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Cryptic diversity in European bats

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Different species of bat can be morphologically very similar. In order to estimate the amount of cryptic diversity among European bats we screened the intra- and interspecific genetic variation in 26 European vespertilionid bat species. We sequenced the DNA of subunit 1 of the mitochondrial protein NADH dehydrogenase (ND1) from several individuals of a species, which were sampled in a variety of geographical regions. A phylogeny based on the mitochondrial (mt) DNA data is in good agreement with the current classification in the family. Highly divergent mitochondrial lineages were found in two taxa, which differed in at least 11% of their ND1 sequence. The two mtDNA lineages in *Plecotus austriacus* correlated with the two subspecies *Plecotus austriacus austriacus* and *Plecotus austriacus kolombatovici*. The two mtDNA lineages in *Myotis mystacinus* were partitioned among two morphotypes. The evidence for two new bat species within Europe is discussed. Convergent adaptive evolution might have contributed to the morphological similarity among distantly related species if they occupy similar ecological niches. Closely related species may differ in their ecology but not necessarily in their morphology. On the other hand, two morphologically clearly different species (*Eptesicus serotinus* and *Eptesicus nilssonii*) were found to be genetically very similar. Neither morphological nor mitochondrial DNA sequence analysis alone can be guaranteed to identify species.

Keywords: cryptic species; mitochondrial DNA; phylogeny; Chiroptera

1. INTRODUCTION

Bats are among the most extensively studied mammals, at least in Europe, and great efforts are made in order to maintain their protection (e.g. the Agreement on the Conservation of Bats in Europe within the Bonn Convention). Nevertheless, their degree of species diversity is still uncertain since cryptic species are likely to exist. Three species, *Plecotus austriacus*, *Myotis brandtii* and the 55 kHz phonic type of *Pipistrellus pipistrellus* (which is referred to as *Pipistrellus pygmaeus/mediterraneus* in this paper) were only discovered during the last four decades and are all common in central Europe (Bauer 1960; Gaukler & Kraus 1970; Hanák 1970; Jones & van Parijs 1993). The existence of further species has been suggested, but their taxonomic status remains unsolved (Tupinier 1977; Hanák & Horáek 1983; Bogdanowicz & Wójcik 1986; Volleth 1987; Benda & Tsytsulina 2000). Cryptic species are assumed to be particularly common in bats (Jones 1997). Correct assignment of individuals to biological species is essential for an understanding of the ecology, behavioural strategies, distribution and conservation status of species.

A large number of typically morphological characters are currently used for the classification and phylogenetic reconstruction of biological species. However, molecular characters such as DNA sequences are rapidly supplementing morphological characters and have proven to be a powerful tool in detecting even morphologically very similar, i.e. cryptic, species (see for example Wilcox *et al*. 1997; Yoder *et al*. 2000).

We tested whether the current classification, which is based primarily on morphological characters, correlates with mitochondrial (mt) DNA sequence data. Eight hundred base pairs (bp) of the mitochondrial *ND1* gene were sequenced in 169 individuals representing 26 out of the 29 European vespertilionid bat species. Whenever possible we investigated individuals from different geographical regions within a species. Special emphasis was placed on taxa of uncertain taxonomic status. The nomenclature of the 55 kHz phonic type of *P. pipistrellus* is currently under discussion (Jones & Barratt 1999; von Helversen *et al*. 2000). We refer to this species by using both proposed names throughout the paper: *P. pygmaeus/mediterraneus*.

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2. MATERIAL AND METHODS

(a) *Sample collection*

Two to 22 individuals from 27 bat species belonging to the family Vespertilionidae were sampled, including 26 of the 36 European bat species (Mitchell-Jones *et al*. 1999). Only those species within European vespertilionid bats that are endemic to islands were not included in this study. One, *Myotis ikonnikovi*, from western Asia was included in the analysis because its possible occurrence in Eastern Europe has been discussed (Abelencev *et al*. 1956). The 169 bats originated from Morocco, Israel and 17 different European countries, ranging from Spain to Russia (table 1). A wing membrane sample was taken from museum specimens or from bats that were caught in the field.

(b) *Genetic analysis*

The tissue was digested for 4 h at 56 °C in TNE buffer (100 mM NaCl, 100 mM Tris−HCl and 2 mM EDTA, pH 8.0) with 200 μg ml^{-1} proteinase K, 2% SDS and 0.04 M DTT. DNA was isolated according to a salt–chloroform procedure (Müllenbach *et al*. 1989).

The complete *ND1* gene was amplified with the primers ER65 (5′-CCTCGATGTTGGATCAGG-3′) and ER66 (5′-GTATGGGCCCGATAGCTT-3′), which are located in the *16s rRNA* and the *tRNA-Met* genes, respectively (A. Janke, personal communication). The amplifications were carried out in a volume of 25 μl containing 0.2% BSA, 2.5 mM $MgCl_2$, 1 μM of each primer,

Table 1. Sequence differences among the first 800 bp of the mitochondrial *ND1* gene within European vespertilionid bat species.

