NMP 91073 (alcoholic preparation with skull extracted). In the island of Sicily, an adult female was netted inside the sanctuary Grotte della Gurfa o dei Saraceni (37° 45' N, 13° 45' E), 5 km SE from Ália (ca. 705 m a.s.l) on June 15th, 2007. The specimen was deposited in the collections of the Charles University in Prague (Department of Zoology).

We have isolated DNA from wing membrane punches by DNeasy blood and tissue kit (Qiagen), sequenced 402 bp part of cytochrome *b* gene as described in H u l v a et al. (2004) and deposited the sequences in the GenBank (Table 1). We used *Pipistrellus kuhlii*
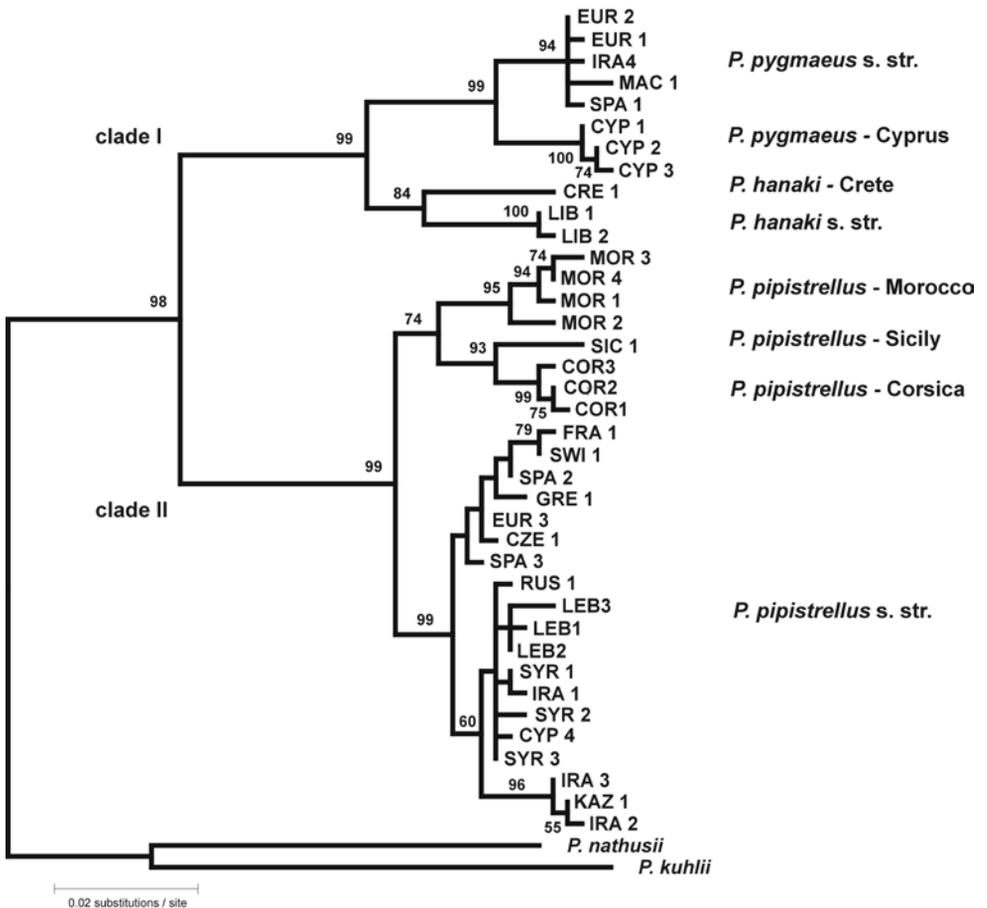

Fig. 1. Phylogeny of *Pipistrellus pipistrellus* complex inferred by neighbor joining method based on 402 bp cytochrome *b* gene data. Only bootstrap values exceeding 50% displayed. Country codes: CRE – Crete, CYP – Cyprus, CZE – Czech Republic, COR – Corsica, EUR – Europe, FRA – France, GRE – Greece, IRA – Iran, KAZ – Kazakhstan, LEB – Lebanon, LIB – Libya, MAC – Macedonia, MOR – Morocco, RUS – Russia, SIC – Sicily, SPA – Spain, SWI – Switzerland, SYR – Syria.

