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Molecular architecture of *Pipistrellus pipistrellus*/*Pipistrellus pygmaeus* complex (Chiroptera: Vespertilionidae): further cryptic species and Mediterranean origin of the divergence

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Abstract

Previous genetic analyses have demonstrated that two phonic types of one of the most common European bats, the Common pipistrelle, belong to distinct species, although they are almost identical morphologically (45 kHz *Pipistrellus pipistrellus* and 55 kHz *Pipistrellus pygmaeus*). To reconstruct the history of the species complex and explain the codistribution of both forms in Europe and the Mediterranean, we performed phylogenetic analysis based on a 402-bp portion of the cytochrome *b* gene. Particular attention was paid to the eastern and southern parts of the range where no data were available. We found further distinctive allopatric haplotypes from Libya and Morocco. The difference of about 6–7% described in the Libyan population suggests the occurrence of a new species in the southern Mediterranean. The species status of Moroccan population is also discussed. The phylogeographic patterns obtained and analysis of fossil records support the hypothesis of expansion of both species into Europe from the Mediterranean region during the Holocene. The allopatric speciation model fits our data best. The paleobiographic scenario envisaged is corroborated also by molecular clock estimations and correlations with Late Neogene environmental changes in the Mediterranean region which ended with the Messinian salinity crisis.

Keywords: Phylogeny; Phylogeography; Cryptic diversity; Allopatry; Cytochrome *b*; mtDNA; *Pipistrellus pipistrellus*; *Pipistrellus pygmaeus*

1. Introduction

The last decade brought very detailed information about genetic variability and phylogeography in many taxa of Palearctic rodents and lipotyphlans (e.g., Brunhoff et al., 2003; Jaarola and Tegelstrom, 1995; Jaarola and Searle, 2002; Michaux et al., 2003; Seddon et al., 2001). The study of these groups has shown a very high standard of mammalian phylogeographic analysis regarding both sample size (generally hundreds of individuals) and geographical scope (the entire range). In comparison, relatively little information is available on the phylogeography of Palearctic bats. This is apparently due to the fact that in most regions, bats rank among the most rare and least known of mammals, resulting partly from the very low local abundance of most species and partly from their way of life—which makes them hardly accessible. Thus, for many species, reliable distributional and taxonomical information is only available from western and central Europe, i.e., a region that is not an essential part of the respective species ranges as a rule. This situation has affected not only the current status of molecular phylogeography of Palearctic bats, but also the traditional taxonomic and distributional information on individual taxa resulting mostly from morphometric comparisons. Horáček et al. (2000) argued that reliable alpha taxonomic information is available for 11 species only, i.e., for only about 10% of Palearctic bats.

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In contrast to the situation with rodents, however, the extensive application of molecular techniques in European bats has revealed an unexpected amount of intraspecific cryptic variability. Over only a few years, the standard list of European bat species, composed of 30 items (comp. Horáček et al., 2000; Mitchel-Jones et al., 1999) has grown by five new species and three other cryptic species have been identified in regions immediately neighboring continental Europe. All of these cryptic species appeared within the four species groups that became the major subject of molecular studies, viz. *Myotis myotis*/*Myotis blythii*, *Myotis mystacinus*, *Plecotus auritus*/*Plecotus austriacus*, and *Pipistrellus pipistrellus*/*Pipistrellus pygmaeus* (Barrat et al., 1997; Benda and Tsytsulina, 2000; Castella et al., 2000; Kiefer et al., 2002; Mayer and von Helversen, 2001b; Spitzenberger et al., 2001).