(The mean (i.e. nucleotide diversity) and maximum differences are given as percentages (*p* distance). The numbers of individuals sequenced (*n*) are partitioned according to the sample location using the following country codes: D, Germany; GR, Greece; RUS, Russia; TR, Turkey; HR, Croatia; H, Hungary; E, Spain; YU, Yugoslavia; BY, Byelorussia; CH, Switzerland; KI, Kirghizia; BG, Bulgaria; NL, The Netherlands; IL, Israel; PL, Poland; MA, Morocco; UK, United Kingdom; F, France; UA, Ukraine; S, Sweden. Sample sizes are given in parentheses.)

species	*n*	percentage pairwise sequence differences within species		sample location and size
		mean	maximum	
Nyctalus leisleri (Kuhl, 1817)	2	0.00	0.00	GR (2)
Nyctalus lasiopterus (Schreber, 1780)	5	0.44	0.88	GR (1) and H (4)
Nyctalus noctula (Schreber, 1774)	22	0.28	1.25	D (14), RUS (5), UK (1) and F (1)
Pipistrellus kuhlii (Kuhl, 1817)	3	0.67	0.88	GR (3)
Pipistrellus nathusii (Keyserling & Blasius, 1839)	4	0.00	0.00	D (2), GR (1) and RUS (1)
Pipistrellus pipistrellus (Schreber, 1774)	18	1.10	2.50	D (9), GR (5), TR (1), E (1), IL (1) and F (1)
Pipistrellus pygmaeus/mediterraneus	15	0.50	1.50	D (2), GR (10), RUS (1), UA (1) and S (1)
Hypsugo savii (Bonaparte, 1837)	3	1.64	2.39	TR (2) and GR (1)
Eptesicus bottae (Peters, 1869)	2	0.13	0.13	GR (2)
Eptesicus serotinus (Schreber, 1774)	3	0.58	0.75	D (1) and GR (2)
Eptesicus nilssonii (Keyserling & Blasius, 1839)	4	1.17	1.50	D (3) and RUS (1)
Vespertilio murinus Linnaeus, 1758	4	0.25	0.38	D (2), CH (1) and RUS (1)
Myotis bechsteinii (Kuhl, 1817)	5	0.64	1.50	D (2), RUS (1), BY (1) and CH (1)
Myotis daubentonii (Kuhl, 1817)	8	2.14	3.88	D (3), GR (1), E (2) and BY (2)
Myotis capaccinii (Bonaparte, 1837)	3	0.50	0.75	GR (3)
Myotis dasycneme (Boie, 1825)	3	0.42	0.63	NL (1) and H (2)
Myotis emarginatus (E. Geoffroy, 1806)	4	0.31	0.63	GR (1) and IL (3)
Myotis nattereri (Kuhl, 1817)	2	1.29	1.29	GR (1) and H (1)
Myotis myotis (Borkhausen, 1797)	5	1.53	2.63	D (1) and PL (4)
Myotis blythii (Tomes, 1857)	4	0.63	1.12	GR (4)
Myotis brandtii (Eversmann, 1845)	8	0.60	1.13	D (2), RUS (2), H (2) and BG (2)
Myotis mystacinus (Kuhl, 1817)				
clade A (*alcathoe* v. Helversen & Heller, 2001)	7	0.67	1.38	GR (5) and H (2)
clade B (*mystacinus*)	13	1.45	4.75	D (1), GR (6), RUS (1), H (1), E (1), BG (2) and MA (1)
Myotis ikonnikovi Ognev, 1912	1	—	—	RUS (1)
Plecotus auritus Linnaeus, 1758	7	0.52	1.13	D (2), RUS (2) and HR (3)
Plecotus austriacus (J.B. Fischer, 1829)				
clade A (*kolombatovici* Dulic, 1980)	4	0.50	0.88	GR (2) and HR (2)
clade B (*austriacus*)	2	0.75	0.75	D (2)
Barbastella barbastellus (Schreber, 1774)	2	1.15	1.15	D (1) and H (1)
Miniopterus schreibersii (Kuhl, 1817)	6	0.88	1.88	GR (2), HR (1), H (1), E (1) and YU (1)

0.25 mM of each dNTP, 0.5 units Goldstar DNA Polymerase and its reaction buffer (Eurogentec, Seraing, Belgium). A temperature cycle of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s was repeated 40 times. The amplification products were cleaned with the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Cycle sequencing reactions were performed with two internal sequencing primers for the L-strand (ER70, 5′-CAGACCG-GAGTAATCCAGGTCGGTT-3′ and ER175, 5′-GGCTGGGC-CTCAAACTCNAAATA-3′) and one internal sequencing primer for the H-strand (ER89, 5′-CTCTATCAAAGTAACTC-TTTTATCAGAC-3′) using the Thermo Sequenase fluorescent

labelled primer cycle sequencing kit (Amersham, Little Chalfont, UK) following the manufacturer's instructions. Thirty cycles were performed at 94 °C for 20 s, 70 °C (ER70), 62 °C (ER89) or 55 °C (ER175) for 15 s and 72 °C for 30 s. The sequences were run on a 4.5% Sequagel XR (National Diagnostics, Atlanta, GA, USA) and analysed using a LI-COR (Lincoln, NB, USA) DNA sequencer (model 4000L). All sequences are available at GenBank (accession numbers AF 065103–AF065109, AF401362–AF401477, AY027832–AY027860, AY033949, AY033950, AY033954, AY033964, AY033966, AY033969, AY033977, AY033978 and AY033984–AY033988).

The first 800 bp of the *ND1* gene were used for pairwise sequence comparisons. The sequence alignment was unequivocal since no insertions or deletions were found. Pairwise DNA sequence differences (*p* distances) were calculated. The neighbour-joining algorithm based on Hasegawa–Kishino–Yano distances (Hasegawa *et al*. 1985) and a heurisic search under the parsimony criterion was used for phylogenetic reconstruction. All positions and substitution types were weighted equally. The computer software PAUP* version 4.0b4a was used for all analyses (Swofford 2000).

3. RESULTS

Four hundred and fifty-nine variable and 410 parsimony-informative positions from 169 DNA sequences from 27 vespertilionid bat species were detected among the first 800 bp of the mitochondrial gene coding for NADH dehydrogenase (ND1). Pairwise sequence comparisons revealed differences ranging from 0 to 4.75% within a species (table 1) and up to 27% between the two vespertilionid subfamilies Miniopterinae and Vespertilioninae (Koopman 1994). A phylogenetic analysis of all 169 sequences using the neighbour-joining algorithm revealed a monophyletic clade (bootstrap values of 100) for almost all sequences from specimens that were assumed to belong to a single species (figure 1).