(AJ504445) and *Pipistrellus nathusii* (AJ504446) sequences as outgroups. We have collapsed the dataset into haplotypes. Since there were no insertions and deletions in our dataset, we aligned sequences by eye. We have tested cladistic information content via skewness of distribution of 1.000,000 randomly generated trees (H i l l i s & H u e l s e n b e c k 1992) and by plotting all pairwise distances uncorrected for multiple substitutions (p-distances) against those corrected for multiple substitutions (GTR) for first, second and third codon positions (for details see e.g., D a u g b j e r g & A n d e r s e n 1997). We inferred model of sequence evolution in Modeltest 3.7 (P o s a d a & C r a n d a l l 1998). We have used PAUP* 4.0b2 (S w o f f o r d 1998) to compute p and corrected distances and to illustrate them on neighbour joining tree with 1000 replication bootstrap (Fig. 1).

For a morphologic comparison, we examined the material of African, European and Middle Eastern pipistrelle bats as listed by B e n d a et al. (2004: 214–215, Appendix 1), i.e. a set of altogether 92 specimens of *Pipistrellus pipistrellus*, *P. pygmaeus* and *P. hanaki*. Measurements were taken with a help of mechanical and optical calliper, in the same way as defined by B e n d a et al. (2004).

Results

G e n e t i c s

Within the genetic dataset with 38 resultant haplotypes, 132 characters were variable and 101 parsimony informative. We have concluded to GTR+I model of sequence evolution under AIC criterion. Skewness (g_1= –0.586) and curtosis (g_2=0.270) of randomly generated trees suggest the data contain phylogenetic signal. This was confirmed by saturation tests (first and second codon positions were far from saturation, the saturation occurring at third codon positions was corrected by proposed model of sequence evolution). The tree building methods conclude to two major clades, corresponding to *P. pipistrellus* (clade I) and *P. pygmaeus* (clade II) lineages (comp. Fig. 1 and H u l v a et al. 2004). Clade I consists of four main lineages:

a) Continental lineage of *P. pygmaeus* (*P. pygmaeus* s. str.). This group exhibits very low genetic variability across the range. We refer *P. pygmaeus* s. str. from Iran, which means that the known range of this species extends more to the South-East than expected and also the zone of sympatry with continental *P. pipistrellus* is broader.

b) The lineage from Cyprus (recently described as *P. pygmaeus cyprius* Benda, 2007), separated from the continental form by relatively deep divergence and exhibiting moderate diversification with three haplotypes.

c) *P. hanaki* lineage, separated by deep divergence from the above mentioned lineages.

d) The Cretan haplotype, positioned in the proximity of *P. hanaki*. It differs from the sister taxon by genetic distance p=4.2–4.5% (corrected distances GTR and K2P= 4.4–4.7).

Clade II contains three main lineages.

a) Continental lineage of *P. pipistrellus* (*P. pipistrellus* s. str.). This group shows moderate genetic variability with two shallow clades – the European and Asiatic one. We refer the first record of *P. pipistrellus* from Cyprus, the Cypriot haplotype is embedded in the Asiatic clade in proximity of haplotypes from Levant. Whereas the Cypriot population of *P. pygmaeus* is relatively deeply isolated from the European conspecifics and is relatively abundant on the island (B e n d a et al. 2007), the Cypriot *P. pipistrellus* haplotype show only shallow diversification from the Levantine population and is known only from one specimen.

b) Moroccan lineage, separated by deep divergence from the European continental form.
c) The clade comprising Sicilian and Corsican lineage, positioned as sister taxon of Moroccan lineage with genetic distance p=3.2–4.5% (corrected distances GTR and K2P= 3.3–4.7).