Perhaps the most illustrious case and the first one among the European mammals where no cryptic variation was expected prior to application of mitochondrial sequence analyses, is that of the Common pipistrelle, *P. pipistrellus* (Schreber, 1774). This is one of most common European bats, ranging from northern Africa, Ireland, and southern Scandinavia to Kashgaria, southern Iran, and northern Arabia. Despite several notes in older literature, suggesting possible categorical variation in the European pipistrelle (Cabrera, 1904; Koch, 1865), *P. pipistrellus* has traditionally been considered to be a single species, monotypic in the western part of its range and with a separate subspecies *P. p. aladdin* Thomas, 1905 (= *P. p. bactrianus* Satunin, 1905) in the eastern part, ranging from Iran to Kashmir and eastern Turkestan (Koopman, 1994). However, routine application of bat detectors in the 1980s indicated that echolocation calls of pipistrelles fall into two distinct frequency bands with terminal frequencies of 45 and 55 kHz, respectively (Ahlén, 1981; Miller and Degn, 1981; Weid and von Helversen, 1987; Zingg, 1990). The extensive study of Jones and van Parijs (1993) demonstrated that, at least in Great Britain, the two phonic types occur in sympatry without sharing the same roosts and suggested that these phonic types may represent separate species. The reproduction isolation proven by Park et al. (1996) and sequence differences in a 308-bp segment of the cytochrome *b* gene demonstrated by Barrat et al. (1995) supported the latter conclusion quite convincingly. Further analyses undertaken with samples from more regions of western Europe with the aid of 630 bp of aligned cytochrome *b* sequence (Barrat et al., 1997) revealed four haplotypes clustered into two distinct clades, with divergence exceeding 11%. Clade I (55 kHz) was found to be allopatric in Sweden and Denmark and in the Iberian Peninsula, while most of Europe was demonstrated to be colonized exclusively by clade II (45 kHz), except of Great Britain, where both species occurred in sympatry. The extensive screening in further parts of Europe and the Middle East summarized by Mayer and von Helversen (2001a) and Benda et al. (2003), by means of both echolocation data and further genetic markers, demonstrated sympatric occurrence of both species in southern Germany, Switzerland, Hungary, and Greece. The cryptic species of pipistrelles were found to be almost indistinct morphologically (Barlow et al., 1997; Häussler et al., 2000), and a heated debate on the most appropriate names for them terminated only recently with opinion 2028 of the International Commission on Zoological Nomenclature (2003), which supported the use of the name *P. pipistrellus* (Schreber, 1774) for the 45 kHz form and adoption of the name *P. pygmaeus* (Leach, 1825) for the 55 kHz form. Thanks to continued interest and multiple reinvestigations, the Common pipistrelles have come to represent perhaps the most comprehensive model for the study of cryptic variation in Palearctic bats.

Despite this, only a minute part (less than one-fifth) of their total range has been sufficiently covered to date, and almost no information is available on actual patterns of genetic variation beyond the scope of western Europe and a few Mediterranean regions (Iberia and Greece). The present study was intended to fill the gap in the data obtained from the marginal regions of the respective ranges, and to provide the first reliable information on genetic architecture of *P. pipistrellus*/*pygmaeus* complex throughout most of its western Palearctic range. We have succeeded in collecting sample specimens from several regions of central Europe, northern Africa, Iberian Peninsula, the Balkans and the Middle East, southern Russia, and Kazakhstan, and have demonstrated that the structure of the species assemblage is actually more complicated than expected.

2. Materials and methods

This molecular study was applied to 68 specimens of *P. pipistrellus*/*P. pygmaeus* (Table 1). In most cases, basic morphometric data and echolocation information on particular specimens were also available. A detailed analysis of morphometric comparisons (operating with both external and cranial characters) will be published elsewhere. Voucher specimens are deposited in the collections of the National Museum (of Natural History), Prague, Czech Republic; the Silesian Country Museum, Opava, Czech Republic; the South-Moravian Museum, Znojmo, Czech Republic; Doñana Biology Station, Seville, Spain, and the Zoological Institute of the Russian Academy of Sciences, St. Petersburg, Russia. Data on echolocation were obtained in the field using heterodyne and time-expansion output from Petersson D240 detectors, partly recorded and later examined with the aid of Petersson Bat Sound 1.1 software.