(a) *Slight sequence differences between species*

Species that are currently recognized as distinct taxa were not reciprocally monophyletic and displayed levels of interspecific sequence difference within the range of intraspecific variation in two cases: *Eptesicus serotinus* plus *Eptesicus nilssonii* and *Myotis myotis* plus *Myotis blythii* clearly form monophyletic groups. The three sequences of *E. serotinus* clustered monophyletically within the genetically more diverse *E. nilssonii* (figure 1). *Eptesicus serotinus* and *E. nilssonii* differed by only 0.7–1.4% while their intraspecific variabilities were up to 0.7 and 1.5%, respectively. *Myotis blythii* lies within the cluster of *M. myotis* (0.25–2.6% interspecific differences). Therefore, *M. myotis* formed a paraphyletic group including the four investigated specimens of *M. blythii* (figure 2). The interspecific sequence differences among all other species pairs were 5.7% or higher.

Specimens within *Myotis mystacinus* B (figure 1) from southeastern Europe are genetically very similar to those from other parts of Europe (figure 2) although they have been regarded as belonging to a separate species (*Myotis* species A by Volleth (1987) and *Myotis aurascens* by Benda & Tsytsulina (2000)) on the basis of differences in the location of their nucleolus organizer regions. Specimens of *Myotis daubentonii* closely resemble each other genetically across Europe, including two specimens from Spain, which belong to a morphotype that is characteristic of *Myotis nathalinae* Tupinier, 1977 (figure 2).

(b) *Large intraspecific sequence differences*

Two clearly distinct mtDNA lineages in *Myotis mystacinus* differed by 13.1–15.9%. The sequence divergence within each lineage was much lower and did not exceed 4.75% (figure 3). Individuals of both mtDNA lineages were sampled at the same locations in Greece and Hungary. Two highly divergent mtDNA lineages were also found in *Plecotus austriacus*, which form a monophyletic clade. Sequences from individuals sampled in Germany differed by 10.6–12.4% from animals found in southeastern Europe (Croatia and Greece). The sequence divergence within a lineage was 10 times lower (figure 3). The sequence differences correlate with the current classification into two different subspecies *Plecotus austriacus austriacus* (J.B. Fischer, 1829) and *Plecotus austriacus kolombatovici* Dulic, 1980, which can also be distinguished morphologically (Dulic 1980).

(c) *Phylogenetic relationships*

Genera belonging to the same tribe always cluster together and are supported by bootstrap analysis (figure 1). The monophyly of the genera *Nyctalus*, *Eptesicus*, *Myotis* and *Plecotus* is supported by bootstrap values of at least 68, which was not the case for *Pipistrellus* (bootstrap support below 50%). The resolution of the phylogenetic relationships at the species level was generally poor (figure 1). Neither an analysis with a single but complete *ND1* sequence (957 base pairs) per species nor a heuristic search using parsimony criteria improved the bootstrap support or changed the phylogenetic relationships with bootstrap support of at least 50% (data are not shown). The cryptic species pair *Pipistrellus pipistrellus and P. pygmaeus/mediterraneus* clearly group as sister species on a common branch. The divergent sequences within each species are clearly separated. *Nyctalus noctula* and *Nyctalus lasiopterus* are more closely related to one another than either is to *Nyctalus leisleri*. The species pair *Eptesicus serotinus/E. nilssonii* and *Eptesicus bottae* also share also a more recent common ancestor than most other species pairs.

4. DISCUSSION

DNA sequence analysis of the mitochondrial *ND1* gene in European vespertilionid bat species in most cases confirms the current taxonomic classification, which is based on morphological characters. All three tribes and the genera *Plecotus*, *Myotis* and *Nyctalus* form monophyletic groups. The genus *Pipistrellus* might be paraphyletic, although this was not supported by bootstrap analysis. Most species are found on long branches, i.e. they diverged during an early period of vespertilionid bat evolution. A more recent divergence is indicated for *N. noctula* and *N. lasiopterus*, *P. pipistrellus* and *P. pygmaeus/mediterraneus* and *M. myotis* and *M. blythii* as well as *E. serotinus*, *E. nilssonii* and *E. bottae*. Short branches connect most species, all genera and tribes. Hence, phylogenetic relationships above species level were not well resolved (bootstrap values below 70).

Only slight genetic differences were found within two pairs of species. The interspecific sequence differences