M o r p h o l o g y

The measurements of the Cretan specimen were as follows: external dimensions: body weight 3.9 g, head and body length 43 mm, tail length 34 mm, forearm length 30.3 mm, auricle length 11.3 mm, tragus length 5.0 mm, measurements of the Sicilian specimen were not taken in the field. Cranial dimensions of the Cretan and Sicilian specimens: greatest length of skull 11.99 and 11.28 mm, condylobasal length 11.64 and 10.89 mm, zygomatic width 7.45 mm (broken in the Sicilian specimen), width of interorbital constriction 3.52 and 3.09 mm, neurocranium width 6.28 and 5.98 mm, neurocranium height 4.48 and 4.16 mm, mastoidal width 6.79 and 6.47 mm, height of skull incl. the bullae tympanicae 5.74 and 5.52 mm, rostral width between upper canines (incl.) 3.58 and 3.62 mm, rostral width between third upper molars (incl.) 4.88 and 4.95 mm, length of the upper tooth-row (CM^3) 4.21 and 4.13 mm, length of the upper molar-row (M^{1}M^3) 2.82 and 2.88 mm, length of the upper unicuspid tooth-row (CP^4) 1.99 and 1.78 mm, mandible length 8.28 and 8.17 mm, coronoid process height 2.33 and 2.21 mm, length of the lower tooth-row (CM$_3$) 4.48 and 4.39 mm, length of the lower molar-row (M$_1$M$_3$) 3.00 and 3.07 mm, length of the lower unicuspid tooth-row (CP$_4$) 1.49 and 1.49 mm, respectively; dental dimensions: mesiodistal length of the upper canine 0.95 and 0.91 mm, palatolabial width of the upper canine 0.68 and 0.59 mm, mesiodistal length of the 1st upper premolar 0.41 and 0.43 mm, mesiodistal length of the 2nd upper premolar 0.87 and 0.81 mm, palatolabial width of the 2nd upper premolar 1.18 and 1.19 mm, mesiodistal length of the 1st upper molar 1.10 and 1.18 mm, mesiodistal length of the 3rd upper molar 0.72 and 0.69 mm, mesiodistal length of the lower canine 0.60 and 0.55 mm, mesiodistal length of the 1st lower premolar 0.49 and 0.40 mm, height of mesiopalatal cingular cusp of the 2nd upper premolar 0.19 and 0.17 mm.

Both bats, the Cretan and Sicilian pipistrelle, resemble in most of their morphological characters (body and skull size, coloration) the representatives of both European species, *P. pipistrellus* and *P. pygmaeus*, however, from the Cyrenaican pipistrelle, *P. hanaki*, they differs, being smaller and slightly darker. Nevertheless, in the dental traits, mainly in the dimensions of the upper canine, the Cretan specimen is similar to the Cyreniacan form, in which the crown of the upper canine is 0.95–1.04 mm long in mesiodistal direction (mean 0.99, n=12) (B e n d a et al. 2004), than to the European continental species (0.61–0.79 mm, incl. their non-European populations, n=74). In the length of the upper unicuspidal tooth-row (CP^4), the Cretan pipistrelle showed the largest value found among the examined comparative set (see B e n d a et al. 2004: Table 1). Cretan specimen showed the *P. pipistrellus* pattern of wing veining (sensu V i e r h a u s 1996 and von H e l v e r s e n & H o l d e r i e d 2003) and no tendency to paling of the wing membrane margin was observable in this bat.

The Sicilian pipistrelle shows close similarity to the European continental samples of *P. pipistrellus* s.str. (comp. data by B e n d a et al. 2004), however, in some characters it presents rather extreme positions. In the skull size, it is small representative of the *pipistrellus* lineage, resembling rather European *P. pygmaeus*, but in the tooth characters and measurements it belongs to the largest examined individuals of the complex. The ratios between largest cranial length and the length of rostrum (IM^3) as well as between largest cranial length and the rostral width across upper canines are in this bat one of the largest

among the compared bats (in these aspects the Cretan specimen shows mean values), so, the bat has an extremely massive rostrum. The molariform teeth are large, on the upper margin of the variation range of *P. pipistrellus* s.str. and of similar size or even larger than in *P. hanaki*, possessing on average the most massive teeth in the complex (B e n d a et al. 2004). The crown of the upper canine is rather long in mesiodistal direction, but very narrow in palatolabial aspect, the relative width of the upper canine crown is smallest from all compared samples (0.64 vs. 0.73–0.99 [mean 0.81] in *P. pipistrellus*, 0.68–0.89 [0.79] in *P. pygmaeus*, and 0.72–0.95 [0.83] in *P. hanaki*).