Table 1
Specimen and sequence information

Species	Haplotype	Locality	Provider/reference	Accession No.
Pipistrellus pygmaeus	E1	Hoperskij zapovednik, Russia	P. P. Strelkov	AY426086
Pipistrellus pygmaeus	E1	Velika Koprüsü, Turkey	P. Benda	AY426087
Pipistrellus pygmaeus	E1	Tarragona, Spain	J. Juste, C. Ibanez	AY582277
Pipistrellus pygmaeus	E2	Betlém, Czech Republic	Z. Řehák	AY316321
Pipistrellus pygmaeus	E2	Třeboň, Czech Republic	V. Hanák	AY316325
Pipistrellus pygmaeus	E2	Malý Ratmírov, Czech Republic	V. Hanák	AY316324
Pipistrellus pygmaeus	E2	Malý Ratmírov, Czech Republic	V. Hanák	AY316323
Pipistrellus pygmaeus	E2	Krávovna, Czech Republic	V. Hanák	AY316326
Pipistrellus pygmaeus	E2	Kolence u Třeboně, Czech Republic	V. Hanák	AY316322
Pipistrellus pygmaeus	E2	Lednice, Czech Republic	Z. Řehák	AY316319
Pipistrellus pygmaeus	E2	Vranovice, Czech Republic	Z. Řehák	AY316320
Pipistrellus pygmaeus	E2	Planá nad Lužnicí, Czech Republic	P. Nová	AY582278
Pipistrellus pygmaeus	E2	Anthiro, Greece	Hanák et al. (2001); Benda et al. (2003)	AY316330
Pipistrellus pygmaeus	E2	Sparta, Greece	Hanák et al. (2001); Benda et al. (2003)	AY426088
Pipistrellus pygmaeus	E2	Artiki, Greece	Hanák et al. (2001); Benda et al. (2003)	AY316327
Pipistrellus pygmaeus	E2	Simopoulo, Greece	Hanák et al. (2001); Benda et al. (2003)	AY316329
Pipistrellus pygmaeus	E2	Dimítra, Greece	Hanák et al. (2001); Benda et al. (2003)	AY316331
Pipistrellus pygmaeus	E2	Velika Koprüsü, Turkey	Hanák et al. (2001); Benda et al. (2003)	AY316328
Pipistrellus pygmaeus	E2	Betfia, Romania	I. Horáček, R. Lučan	AY582279
Pipistrellus pygmaeus	E2	Sevilla, Spain	J. Juste, C. Ibanez	AY582280
Pipistrellus pygmaeus	E2	Logrono, Spain	J. Juste, C. Ibanez	AY582281
Pipistrellus pygmaeus	ES1	Malaga, Spain	J. Juste, C. Ibanez	AY582282
Pipistrellus pygmaeus	MC1	Rendina, Greece	Stadelmann et al. (2004)	AJ504441
Pipistrellus pygmaeus	CY1	Kryos River, Trodos Mt., Cyprus	Stadelmann et al. (2004)	AJ504442
Pipistrellus sp. I	LI1	Wadi al Minshiyah, Libya	P. Benda	AY316333
Pipistrellus sp. I	LI1	Arqub ash Shafshaf, Libya	P. Benda	AY426091
Pipistrellus sp. I	LI1	Arqub ash Shafshaf, Libya	P. Benda	AY426092
Pipistrellus sp. I	LI2	Wadi al Kuf, Libya	P. Benda	AY426089
Pipistrellus sp. I	LI2	Wadi al Kuf, Libya	P. Benda	AY316334
Pipistrellus sp. I	LI2	Wadi al Kuf, Libya	P. Benda	AY426090