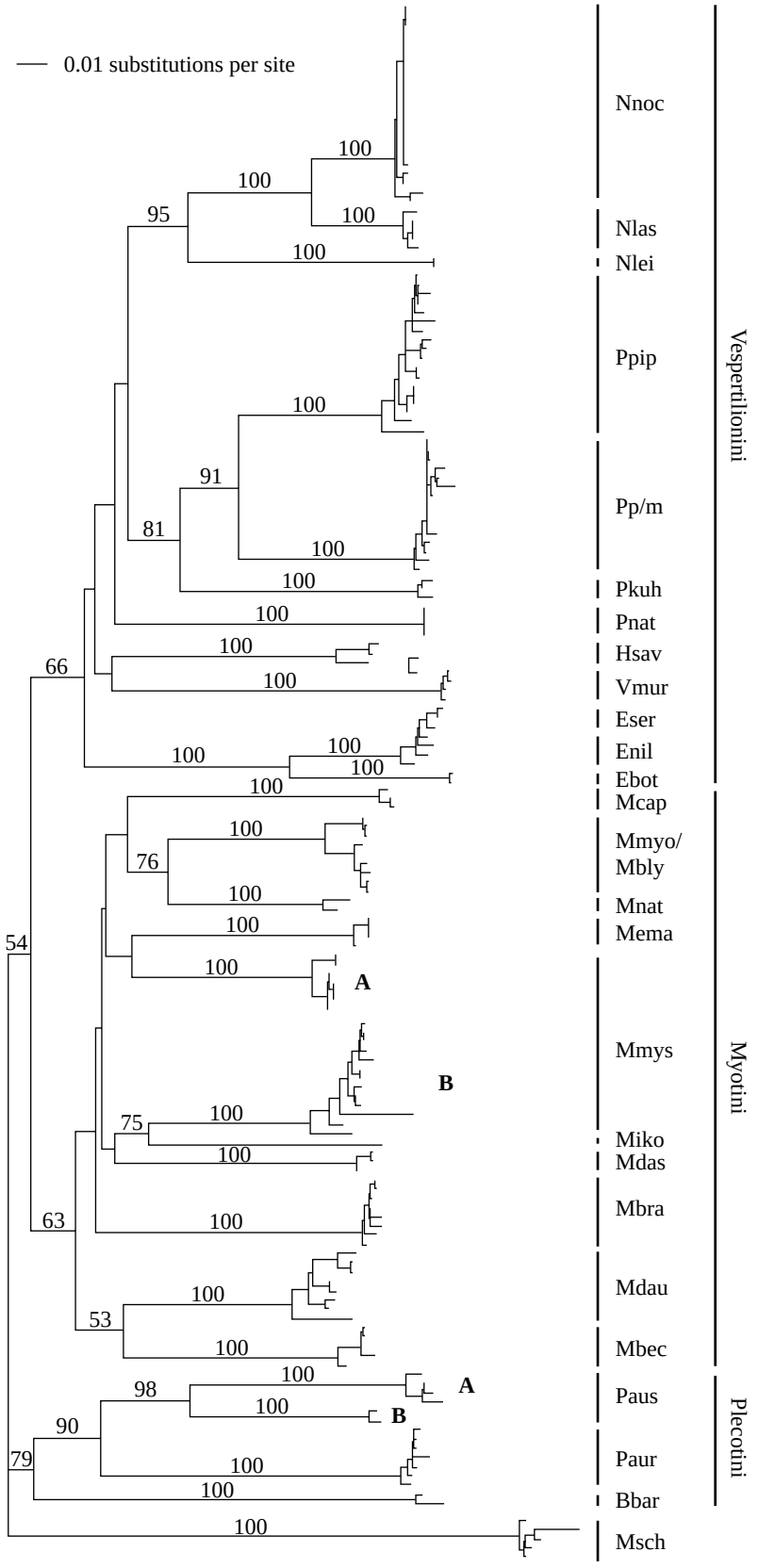

Figure 1. The phylogenetic relationships among all 169 sequences using the neighbour-joining algorithm. The support of particular branches was evaluated by 100 bootstrap replicates. The two mtDNA lineages possibly representing cryptic species in *M. mystacinus* and *P. austriacus* are labelled A and B. The following species codes were used: Nleis, *Nyctalus leisleri*; Nlas, *N. lasiopterus*; Nnoc, *N. noctula*; Pkuh, *Pipistrellus kuhlii*; Pnat, *P. nathusii*; Ppip, *P. pipistrellus*; Pp/m, *P. pygmaeus*/*mediterraneus*; Hsav, *Hypsugo savii*; Ebot, *Eptesicus bottae*; Eser, *E. serotinus*; Enil, *E. nilssonii*; Vmur, *Vespertilio murinus*; Mbec, *Myotis bechsteinii*; Mdau, *M. daubentonii*; Mcap, *M. capaccinii*; Mdas, *M. dasycneme*; Mema, *M. emarginatus*; Mnat, *M. nattereri*; Mmyo, *M. myotis*; Mbly, *M. blythii*; Mbra, *M. brandtii*; Mmys, *M.mystacinus*, Miko, *M. ikonnikovi*; Paur, *Plecotus auritus*; Paus, *P.austriacus*; Bbar, *Barbastella barbastellus*; Msch, *Miniopterus schreibersii*.

