Biological Journal of the Linnean Society, 2006, **87**, 577–582. With 3 figures

Molecular taxonomy and phylogeography of *Miniopterus schreibersii* (Kuhl, 1817) (Chiroptera: Vespertilionidae), in the Eurasian transition

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Received 16 July 2004; accepted for publication 29 April 2005

Miniopterus schreibersii is a polytypic bat species, with one of the widest distribution ranges among the mammals. We studied the genetic differentiation and taxonomy of this species in the transition zone between south-eastern Europe and Anatolia (in Asia), where two subspecies have been described. The results indicated a sharp genetic break between the samples from western Anatolia and south-eastern Europe and those of eastern Anatolia. In addition, the samples from western Anatolia and south-eastern Europe were seen to be reciprocally monophyletic, although the differentiation was less drastic. These patterns of genetic differentiation suggest the presence of two distinct groups within the *M. schreibersii* complex in the region, concordant with previous subspecific recognition. The cause of this genetic break is most likely differentiation in separate glacial refugia followed by secondary contact. However, more samples are needed to assess whether these represent different species, as well as to understand more clearly the causes of this differentiation. © 2006 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2006, **87**, 577–582.

ADDITIONAL KEYWORDS: Anatolia – Chiroptera – D-loop – mtDNA – phylogeography – south-eastern Europe – taxonomy.

Miniopterus schreibersii (Kuhl, 1817), the bent-winged bat, is a polytypic species with one of the largest distribution ranges among the mammals (Macdonald & Barrett, 1998; Hutson, Mickleburgh & Racey, 2001). Recent compendia such as that by Koopman (1994) recognize 15 subspecies within this species complex, spanning Palearctic, Australian, Oriental and African populations. However, Koopman (1994) also noted that 'the boundaries are not clear' (thus some subspecies may be consolidated) and that 'some populations have not been allocated subspecifically', the allocation of which would result in an increase in the number of defined subspecies. For example, Cardinal & Christidis (2000) used molecular and morphological methods to indicate the presence of three subspecies in Australia, instead of the two previously considered to be present (Maeda, 1982). Furthermore, recent studies suggest that the '*M. schreibersii* complex' may comprise more than one species, as well as the multiple aforementioned subspecies. For instance, Tian *et al*. (2004) have suggested the division of *M. schreibersii* into three species in Europe, Asia and Australia, while Appleton, McKenzie & Christidis

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(2004) have showed that *M. schreibersii* was actually a paraphyletic species complex, emphasizing the need for revision of its taxonomy.

One area where multiple subspecific classifications have been proposed for *M. schreibersii* is in the transition zone between Europe and Asia, where the two continents are separated by the Marmara Sea (Fig. 1) (Benda & Horacek, 1998). Thirty-six bat species are present in this region, ten of which (e.g. *Rousettus aegyptiacus*) reach their global distribution boundaries. Species from Africa, Asia and Europe converge in the area, resulting in elevated levels of diversity compared to neighbouring countries (Veith *et al*., 2003). In addition, patterns observed in subspecific taxonomy of the bats also reflect factors affecting the differentiation of species and populations, providing an array of ideas and testable hypotheses with regard to the potential causes of this diversification.

In terms of the taxonomy of *M. schreibersii* in this region, western Turkey in Asia extending into continental Europe and North Africa is inhabited by the nominate form, *M. schreibersii schreibersii* (Corbet, 1978; Benda & Horacek, 1998), while eastern Turkey extending into Afghanistan is inhabited by the subspecies *M. schreibersii pallidus* (Thomas, 1907) (DeBlase, 1980; Koopman, 1994). Although Heinrich (1936) suggested the inclusion of Balkan populations under the subspecies *M. s. inexpectatus*, this name has been generally considered a junior synonym of the nominotypical form (Hanak *et al*., 2001). In the zone where the two 'subspecies' are in contact in Turkey, Albayrak & Coskun (2000) have suggested that subspecific differentiation takes place with geographical distance as one moves from Turkish Thrace (the part of Turkey in Europe) into Anatolia (the part of Turkey in Asia) (Fig. 1). No metric differences have been recorded for the two 'subspecies'; the differentiation of the morphotypes is mainly based on fur coloration, with that of the ventral side of *M. schreibersii schreibersii* being darker (brownish) than that of *M. schreibersii pallidus* (greyish) (Steiner & Gaisler, 1994; Albayrak & Coskun, 2000; Karataş & Sözen, 2004). Wilson & Reeder (1993), on the other hand, suggested that *M. s. pallidus* should be considered a younger synonym of *M. s. schreibersii*.