Pipistrellus sp. I	LI2	Wadi al Kuf, Libya	P. Benda	AY316332
Pipistrellus sp. II	MO1	Gorges du Todra, Morocco	Z. Řehák	AY426093
Pipistrellus sp. II	MO2	Gorges du Todra, Morocco	Z. Řehák	AY426094
Pipistrellus sp. II	MO3	Bekrite (Azrou), Morocco	P. Benda	AY582283
Pipistrellus sp. II	MO4	Gorges du Dedes, Morocco	P. Benda	AY582284
Pipistrellus sp. II	MO4	Gorges du Todra, Morocco	P. Benda	AY582285
Pipistrellus pipistrellus	KA1	Balkhash Lake, Kazakhstan	P. P. Strelkov	AY426095
Pipistrellus pipistrellus	IR1	Yazd, Iran	Benda et al.	AY316335
Pipistrellus pipistrellus	SY1	Dimashq, Syria	Benda et al.	AY316337
Pipistrellus pipistrellus	SY2	Slinfeh, Syria	Benda et al.	AY316336
Pipistrellus pipistrellus	SY3	Banyas, Syria	Benda et al.	AY426096
Pipistrellus pipistrellus	RU1	Adler, Russia	P. P. Strelkov	AY426097
Pipistrellus pipistrellus	E3	Blansko, Czech Republic	Z. Řehák	AY316346
Pipistrellus pipistrellus	E3	Hranice, Czech Republic	Z. Řehák	AY316345
Pipistrellus pipistrellus	E3	Brno, Czech Republic	Z. Řehák	AY316344
Pipistrellus pipistrellus	E3	Boskovštejn, Czech Republic	A. Reiter	AY316342
Pipistrellus pipistrellus	E3	Čížov, Czech Republic	A. Reiter	AY316341
Pipistrellus pipistrellus	E3	Bavorov, Czech Republic	R. Lučan	AY316349
Pipistrellus pipistrellus	E3	Plzeň, Czech Republic, juv.	V. Říš	AY582286
Pipistrellus pipistrellus	E3	Nesvačily, Czech Republic	P. Nová	AY582287
Pipistrellus pipistrellus	E3	Jaroměř, Czech Republic	I. Horáček	AY582288
Pipistrellus pipistrellus	E3	Křivoklát, Czech Republic	I. Horáček	AY582289
Pipistrellus pipistrellus	E3	Křivoklát, Czech Republic	I. Horáček	AY582290
Pipistrellus pipistrellus	E3	Tisovec, Slovakia	P. Benda	AY426098
Pipistrellus pipistrellus	E3	Tisovec, Slovakia	P. Benda	AY426099
Pipistrellus pipistrellus	E3	Erňa cave, Zadiel, Slovakia	P. Benda	AY426100
Pipistrellus pipistrellus	E3	Krumovgrad, Bulgaria	R. Lučan	AY582291
Pipistrellus pipistrellus	CR1	Velký Jindřichov, Czech Republic	V. Hanák	AY316347
Pipistrellus pipistrellus	CR1	Chvalčov, Czech Republic	R. Lučan	AY316350
Pipistrellus pipistrellus	CR2	Raspenava, Czech Republic	Z. Řehák	AY316338
Pipistrellus pipistrellus	CR3	Kašperské Hory, Czech Republic	J. Červený	AY316339
Pipistrellus pipistrellus	CR4	Onšov, Czech Republic	A. Reiter	AY316343
Pipistrellus pipistrellus	CR5	Čížov, Czech Republic	A. Reiter	AY316340