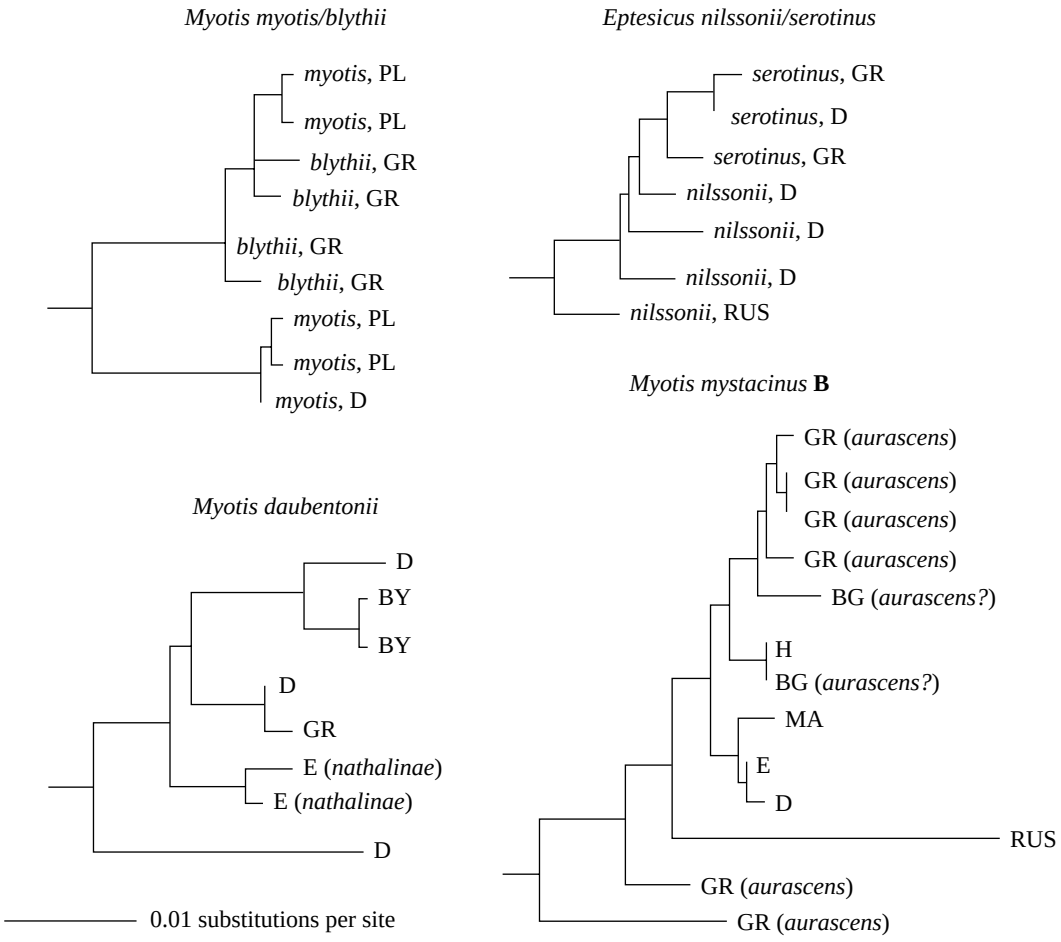

Figure 2. Enlargements of three groups from the neighbour-joining tree in figure 1. The country of sampling is given for all individuals using the country codes listed in table 1.

between *Eptesicus serotinus* and *E. nilssonii* were in the range of intraspecific variation. All sequences of *E. serotinus* formed a monophyletic group within the genetically more divergent *E. nilssonii*. This was confirmed in a larger dataset by sequencing the mitochondrial control region (data not shown). Both species are well-recognized species that differ in many morphological characters. For example, *E. serotinus* is twice as heavy as *E. nilssonii* and differs in coloration. Their surprising genetic similarity may have resulted in two different ways: either the two species diverged rather recently or an introgression of mitochondrial genome secondarily caused the similarity. Indistinguishable karyotypes of both species but clear differences in the banding patterns from the karyotype of *E. bottae* (Volleth *et al*. 2001) suggest a recent split and rapid morphological divergence of *E. serotinus* and *E. nilssonii*. *Myotis blythii* was placed within *M. myotis* (figure 2). Gene flow does not seem to occur between the two species (Ruedi *et al*. 1990; Castella *et al*. 2000) although they roost in mixed clusters (Arlettaz 1999). Both species can even have the same mitochondrial cytochrome *b* haplotype (Castella *et al*. 2000). This indicates a very recent split of the two morphologically similar species, probably during the glaciations of the Pliocene era (Ruedi *et al*. 1990).

(a) *Cryptic species diversity*

Genetically highly divergent mitochondrial lineages raise the question as to whether as yet unknown cryptic species are present because genetic drift eliminates genetic diversity. It was recently shown that the pipistrelle bat represented two cryptic species that had been unrecognized for more than 200 years (Jones & Van Parijs 1993): Both species are morphologically very similar (Barlow *et al*. 1997; Häussler *et al*. 1999), but they clearly differ in their mitochondrial genome (at least 11% in the cytochrome *b* gene (Barratt *et al*. 1997) and 10–13% in the *ND1* gene (this paper)). A similar situation was found in mouse-eared bat species (Castella *et al*. 2000) and in the two taxa *Myotis mystacinus* and *Plecotus austriacus* (this paper). The DNA sequence differences in both species exhibited a bimodal pattern (figure 3). The two mitochondrial lineages differed by between 11 and 13% in both species, while the sequence differences within a lineage were less than 1% and 5%, respectively. Sequence differences of at least 11% between clades A and B are clearly in the range of interspecific differences and above the mitochondrial sequence divergence of hybridizing taxa (up to 7% in *Bombina* toads) (Szymura 1993; see also Avise 2000).

High sequence divergence in *P. austriacus* was observed between a specimen from Germany (clade B in figure 1) and four individuals caught in Croatia and Greece (clade A in figure 1), which showed the morphological characters of *P. a. kolombatovici*. This subspecies is smaller and more brownish than the larger grey *P. a. austriacus*, which is found further to the north (Dulic 1980). Large genetic as well as