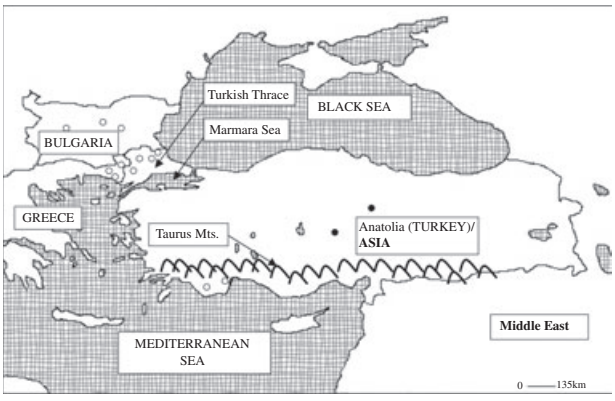

Figure 1. Map of the research area, spanning the transition from Europe into Asia. The black dots represent the caves in eastern Anatolia, the white dots the caves in western Anatolia and southeastern Europe, where samples were collected.

In this study, we used molecular genetic methods for evaluating various subspecific nominations of *M. schreibersii* in south-eastern Europe and Anatolia. Patterns of differentiation in mitochondrial DNA (mtDNA) were used to determine whether the different morphological variations (especially pelage coloration) coincided with mtDNA structure. mtDNA was used because its maternal mode of inheritance makes it possible to build gene trees which are hierarchically branched even within sexually reproducing species (Avise, 1994, 2000), while its lack of recombination is helpful in providing a clean branching structure of its gene trees (Hartl & Clark, 1997).

MATERIAL AND METHODS

Sampling was carried out in ten caves in south-eastern Europe [three in Bulgaria (18 individuals), three in Greece (17 individuals), and four in Turkish Thrace (18 individuals)] that were known to host *M. schreibersii* (Fig. 1). Two caves were visited in western Anatolia and samples were collected from a total of 12 individuals of *M. s. schreibersii*. Two individuals from eastern Anatolia, classified as *M. s. pallidus* (based on fur coloration), were also included in the analysis. A sample of *M. schreibersii* from Indonesia was included in the analysis as same-species outgroup. Samples of *Rhinolophus ferrumequinum* and *R. euryale* were used as outgroups from a different family. Sequences of *Myotis evotis* and *M. californicus*, obtained from GenBank (Rodriguez & Ammermann, 2004), were used as within-family outgroups. Tissue for genetics analysis was collected from individuals using a nonlethal sampling method, from each of the wing membranes (plagiopatagium) of individual bats with biopsy punches (3 mm diameter), as outlined by Worthington Wilmer & Barratt (1996).

The hyper variable region I of control region (D-loop), tRNA proline and tRNA threonine genes, and a partial cytochrome *b* sequence of mtDNA were sequenced using the following methods. Half of a biopsy punch was used for each individual's DNA extraction, with a QIAGEN DNeasy Extraction Kit, following the manufacturer's protocols (Qiagen, Valencia, CA). The primers C and E and the PCR conditions were used as described in Wilkinson & Chapman (1991) for DNA amplification. This was followed by

cycle sequencing, both in 5′ and 3′ directions, using the primers C and E, respectively. This involved 25 cycles in 10 L reactions, which were composed of 1 L of PCR product, 5.7 L of H_20, 0.3 L of primer (20 pM/ L), 1 L of fluorescent dye (ABI Big Dye) and 2 L of 5X buffer (provided with the dye). The cycle sequencing reactions were cleaned up using ethanol precipitation and run in an ABI 3730 XL automated sequencer according to the manufacturer's protocol (Applied Biosystems Inc.). The obtained sequences were cleaned manually using Sequencher v. 4.1 (Gene Codes Corp.), and aligned using Clustal X (Thompson *et al*., 1997).