Table 1 (*continued*)

Species	Haplotype	Locality	Provider/reference	Accession No.
Pipistrellus pipistrellus	CR6	Bavorov, Czech Republic	R. Lučan	AY316348
Pipistrellus pipistrellus	GR1	Greece	Stadelmann et al. (2004)	AJ504443
Pipistrellus pipistrellus	ES2	Logrono, Spain	J. Juste, C. Ibanez	AY582292
Pipistrellus pipistrellus	ES3	Cádiz, Spain	J. Juste, C. Ibanez	AY582293
Pipistrellus pipistrellus	ES3	Cádiz, Spain	J. Juste, C. Ibanez	AY582294

Genomic DNA was extracted from alcohol-preserved or dry tissue samples (pectoral muscle or wing membrane) by proteinase K digestion and standard phenol–chloroform extraction (Hoelzel and Green, 1992) in PLG tubes (Eppendorf). Museum specimens were lysed in proteinase K + Chelex (Biorad) solution and the DNA from the supernatant was purified using QIA Quick PCR Purification Kit (Qiagen) (Spitzenberger et al., 2001). Portion of the cytochrome *b* gene (402 bp, 5′ end) was amplified by PCR in 25 μl reaction mixtures contained 1× *Taq* buffer (Promega), 2.5 mM $MgCl_2$, each dNTP at 200 μM, primers at 0.5 μM (MVZ04—5′ GCA GCC CCT CAG AAT GAT ATT TGT CCT C 3′ and MVZ05—5′ CGA AGC TTG ATA TGA AAA ACC ATC GTT G 3′; Smith and Patton, 1992), 1 U *Taq* polymerase (Promega) and 100 ng template DNA. Amplifications were performed in a PTC200 thermal cycler employing predenaturation (94 °C, 3 min), then 40 cycles of denaturation (94 °C, 1 min), annealing (45 °C, 1 min), and extension (72 °C, 1 min), followed by a final extension step (72 °C, 4 min). Amplicons were excised from agarose gels, purified with a QIAquick Gel Extraction Kit (Qiagen), and sequenced by BigDye Cycle Sequencing Kit (with the same primers as for the PCR) in an automated DNA sequencer (PE310). Chromatograms were edited with Chromas (McCarthy, 1996) and the resultant sequences were aligned using ClustalW version 1.8 (Thompson et al., 1994).

GenBank Accession Nos.: AY316319–AY316350, AY426086–AY426100, and AY582277–AY582294. Sequences retrieved from GenBank: AJ504441–AJ504443, Outgroups: *Pipistrellus kuhli* (AJ504444), *Pipistrellus nathusii* (AJ504446), *Myotis nattereri* (AF376863), *Myotis schaubi* (AF376868), *Nyctalus leisleri* (AF376832), *Sorex ornatus* (AF238035), and *Echinosorex gymnura* (AF348079).

2.1. Sequence analysis

The whole data set was collapsed into haplotypes (Table 1). Standard g_1 statistics for measuring the skewness of tree lengths of alternative trees (Hillis and Huelsenbeck, 1992) and saturation tests (plots of pairwise substitutions uncorrected for multiple substitutions against those corrected for multiple substitutions; see, e.g., Daugbjerg and Andersen, 1997) were used to test phylogenetic information content (Xia et al., 2003). Phylogenetic analyses were performed with PAUP 4.0b10 (Swofford, 2001). Distance analysis was computed using *p* values (Table 2). Initial phylogenetic hypotheses were constructed using neighbor-joining, maximum parsimony (heuristic search with 100 random-addition sequences, and tree bisection reconnection (TBR) branch-swapping algorithm; Fig. 1) and weighted parsimony methods with 1000 bootstrap replicates. Codon positions were weighted as the inverse of the total number of differences in the mutation rate observed in each position in a pairwise comparison of all taxa standardized against the third position (Kennedy et al., 1999; Sudman et al., 1994). Modeltest 3.06 (Posada and Crandall, 1998) was used to analyze the pattern of sequence evolution and we used the resulting model to compute maximum likelihood and a Bayesian tree. We ran the Bayesian phylogenetics method (Huelsenbeck and Ronquist, 2001) with four chains in MCMC analysis with 10,000,000 replicates and burnin set at 100,000 according to the number of trees that were sampled, while the chain was not at stationarity (Fig. 2). The results of all methods were combined in the strict consensus tree in order to reconstruct robust nodes (Fig. 1). Geographic arrangements of parsimony networks were used to demonstrate relationships among haplotypes (Avise, 2000; Fig. 3). In molecular clock estimations, we worked with ingroup taxa and *P. nathusii*, *M. nattereri*, and *M. schaubi* as outgroups to prevent rate heterogeneity in our data set. In agreement with Ruedi and Mayer (2001), we used our extensive fossil data estimating the divergence time of *M. nattereri* (Kuhl, 1817) and *M. schaubi* Kormos, 1935 (Horáček and Hanák, 1984) to calibrate the molecular clock. This model was chosen due to its similarity with pipistrelles in amount of genetic divergence and thus expected divergence time (6 Myr), taxonomic proximity, and body size (thus fitting the presumptions of the metabolic rate hypothesis). It should also be remembered that the fossil record of *Pipistrellus* as well as that of closely related clades such as *Nyctalus* is quite poor and the suprageneric divergences such as *Pipistrellini* vs. *Hypsugo-Miostrellus* or *Eptesicus*, (for rough estimation of which the fossil record may exist, eventually: cf. Hoofer and Van Den Bussche, 2003; Horáček, 2001; Rachl, 1983) are so deep that they could not provide a realistic calibration for shallow divergences between sibling species. The calibration was based on corrected distances (with