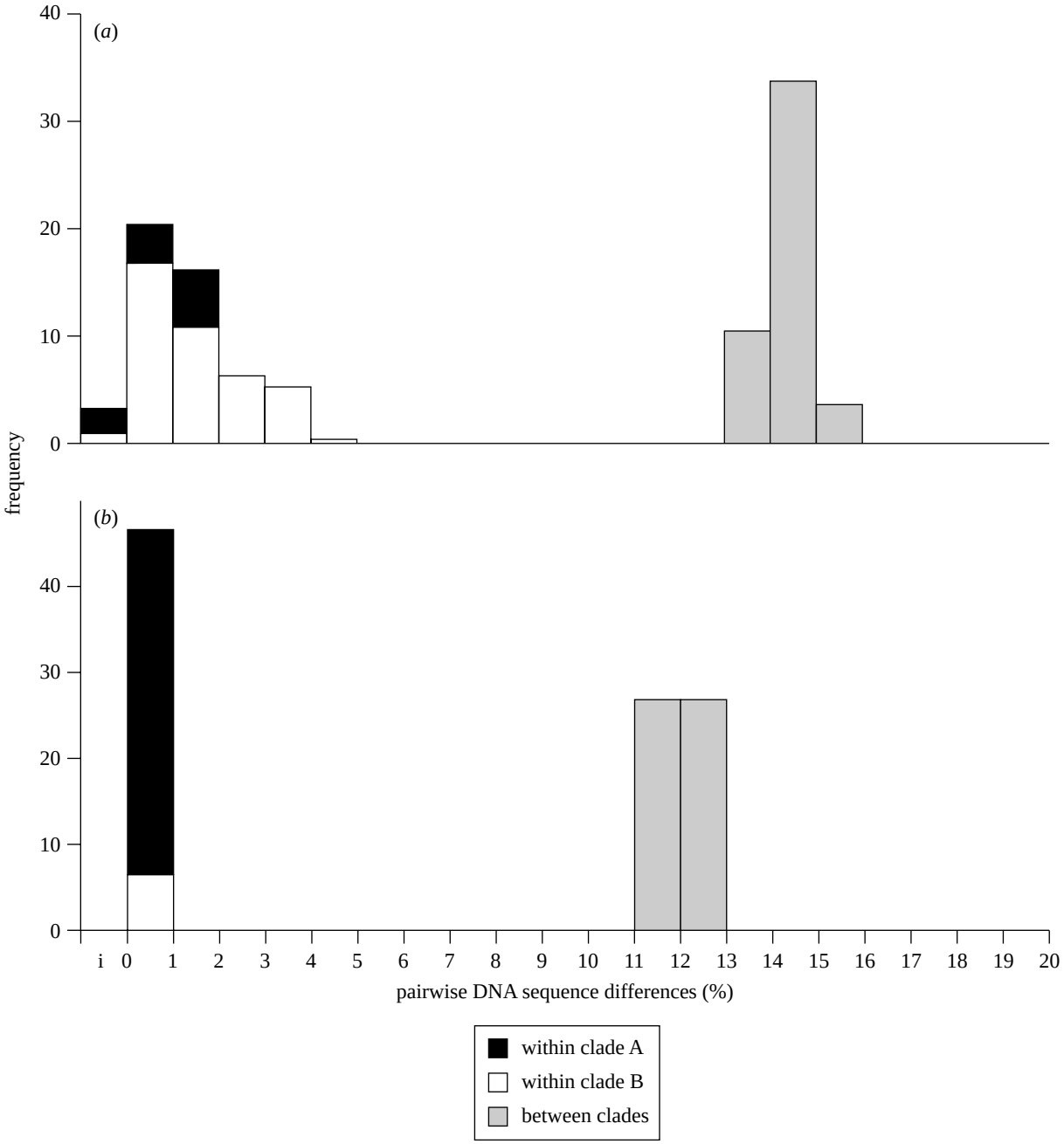

Figure 3. Distributions of the pairwise DNA sequence differences (percentage of 800 bp) in the mitochondrial *ND1* gene within (*a*) *Myotis mystacinus* and (*b*) *Plecotus austriacus*. Differences within and between clades A and B (figure 1) were distinguished. The frequency of identical (i) sequences is given in the first column.

morphological differences between the two subspecies suggest that *P. austriacus* does indeed represent two separate species. In order to prove a full species rank of both taxa according to the biological species concept (Mayr 1963) a denser sampling in the putative contact zone in Croatia is needed for resolving the question of whether both forms occur in sympatry and do not interbreed.

The two mtDNA lineages in the whiskered bat (*Myotis mystacinus*) did not group together in a phylogenetic analysis (clades A and B in figure 1), although statistically this is not well supported. The mitochondrial DNA sequences of other species (*M. ikonnikovi* and *M. emarginatus*) are more similar to either one of the two mitochondrial lineages found in *M. mystacinus* than the two lineages are to each other. This implies that (i) the two lineages represent two different species and (ii) the two species might not even be sister taxa. Volleth (1987) even distinguished three species within European *M. mystacinus* according to the location of their active nucleolus organizer regions. *Myotis* species B in Volleth (1987) corresponds to clade A of *M. mystacinus* in figure 1 and is now described as *Myotis alcathoe* (von Helversen *et al*. 2001) according to differences from *M. mystacinus* in their mitochondrial genome, morphology and location of nucleolus organizer regions and the sympatric occurrence of both species in Hungary and Greece.

Is there a further species of whiskered bat in Europe? Stubbe & Chotolchu (1968) first recognized morphological

differences between *Myotis mystacinus mystacinus* from central Europe and specimens from the Balkans, for which they suggested the name *Myotis mystacinus przewalskii* (Bobrinski 1926). These bats are slightly larger than the whiskered bats from central Europe and they have longer hind feet, a larger baculum (Strelkov & Buntova 1982; Benda & Tsytsulina 2000; own observations) and differ in the location of their active nucleolus organizer regions (*Myotis* species A in Volleth 1987). Benda & Tsytsulina (2000) suggested that this form should be regarded as a species, namely *M. aurascens* Kuzyakin 1935. Our results show that, according to their mitochondrial DNA sequences, the specimens of *M. aurascens* cluster together with western and central European *M. m. mystacinus* on one branch (figure 2) and that their sequence differences are below 5%, i.e. within the range of intraspecific variation. However, this does not of course exclude the possibility that the central European and Balkan populations belong to two different species, as shown here for *Myotis blythii* and *M. myotis* as well as *Eptesicus serotinus* and *E. nilssonii*. Recently diverged species might not have evolved substantial genetic differences in their mitochondrial genome and more sensitive techniques are required for measuring gene flow. The decisive question will be whether the two forms *mystacinus* and *aurascens* interbreed in the range where populations come into contact. Benda & Tsytsulina (2000) did not realize that the southern Bulgarian *mystacinus* and possibly also the specimens from Macedonia and Greece that they investigated are likely to be *M. alcathoe*. Up to now there are no undoubted records of *M. m. mystacinus* from the southern Balkans.

A species rank of *M. nathalinae* Tupinier 1977 was not supported by our data, and it has already been called into question because of genetic (allozymes) and morphological similarity to *M. daubentonii* (Bogdanowicz & Wójcik 1986). The two Spanish specimens of the *M. nathalinae* type clustered within *M. daubentonii* from various geographic regions (figure 2).