Data analysis

Genetic vs. geographical distance plots

In the absence of any barriers (geographical, social, climatic, etc.) to gene flow, genetic distances would be expected to increase with an increase in geographical distances within the same species, the phenomenon referred to as isolation by distance (Rousset, 1997). According to this perspective, the linear regression of a plot of genetic vs. geographical distances between species would be expected to result in a regression line that does not go through the origin (Good & Wake, 1992). A genetic distance matrix was generated for pairwise comparisons of individuals using GenAlEx v. 5.1 (Peakall & Smouse, 2001); the genetic distances (uncorrected P), calculated using PAUP* 4.0 (Swofford, 2001), were plotted against geographical distances between individuals. The significance of the association was checked through Mantel tests (Mantel, 1967), which permute the geographical and genetic distance matrices to test whether the observed associations between genetic and geographical distances are significant. This analysis also makes it possible to visually observe any genetic breaks that occur, in particular with regard to geographical distance, for different species/subspecies.

Phylogenetic trees

The mtDNA data were used to construct trees that depict the relationships of individuals. To provide the trees, maximum parsimony (MP) (unweighted) and maximum likelihood (ML) analyses, discrete tree-building methods that operate directly on the nucleotide differences between the sequences of individuals (Page & Holmes, 2000), were made using PAUP* 4.0. TBR (tree bisection and reconnection) was used as the branch-swapping algorithm, as it is the most effective routine for recovering an optimum set of cladograms (Kitching *et al*., 2000). Gaps were treated as missing data. For ML analysis, Modeltest v. 3.06 (Posada & Crandall, 1998) was used to determine the best model of evolution for the subsequent analysis, based on the hierarchical likelihood ratio criterion. The confidence limits for the nodes in ML and MP trees were obtained by bootstrapping 1000 times using PAUP* 4.0.

RESULTS

In total, six haplotypes were found in south-eastern Europe, two in western Anatolia and two in eastern Anatolia. The sequences have been deposited in GenBank with the accession numbers AY923061–AY923073. The number of fixed differences and percent nucleotide differences between the haplotypes from different regions, calculated using DNASP (Rozas & Rozas, 1995), is provided in Table 1. The percent nucleotide difference between south-eastern Europe and western Anatolia is small (1%) when compared with eastern Anatolia (6.7% and 7%, respectively). These differences between regions are important and indicative of differentiation when compared to the percent nucleotide differences within each region, which range between 0.2% and 0.4%. All populations from this area show an average nucleotide differentiation of 9.9% when compared to the sample from Indonesia.

A plot of genetic and geographical distances (excluding the sample from Indonesia) supports the general pattern shown in the nucleotide differences (Fig. 2). All of the comparisons represented by grey squares indicate those including individuals from eastern Anatolia and show the drastic jump in genetic differentiation of the samples at comparable geographical distances to that of the individuals within western Anatolia and south-eastern Europe. The genetic distances between individuals from western Anatolia and south-eastern Europe are less than those of either region with eastern Anatolia at similar geographical

Table 1. The percentage and fixed (in parentheses) nucleotide differences, between and within the regions of interest

	south-eastern Europe	western Anatolia	eastern Anatolia	Indonesia
south-eastern Europe	0.4%			
western Anatolia	1% (3)	0.2%		
eastern Anatolia	6.7% (30)	7% (32)	0.2%	
Indonesia	9.3% (42)	8.9% (41)	11.4% (53)	0