Table 2
Pairwise comparisons of sequence divergence (p values)

	Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Clade I	1 E1																																		
	2 E2	0.002																																	
	3 ES1	0.005	0.002																																
	4 MC1	0.010	0.007	0.010																															
	5 CY1	0.032	0.030	0.032	0.037																														
	6 LI2	0.067	0.065	0.067	0.072	0.070																													
	7 LI1	0.065	0.062	0.065	0.070	0.067	0.002																												
Clade II	8 MO1	0.109	0.107	0.104	0.110	0.117	0.107	0.104																											
	9 MO2	0.114	0.112	0.109	0.115	0.119	0.112	0.109	0.015																										
	10 MO3	0.109	0.107	0.104	0.110	0.117	0.112	0.109	0.010	0.017																									
	11 MO4	0.104	0.102	0.100	0.105	0.117	0.107	0.104	0.005	0.015	0.005																								
	12 KA1	0.112	0.109	0.107	0.112	0.124	0.104	0.102	0.057	0.052	0.057	0.052																							
	13 IR1	0.119	0.117	0.119	0.120	0.127	0.107	0.104	0.050	0.045	0.055	0.050	0.017																						
	14 SY1	0.122	0.119	0.117	0.122	0.129	0.109	0.107	0.047	0.042	0.052	0.047	0.015	0.002																					
	15 SY2	0.119	0.117	0.114	0.120	0.127	0.107	0.104	0.045	0.045	0.050	0.045	0.022	0.010	0.007																				
	16 SY3	0.119	0.117	0.114	0.120	0.127	0.107	0.104	0.045	0.040	0.050	0.045	0.017	0.005	0.002	0.005																			
	17 RU1	0.117	0.114	0.112	0.117	0.124	0.104	0.102	0.042	0.037	0.047	0.042	0.020	0.007	0.005	0.007	0.002																		
	18 GR1	0.119	0.117	0.114	0.120	0.132	0.112	0.109	0.050	0.045	0.055	0.050	0.032	0.020	0.017	0.020	0.015	0.012																	
	19 CR1	0.114	0.112	0.109	0.115	0.122	0.102	0.100	0.040	0.035	0.045	0.040	0.027	0.015	0.012	0.015	0.010	0.007	0.010																
	20 CR2	0.119	0.117	0.114	0.120	0.127	0.107	0.104	0.050	0.045	0.055	0.050	0.027	0.020	0.017	0.020	0.015	0.012	0.015	0.010															
	21 CR3	0.119	0.117	0.114	0.120	0.127	0.107	0.104	0.040	0.035	0.045	0.040	0.027	0.015	0.012	0.015	0.010	0.007	0.010	0.005	0.010														
	22 CR4	0.119	0.117	0.114	0.120	0.127	0.107	0.104	0.045	0.040	0.050	0.045	0.027	0.015	0.012	0.015	0.010	0.007	0.010	0.005	0.010	0.005													
	23 CR5	0.119	0.117	0.114	0.120	0.127	0.107	0.104	0.045	0.040	0.050	0.045	0.027	0.015	0.012	0.015	0.010	0.007	0.010	0.005	0.010	0.005	0.005												
	24 CR6	0.114	0.112	0.109	0.115	0.122	0.102	0.100	0.045	0.040	0.050	0.045	0.027	0.015	0.012	0.015	0.010	0.007	0.010	0.005	0.010	0.005	0.005	0.005											
	25 E3	0.117	0.114	0.112	0.117	0.124	0.104	0.102	0.042	0.037	0.047	0.042	0.025	0.012	0.010	0.012	0.007	0.005	0.007	0.002	0.007	0.002	0.002	0.002	0.002										
	26 ES2	0.122	0.119	0.117	0.122	0.129	0.109	0.107	0.047	0.042	0.052	0.047	0.030	0.017	0.015	0.017	0.012	0.010	0.007	0.007	0.012	0.007	0.007	0.007	0.007	0.005									
	27 ES3	0.117	0.114	0.112	0.117	0.124	0.104	0.102	0.042	0.037	0.047	0.042	0.025	0.017	0.015	0.017	0.012	0.010	0.012	0.007	0.012	0.007	0.007	0.007	0.007	0.005	0.010								
Outgroups	28 *Pipistrellus nathusii*	0.162	0.160	0.157	0.168	0.162	0.152	0.150	0.157	0.152	0.157	0.157	0.165	0.167	0.165	0.167	0.162	0.160	0.162	0.162	0.167	0.162	0.167	0.162	0.162	0.165	0.165	0.160							
	29 *Pipistrellus kuhli*	0.154	0.154	0.157	0.160	0.162	0.154	0.152	0.162	0.164	0.169	0.164	0.159	0.162	0.164	0.162	0.162	0.164	0.172	0.162	0.167	0.162	0.167	0.167	0.164	0.164	0.169	0.164	0.147						
	30 *Nyctalus leisleri*	0.163	0.163	0.165	0.165	0.165	0.157	0.160	0.164	0.167	0.169	0.167	0.172	0.170	0.172	0.170	0.170	0.172	0.180	0.170	0.173	0.175	0.175	0.175	0.175	0.170	0.172	0.178	0.172	0.191	0.138				
	31 *Myotis nattereri*	0.189	0.189	0.187	0.197	0.199	0.177	0.174	0.184	0.187	0.189	0.184	0.192	0.204	0.201	0.199	0.199	0.197	0.194	0.194	0.199	0.199	0.199	0.194	0.194	0.197	0.197	0.197	0.187	0.211	0.189				
	32 *Myotis schaubi*	0.191	0.191	0.189	0.199	0.199	0.209	0.206	0.196	0.186	0.196	0.196	0.204	0.206	0.204	0.204	0.201	0.204	0.201	0.196	0.201	0.196	0.201	0.196	0.196	0.199	0.199	0.199	0.189	0.217	0.186	0.163			
	33 *Sorex ornatus*	0.236	0.234	0.236	0.239	0.226	0.234	0.231	0.229	0.231	0.236	0.231	0.226	0.216	0.219	0.214	0.219	0.221	0.229	0.224	0.224	0.224	0.224	0.219	0.219	0.221	0.221	0.226	0.254	0.266	0.257	0.221	0.259		
	34 *Echinososorex gymnura*	0.266	0.266	0.264	0.272	0.281	0.269	0.266	0.259	0.259	0.259	0.259	0.261	0.266	0.264	0.261	0.261	0.264	0.259	0.259	0.261	0.259	0.264	0.259	0.264	0.261	0.261	0.266	0.272	0.246	0.283	0.246	0.232	0.236	