(b) *Ecology and convergent evolution*

Morphological similarity among recently diverged sibling species can be explained by a lack of disruptive selection on morphological characters or a lack of sufficient time for morphological divergence. But why are several bat species morphologically so similar although they diverged a long time, i.e. several million years, ago? An answer might come from the species' ecology: genetically well-differentiated cryptic species pairs resemble each other in diet and habitat selection, and faecal analysis revealed that Lepidoptera comprise the main diet in *Plecotus auritus* and *P. austriacus*, although the slightly larger *P. austriacus* can handle larger prey and is less inclined to pick prey from surfaces (Bauerova 1982; Beck 1995). Both species can be found in the same roost type and feeding habitat. *Myotis mystacinus* and *M. brandtii* had an almost identical prey spectrum and this was not only when both species were caught at the same location during a single night (Taake 1992). So far, little is known about the ecology of *M. alcathoe*. This species is usually caught together with *M. mystacinus* in Greece and Hungary, which indicates use of the same feeding habitat (von Helversen *et al*. 2001). The two cryptic species within *Pipistrellus* differed in their dietary composition, which can be explained by differences in habitat use (Barlow 1997; Vaughan *et al*. 1997). *Pipistrellus pygmaeus/mediterraneus* feeds predominantly close to habitats associated with water while *P. pipistrellus* uses a wider range of habitats. Despite these differences the food spectrum largely overlapped in the two species (Barlow 1997) and might have been even more similar if individuals hunting in the same habitat had been compared.

The use of rather similar but not identical ecological niches might have assimilated many morphological characters including body size and wing shape by convergent adaptive evolution or prevented morphological divergence, i.e. common morphological character states are plesiomorphic. The latter hypothesis is a likely explanation for the sibling species *P. pipistrellus* and *P. pygmaeus/mediterraneus* and the genus *Plecotus* because these morphologically similar species form a monophyletic group. Adaptive convergent evolution is more likely to have contributed to morphological similarity among *Myotis mystacinus*, *M. brandtii* and *M. alcathoe*. They diverged at an early stage of evolution in the genus *Myotis* and all three species are more closely related to other morphologically distinct species than to each other (figure 1).

5. CONCLUSIONS

The analysis of mitochondrial DNA is a useful tool in identifying species, but neither morphological nor DNA sequence analysis alone can be guaranteed to identify all species. Current evidence shows that species that diverged several million years ago can closely resemble each other in morphology. Clear genetic differences among cryptic species allow species' identification and, hence, the separation of intraspecific from interspecific morphological variation. This could make it possible to find morphological characters that can be used for species' identification. The case of *Myotis myotis* and *M. blythii* exemplifies that two species can resemble each other morphologically as well as genetically. Detailed studies on morphology, ecology, echolocation (reviewed by Jones 1997) and gene flow are required in order to resolve the species status in such cases. Unambiguous species identification is essential in order to study their distribution, abundance or ecology and, hence, is also important from a conservation perspective.

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REFERENCES

Abelencev, V. I., Pidoplitschko, I. G. & Popov, B. M. 1956 *Fauna Ukraini*. Kijev: Tom 1. Ssavci. Vipusk 1.

Arlettaz, R. 1999 Habitat selection as a major resource partitioning mechanism between the two sympatric sibling bat species *Myotis myotis* and *Myotis blythii*. *J. Anim. Ecol.* **68**, 460–471.

Avise, J. C. 2000 *Phylogeography: the history and formation of species*. Cambridge, MA: Harvard University Press.

Barlow, K. 1997 The diets of two phonic types of the bat *Pipistrellus pipistrellus* in Britain. *J. Zool.* **243**, 597–609.

Barlow, K., Jones, G. & Barratt, E. M. 1997 Can skull morphology be used to predict ecological relationships between bat species? A test using two cryptic species of pipistrelle. *Proc. R. Soc. Lond.* B **264**, 1695–1700.

Barratt, E. M., Deaville, R., Burland, T. M., Bruford, M. W., Jones, G., Racey, P. A. & Wayne, R. K. 1997 DNA answers the call of pipistrelle bat species. *Nature* **387**, 138–139.

Bauer, K. 1960 Die Säugetiere des Neusiedlersee-Gebietes. *Bonn. Zool. Beiträge* **11**, 141–344.

Bauerova, Z. 1982 Contribution to the trophic ecology of the grey long-eared bat *Plecotus austriacus*. *Folia Zool.* **31**, 113–122.

Beck, A. 1995 Fecal analysis of European bat species. *Myotis* **32/33**, 109–119.

Benda, P. & Tsytsulina, K. A. 2000 Taxonomic revision of *Myotis mystacinus* group (Mammalia: Chiroptera) in the western Palearctic. *Acta Soc. Zool. Bohem.* **64**, 331–398.

Bogdanowicz, W. & Wójcik, A. M. 1986 Morphological and electrophoretic differentiation between *Myotis daubentoni* (Kuhl, 1819) and *Myotis nathalinae* Tupinier, 1977. *Myotis* **23/24**, 17–30.

Castella, V., Ruedi, M., Excoffier, L., Ibánez, C., Arlettaz, R. & Hausser, J. 2000 Is the Gibraltar Strait a barrier to gene flow for the bat *Myotis myotis* (Chiroptera: Vespertilionidae)? *Mol. Ecol.* **9**, 1761–1772.

Dulic, B. 1980 Morphological characteristics and distribution of *Plecotus auritus* and *Plecotus austriacus* in some regions of Yugoslavia. In *Proceedings of the Fifth International Bat Research Conference* (ed. D. E. Wilson & A. L. Gardner), pp. 151–161. Lubbock, TX: Texas Tech Press.