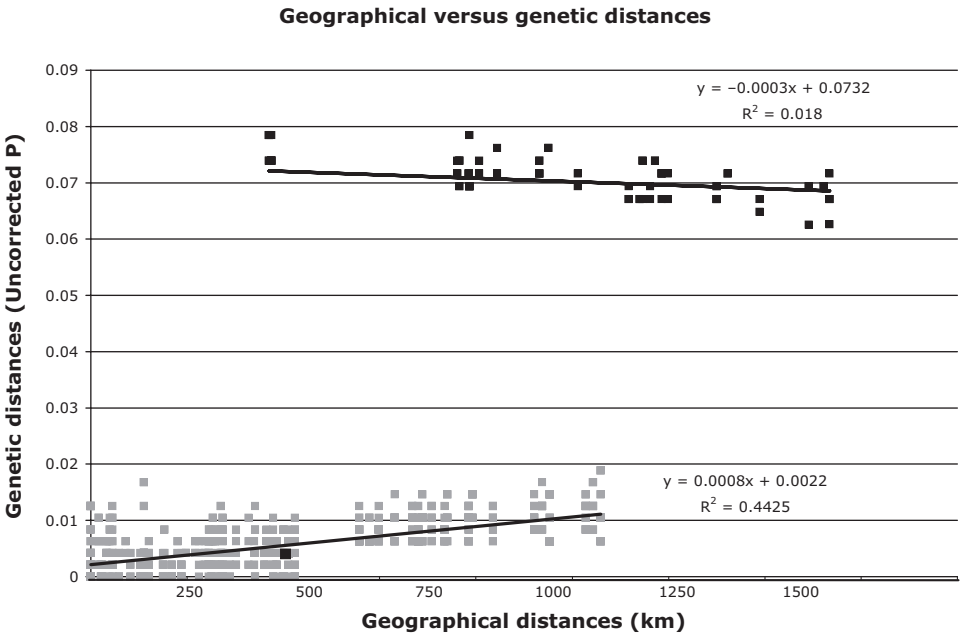

Figure 2. Pairwise geographical vs. genetic distance plot (comparisons with central and eastern Anatolia are shown as black squares).

distances. In Figure 2, it is seen that at between *c.* 750–1000 km, the genetic distances between individuals from eastern Anatolia and south-eastern Europe are at least four times greater than those between western Anatolia and south-eastern Europe. Also, the regression line through the comparisons of eastern Anatolian samples vs. the remainder does not pass through the origin, while that for comparisons of samples within south-eastern Europe and western Anatolia comes close to doing so.

The partial D-loop, tRNA proline, tRNA threonine and partial cytochrome *b* sequences that were analysed together as a partition-homogeneity test (Farris *et al*., 1995) did not indicate any conflicting phylogenetic signals ($P = 0.55$). The ML and MP methods gave identical tree topologies. Including the outgroups, there were 307 variable sites of which 249 were parsimony informative. The CI and RI values were 0.919 and 0.927, respectively, for the parsimony analysis. The MP phylogram is illustrated in Figure 3, where nodes that have greater than 70% bootstrap support are indicated for both ML and parsimony analyses. For ML analysis, the HKY85+ Γ model (Hasegawa-Kishino-Yano, 1985 with gamma) was chosen as the model of sequence evolution, via Modeltest v. 3.06, with a transition transversion ratio of 1.7845. The best tree had a likelihood of –lnl = 2486.81841 and $\alpha = 0.8739$. These phylogenetic analyses showed two main, reciprocally monophyletic, clades (represented as S and P in Fig. 3) in the region of interest. Clade S includes the *M. schreibersii* samples from western

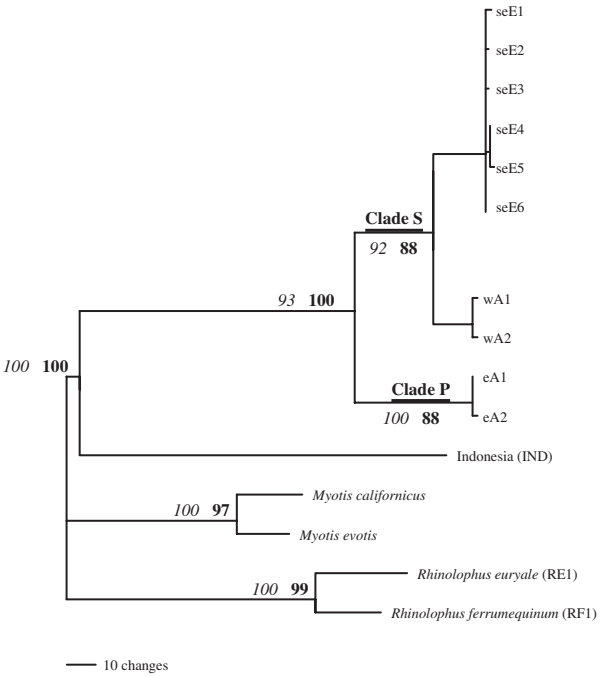

Figure 3. Maximum parsimony phylogram, showing respective branch lengths. Clade S: southeastern Europe (haplotypes seE1–6), western Anatolian samples (wA1–2). Clade P: eastern Anatolian samples (eA1–2). *Myotis evotis, M. californicus, Rhinolophus euryale* (haplotype RE1), *R. ferrumequinum* (RF1) and an individual of *M. schreibersii* from Indonesia (IND) were used as outgroups. The bootstrap values are indicated in italics for parsimony, and in bold for maximum likelihood analysis.