Discussion

P h y l o g e o g r a p h y

The discovery of Cretan, Sicilian and Corsican lineages represents further arguments for importance of the Mediterranean Basin for the phylogeography of *P. pipistrellus* species complex. According to molecular data available to date, we know five relatively isolated allopatric lineages (Libya, Crete, Sicily, Corsica and Morroco) and one isolated lineage sympatric with *P. pipistrellus* s.str. (Cyprus) from this region. The ranges of both continental forms (*P. pipistrellus* s.str. and *P. pygmaeus* s.str. extends to the Mediterranean as well.

The western dispersal center is represented by Maghreb. This region remains isolated from the Iberian peninsula since the end of the Messinian Salinity Crisis at about 5.33 Ma by the Strait of Gibraltar, and according to the studies in other taxa, it represents more often the refugium than the Iberian peninsula (e.g. C a r r a n z a & A r n o l d 2004, L e n k et al. 1999, P a u l o et al. 2002). By discovery of the Sicilian and Corsican lineages, we can refer to the refugium in central Mediterranean in the *P. pipistrellus* complex for the first time. As the total land mass and geographical situation of peninsular Italy is less than in other Mediterranean subcentres, the phylogeographic pattern within this region is usually simple with no difference between Sicily an Calabria (H o r n et al. 2003, C a n e s t r e l l i et al. 2006). However, the peculiar exception was referred in the hedgehog *Erinaceus europaeus* Linnaeus, 1758, with pronounced genetic gap beween Sicily and Calabria in mtDNA (S a n t u c c i et al. 1998, S e d d o n et al. 2001). Using cytochrome *b* and MHC alleles (S a n t u c c i et al. 1998, S e d d o n et al. 2001, B e r g g r e n et al. 2005), the relation between Sicilian and Iberian lineages was revealed. On the contrary, using allozymes, beta-fibrinogen and myoglobin introns led to the phylogenetic reconstruction clustering Sicily with Calabria (F i l i p p u c c i & S i m s o n 1996, S e d d o n et al. 2001). We will need more data from Apenine peninsula to assess the distance between Sicily and Calabria and to state the situation on Sardinia in pipistrelles. Regarding the topology of phylogenetic tree, the lineages from central Mediterranean cluster with those from western Mediterranean. This phylogeographic motif is similar to the situation of mitochondrial lineages in western hedgehogs (S a n t u c c i et al. 1998, S e d d o n et al. 2001). However, since the pipistrelle sibling species are sympatric, the status of representatives of *P. pygmaeus* lineage from central Mediterranean should be assessed in future. The eastern radiation centre of the *P. pipistrellus* complex is represented by Libya, Crete and Cyprus. Considering phylogenetic position of the Cretan population in proximity to *P. hanaki* s.str. and the fact that the occurrence of *P. hanaki* in Europe was not referred to date, our record represents a discovery of new species for European bat fauna. These data suggest also the faunal exchange between

territories of Crete and Cyrenaica (Libya). Since the island of Crete was never in direct contact with Cyrenaica (e.g. B e n - A v r a h a m et al. 2006), the phylogeographic pattern of *P. hanaki* and Cretan lineages could be explained by a colonization event during the Pleistocene history of the Mediterranean Basin. Pipistrelle bats as migratory species show considerable degree of genetic homogeneity in central European populations (e.g. R a c e y et al. 2007). The extent of potential recent genetic flow between Crete and Libya remains unknown. The phylogeographic break in mtDNA reflect certain stage of evolution in allopatry in terms of matrilinear genealogy. The empirical study with nuclear markers will be necessary to interpret the population history in terms of lineage sorting and to asses the paternal contribution to gene flow and potential sex biased philopatry. Moreover, the genetic substructure within real population mirrors rather complex characteristics of the species like ecology and social tradition, than the ability to long distance migrations solely. E.g. in migratory bats from genus *Miniopterus*, a strong population substructure was detected in a geographically relatively homogenous area in South Africa and interpreted as an outcome of habitat preferences and philopatry in both sexes (M i l l e r - B u t t e r w o r t h et al. 2003).