Gaukler, A. & Kraus, M. 1970 Kennzeichen und Verbreitung von *Myotis brandti* (Eversman, 1845). *Zeitschrift für Säugetierkunde* **35**, 113–124.

Hanák, V. 1970 Notes on the distribution and systematics of *Myotis mystacinus* Kuhl, 1819. *Bijdr. Dierk.* **40**, 40–44.

Hanák, V. & Horácek, I. 1983 Some comments on the taxonomy of *Myotis daubentoni* (Kuhl, 1819) (Chiroptera, Mammalia). *Myotis* **21/22**, 7–19.

Hasegawa, M., Kishino, H. & Yano, T. 1985 Dating of the human–ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **21**, 160–174.

Häussler, U., Nagel, A., Braun, M. & Arnold, A. 1999 External characters discriminating sibling species of European pipistrelles, *Pipistrellus pipistrellus* (Schreber, 1774) and *P. pygmaeus* (Leach, 1825). *Myotis* **37**, 27–40.

Jones, G. 1997 Acoustic signals and speciation: the roles of natural and sexual selection in the evolution of cryptic species. *Adv. Study Behav.* **26**, 317–354.

Jones, G. & Barratt, E. 1999 *Vespertilio pipistrellus* Schreber, 1774 and *V. pygmaeus* Leach, 1825 (currently *Pipistrellus pipistrellus* and *P. pygmaeus*; Mammalia, Chiroptera): proposed designation of neotypes. *Bull. Zool. Nomenclat.* **56**, 182–186.

Jones, G. & van Parijs, S. M. 1993 Bimodal echolocation in pipistrelle bats: are cryptic species present? *Proc. R. Soc. Lond.* B **251**, 119–125.

Koopman, K. F. 1994 Chiroptera: systematics. *Handbook Zool.* **8**, 1–217.

Mayr, E. 1963 *Animal species and evolution*. Cambridge, MA: Harvard University Press.

Mitchell-Jones, A. J., Amori, G., Bogdanowicz, W., Krystufek, B., Reijnders, P. J. H., Spitzenberger, F., Stubbe, M., Thissen, J. B. M., Vohralík, V. & Zima, J. 1999 *The atlas of European mammals*. London: Academic Press.

Müllenbach, R., Lagoda, P. J. L. & Welter, C. 1989 An efficient salt–chlorophorm extraction of DNA from blood and tissues. *Trends Genet.* **5**, 391.

Ruedi, M., Arlettaz, R. & Maddalena, T. 1990 Distinction morphologique et biochimique de deux espèces jumelles de chauves-souris: *Myotis myotis* (Bork.) et *Myotis blythi* (Tomes) (Mammalia; Vespertilionidae). *Mammalia* **54**, 415–429.

Strelkov, P. P. 1983 Usataja nocnica (*Myotis mystacinus*) i nocnica Brandta (*Myotis brandti*) v SSSR i vzaimootnosenija etih vidov. Soobscenie 2. *Zool. Z.* **62**, 259–270.

Stubbe, M. & Chotolchu, N. 1968 Zur Säugetierfauna der Mongolei. *Mitteilungen Zool. Mus. Berlin* **44**, 5–121.

Swofford, D. L. 2000 *PAUP*. Phylogenetic analysis using parsimony (*and other methods)*. Sunderland, MA: Sinauer.

Szymura, J. M. 1993 Analysis of hybrid zones with *Bombina*. In *Hybrid zones and the evolutionary process* (ed. R. G. Harrison), pp. 261–289. New York: Oxford University Press.

Taake, K. H. 1992 Strategien der Ressourcennutzung an Waldgewässern jagender Fledermäuse (Chiroptera: Vespertilionidae). *Myotis* **30**, 7–74.

Tupinier, Y. 1977 Description d'une chauve-souris nouvelle: *Myotis nathalinae* nov. sp. (Chiroptera-Vespertilionidae). *Mammalia* **41**, 327–340.

Vaughan, N., Jones, G. & Harris, S. 1997 Habitat use by bats (Chiroptera) assessed by means of a broad-band acoustic method. *J. Appl. Ecol.* **34**, 716–730.

Volleth, M. 1987 Differences in the location of nucleolus organizer regions in European vespertilionid bats. *Cytogenet. Cell Genet.* **44**, 186–197.

Volleth, M., Bronner, G., Göpfeert, M. C., Heller, K.-G., von Helversen, O. & Yong, H.-S. 2001 Karyotype comparison and phylogenetic relationships of *pipistrellus*-like bats (Vespertilionidae; Chiroptera; Mammalia). *Chromosome Res.* **9**, 25–46.

von Helversen, O., Mayer, F. & Kock, D. 2000 Comments on the proposed designation of single neotypes for *Vespertilio pipistrellus* Schreber, 1774 (Mammalia, Chiroptera) and for *Vespertilio pygmaeus* Leach, 1825. *Bull. Zool. Nomenclat.* **57**, 113–115.

von Helversen, O., Heller, K.-G., Mayer, F., Nemeth, A., Volleth, M. & Gombkötö, P. 2001 Cryptic mammalian species: a new species of whiskered bat in Europe. *Naturwissenschaften*. (In the press.)

Wilcox, T. P., Hugg, L., Zeh, J. A. & Zeh, D. W. 1997 Mitochondrial DNA sequencing reveals extreme genetic differentiation in a cryptic species complex of Neotropical pseudoscorpions. *Mol. Phylogenet. Evol.* **7**, 208–216.

Yoder, A. D., Rasoloarison, R. M., Goodman, S. M., Irwin, J. A., Atsalis, S., Ravosa, M. J. & Ganzhorn, J. U. 2000 Remarkable species diversity in Malagasy mouse lemurs (primates, *Microcebus*). *Proc. Natl Acad. Sci. USA* **97**, 11 325–11 330.

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