Anatolia and south-eastern Europe, while clade P includes the two samples from eastern Anatolia.

DISCUSSION

The number of fixed differences and the plot of pairwise genetic and geographical differentiation of populations suggest that the western Anatolian and south-eastern European samples are marginally differentiated, whereas there exists a greater degree of differentiation between these and the samples from eastern Anatolia. The phylogenetic analysis also supports this notion of differentiation, where two clades (S and P) with very high bootstrap support are observed. The S clade corresponds to the distribution area of *M. s. schreibersii*, which in our samples is represented by individuals from Bulgaria, Greece, Turkish Thrace and western Anatolia. The P clade, although underrepresented in our sampling, corresponds to the subspecies *M. s. pallidus* from eastern Anatolia.

Possible features of the landscape that may limit gene flow in the study area include the Taurus Mountains that separate the samples from western and eastern Anatolia, and the Sea of Marmara between Thrace and Anatolia (Fig. 1). However, these two features are unlikely to represent major barriers to gene flow among these bats. If the Taurus Mountains constituted a barrier, then the degree of differentiation observed between eastern Anatolia and western Anatolia would also be expected to be seen between western Anatolia and south-eastern Europe, which was not the case. With regard to the Sea of Marmara, which separates both western and eastern Anatolia from Europe, the western Anatolian populations do not show as marked a difference with those of Europe as do those of eastern Anatolia.

An alternative and more likely explanation is secondary contact after differentiation, possibly due to a range expansion of the European/western Anatolian or eastern Anatolian clades, where these clades actually represent frontiers of expansion. An analysis of Anatolian mountain frogs (Anura: Ranidae) (Veith *et al*., 2003) suggested that two Pliocene refugia existed in south-western and north-eastern Turkey, and that the current populations were formed by expansions from these refugia. Nested clade analysis (Templeton, 1998) could be used to ascertain whether a similar case is presented by differentiation of *M. schreibersii*; however, more extensive sampling within eastern Anatolia is necessary .

These results support rejection of the idea of the presence of a single subspecies (or no differentiation) in the region, as well as of the view that *M. s. pallidus* should be considered a younger synonym of *M.* s. *schreibersii*. They support the suggestion of Albayrak & Coskun (2000) that the populations in south-eastern Europe are differentiated from those in Anatolia. However, it should be noted that differentiation within Anatolia appears to be much greater than it is between western Anatolia and south-eastern Europe. Therefore, the idea of the existence of a single subspecies in Anatolia does not seem to be supported by our analysis, as two separate taxa appear to be present in the region.

From the phylogenetic species concept (Cracraft, 1983) point of view, these two taxa can qualify as two separate phylogenetic species. In terms of the biological species concept, the current pattern of differentiation of samples supports the presence of two subspecies: *M. s. schreibersii* in south-eastern Europe and western/southern Anatolia and *M. s. pallidus* in eastern Anatolia. This differentiation is also concordant with the paler fur coloration of the two specimens used in this study from eastern Anatolia.

However, the extent of differentiation could also indicate two different biological species. The regression line of genetic and geographical distance plots between individuals from eastern Anatolia and the other two regions not passing through the origin also supports this notion. More extensive and thorough geographical sampling and analysis are required to warrant elevation of *M. s. pallidus* to specific status. If they show that individuals with clade S and P haplotypes and diagnostically different fur coloration coexist in certain caves, this would definitely support the idea of the existence of two separate biological species in eastern Anatolia and south-eastern Europe/western Anatolia.

ACKNOWLEDGEMENTS

This paper is dedicated to the memory of İsmail Çoraman who supported our work whole-heartedly. His memory will be with us always. We thank Elizabeth Hemond and an anonymous reviewer for their comments on the original manuscript. Financial support for this project was partially provided by a Bat Conservation International Student Scholarship Grant and a CERC/E3B Doctoral Dissertation Start-up Grant to RB.

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