Comparison could be done with data from Cyprus, the island of similar size and degree of isolation as Crete. The pattern in Cyprus (with isolated and rather widespread lineage of the subspecies *P. pygmaeus cyprius* and only single known Levantine haplotype of *P. pipistrellus*) could be interpreted as a long term residence of *P. pygmaeus* and recent colonization event in *P. pipistrellus* lineage and could provide an interesting natural experiment of secondary contact of both lineages.

Consulting the situation in the rest of the continental range, only two lineages (*P. pipistrellus* s. str. and *P. pygmaeus* s. str.) exist in this area. With regard to phylogeographic patterns of this group (H u l v a et al. 2004, this paper) and the fossil record, it is considerable to conclude that only populations from western and eastern source areas contributed to the genetic diversity of continental Europe. Regarding the refugium in the central Mediterranean, it seems to be true that the Apenine population was trapped by the Alps. However, it was in communication with or even gave rise to the western source area population, as could be deduced also from paleobiogeographic situation of the Meditarranean region in respective times.

These data represent further supply for the hypothesis about episodic, quick speciation scenarios in allopatry with secondary overlap of continental lineages (H u l v a et al. 2004) rather than gradual sympatric change (J o n e s 1997, B a r r a t et al. 1997). Moreover, the Mediterranean populations, compared to the continental ones, seem to prefer mountain habitats. According to presumed Mediterranean origin of pipistrelles of the *P. pipistrellus* complex, such preference could be considered as a plesiomorphic state. In parallel with island biogeography, this preference could strengthen the isolation effect on montane pipistrelle populations in fragmented areas during the history of the Mediterranean Basin. After diversification of the complex in the Mediterranean region, the transition to preference of lowlands happened in population which have spread to central and northern Europe.

T a x o n o m y

These findings could have also taxonomic consequences. With regard to the distance of Cretan lineage from the Cyrenaican *P. hanaki* and the morphological distinctness (in more aspects, it rather resembles true *P. pipistrellus* than *P. hanaki*) and the distance of the clade comprising Sicily and Corsica from the rest of *P. pipistrellus* s.l. clade, the species status of

these populations should be discussed. However, apart from the fact that the consensus in species concept definition was not reached yet (e.g. H e y 2001, listed 24 different species concepts), assessing the status of closely related forms could be complicated especially in the forms living in allopatry (e.g. A v i s e 2004). The reproductive isolating barriers evolve in such cases as non adaptive by-product of genomic divergence of both lineages. The intrinsic cohesion of such population units was often not "tested" in natural conditions in such cases. Since the speciation process is continual, the degree of divergence defining species should be set arbitrarily. However, neutral markers could provide useful genetic signatures of genealogical, demographic, geographic and time settings of speciation events. For example, to provide a formalised method for genetic species identification, M a y e r et al. (2007) have set the cutoff to 5% of sequence divergence for mtDNA. However, for example the other island species, *Plecotus sardus* Mucedda, Kiefer, Pidinchedda et Veith, 2002, was described based on 4.1% of divergence from *P. macrobullaris alpinus* Kiefer et Veith, 2002 (M u c e d d a et al. 2002). In the case of *Eptesicus serotinus* (Schreber, 1774) and *E. nilssonii* (Keyserling et Blasius, 1839), these two traditionally recognized species with pronounced morphological distinctness show differences in the range of intraspecific variability in mtDNA (M a y e r & von H e l v e r s e n 2001b). The message of this case is that the complex view on biology of respective taxon including genetics is necessary for species identification. However, the pronounced genetic isolation in mitochondrial genealogy of the Cretan lineage supports species status within concepts stressing actual degree of genetic isolation (e.g. B r a d l e y & B a k e r 2001, B a k e r & B r a d l e y 2006).

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