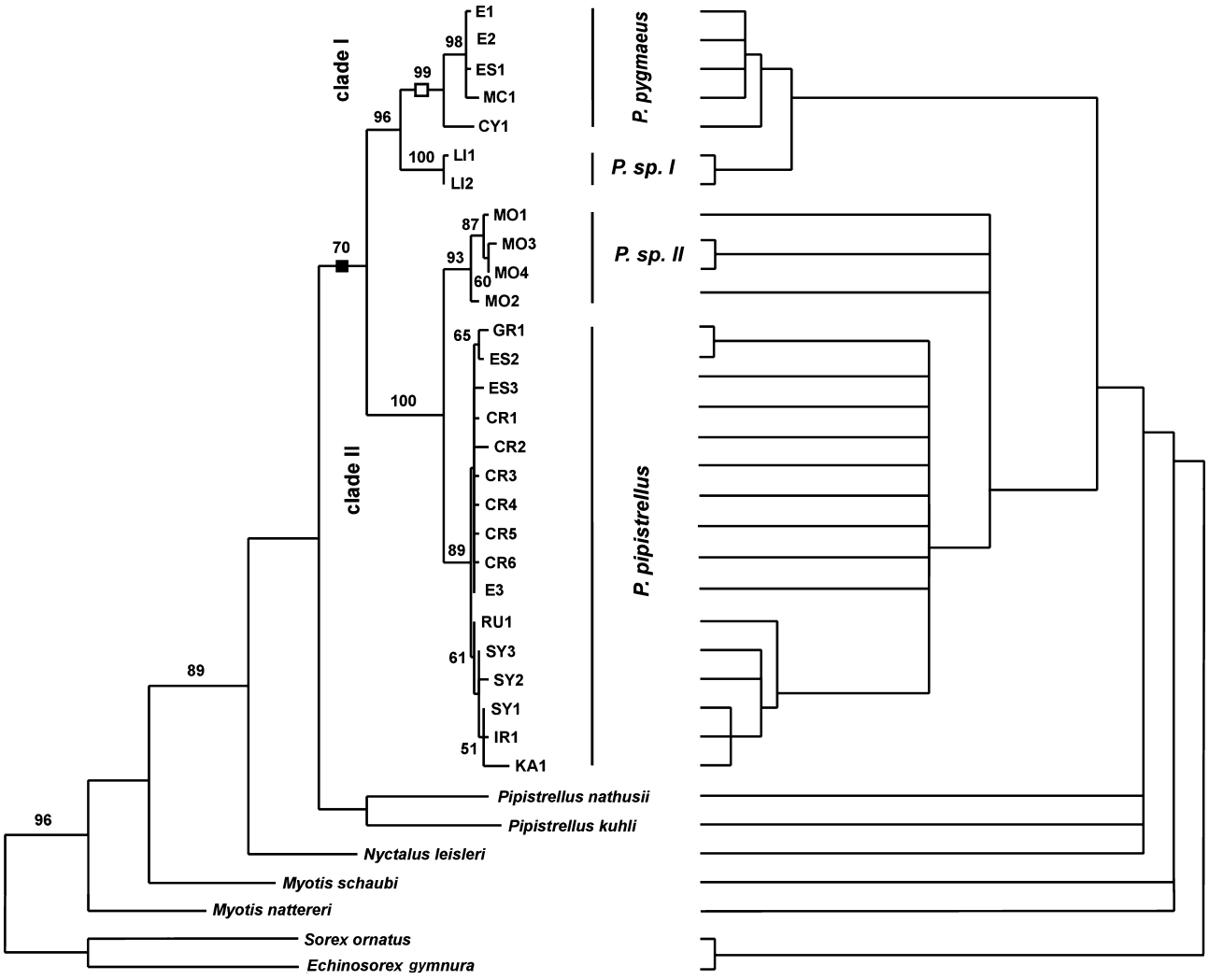

Fig. 1. (Left) Maximum parsimony tree based on 402-bp part of cytochrome *b* showing relationships among the W-Palearctic species of *P. pipistrellus*/*P. pygmaeus* complex. The tree has a length of 463 mutations, consistency index excluding uninformative characters = 0.52 (Kluge and Farris, 1969); retention index = 0.72 (Farris, 1989); rescaled consistency index = 0.41 (Farris, 1989). Numbers at the nodes indicate 1000 replication bootstrap support. Full square—plesiomorphic (45 kHz); open square—apomorphic (55 kHz) state of echolocation frequency. (Right) Strict consensus tree obtained by combination of outcomes from neighbor-joining, maximum parsimony, weighted parsimony, maximum likelihood, and Bayesian methods.

the model that resulted from Modeltest for reduced data set) to avoid mutational saturation at the deeper nodes (Excoffier and Yang, 1999). Alternatively, we used the linearized tree approach (Takezaki et al., 1995; Fig. 4). The goodness-of-fit of the molecular clock assumption was tested via likelihood ratio test (see, e.g., Felsenstein, 1981) comparison of maximum likelihood trees generated with and without molecular clock enforcement.

3. Results

The whole data set contained 402-bp cytochrome *b* sequences of 75 bats (68 ingroup). Within the ingroup, 89 characters were variable and 67 were parsimony informative. All variable positions involved single base substitutions. Of the polymorphism observed, 20.23% occurred at first codon positions, 3.37% at second, and 76.40% at third codon positions. Based on nucleotide states at variable positions, we found 27 distinct haplotypes (Table 1). The sequences exhibited a low proportion of guanine residues (A = 0.342, C = 0.272, G = 0.110, T = 0.276) which is typical for mammals (Irwin et al., 1991). The considerable skewness of random tree length distribution showed that our data contained phylogenetic information. This was supported also by saturation tests. The relationships between uncorrected and corrected substitutions were linear for first and second codon positions, indicating that these positions have not yet reached mutational saturation. The deflection from linearity apparent for third codon positions suggests that multiple substitutions at this site are

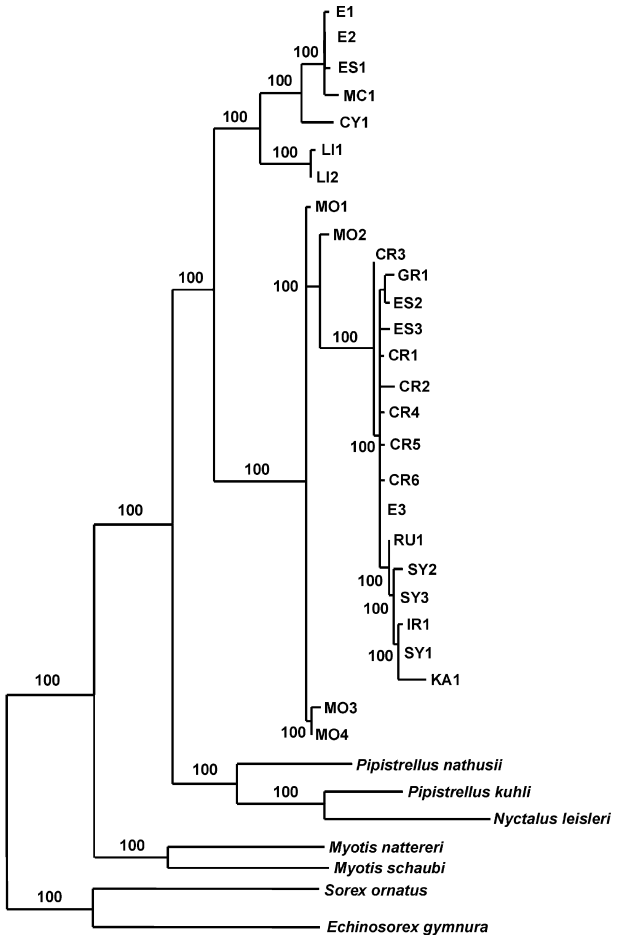

Fig. 2. The result of Bayesian method under the TrN + I + G model of DNA substitution with four chains and 10,000,000 replicates. Numbers at the nodes indicate posterior probabilities.

increasing more rapidly. This phenomenon was reflected in weighted parsimony and maximum likelihood analysis. We obtained codon position weightings 5:18:1 for weighted parsimony and TrN + I + G model with gamma distribution shape parameter = 3.83 for maximum likelihood analysis.

All tree-building methods resulted in two main lineages (clades I and II, 10–13% genetic distance), highly supported by bootstrap values and posterior probabilities. We observed additional cryptic diversity within these clades (Figs. 1–3; Table 2).

Clade I consists of three lineages, which were highly supported by all methods: (a) European samples of *P. pygmaeus* (= *P. pygmaeus* s. str.), (b) a haplotype from Cyprus (3% from *P. pygmaeus* s. str.; cf. Stadelmann et al., 2004), and (c) haplotypes from Cyrenaica, north-eastern Libya (6–7% from *P. pygmaeus* s. str.). The range of clade I includes Europe, Libya, and Cyprus. The geographical distribution of genetic variation in this lineage is quite disproportionate—high in the eastern Mediterranean [Libya, Cyprus, and Greece (for Greece see Mayer and von Helversen, 2001a)] and low in the rest of the European range. In the Czech Republic, we analyzed nine specimens of *P. pygmaeus* s. str. from different localities and all belonged to only one haplotype (E2).

Clade II consists of: (a) a lineage representing European and Asian (Syrian, Caucasian, Irani, and Kazakhi) *P. pipistrellus* (= *P. pipistrellus* s. str.) and (b) haplotypes from Morocco (3–5% from *P. pipistrellus* s. str.). All tree-building methods produced trees with sister position of these two phylogroups, except for the Bayesian method, which put Moroccan samples close to the root of clade II (Fig. 2). *P. pipistrellus* s. str. was subdivided into a European and Asian clade by neighbor-joining and maximum parsimony, while weighted parsimony, maximum likelihood, and the Bayesian method put the European haplotypes close to and the Asian haplotypes distal to the root of *P. pipistrellus* s. str. divergence. The total range of clade II is larger than that of clade I and includes Europe except for Scandinavia, Asia to the Balkhash Lake, and north-western Africa (Maghreb). Our analyses confirmed the occurrence of *P. pipistrellus* in southern Iberia (haplotype ES3). The geographic pattern of genetic diversity exhibits: (a) hiatus between Morocco and Iberia and (b) a shallow cline of phylogenetic transformations (concordant with the divergence-by-distance model) throughout the entire continental range, from Spain to Central Asia. In the Czech Republic (where 18 specimens were analyzed), we found 1 major haplotype (E3; 11 specimens) and 6 less abundant haplotypes that differed from E3 by 1 (CR1, 3, 4, 5, and 6) and 3 (CR2) substitutions.

Based on maximum likelihood corrected HKY + G divergence (24.1%) and the fossil record calibration (6 Mya) of *M. nattereri* and *M. schaubi* split, we obtained a value 4.0%/Myr for molecular clock rate. We used this value to estimate approximate times of major divergence events within pipistrelles from maximum likelihood corrected distances matrix. The difference in log-likelihoods of clock-like and non-clock-like trees ($\log L_{\text{clock}} = -1720.48$, $\log L = -1700.37$) was not significant at the 5% level, and thus the molecular clock assumption could not be rejected. The results of the linearized tree method is depicted in Fig. 4.

4. Discussion

4.1. Genetic architecture of *P. pipistrellus*/*pygmaeus* complex

The structure of the *P. pipistrellus*/*P. pygmaeus* complex obtained, consisting of clades I and II, represents a very similar phylogenetic pattern to that described in previous studies (Barrat et al., 1995, 1997; Mayer and von Helversen, 2001a). In contrast to those, we demonstrated an unexpected amount of a large-scale

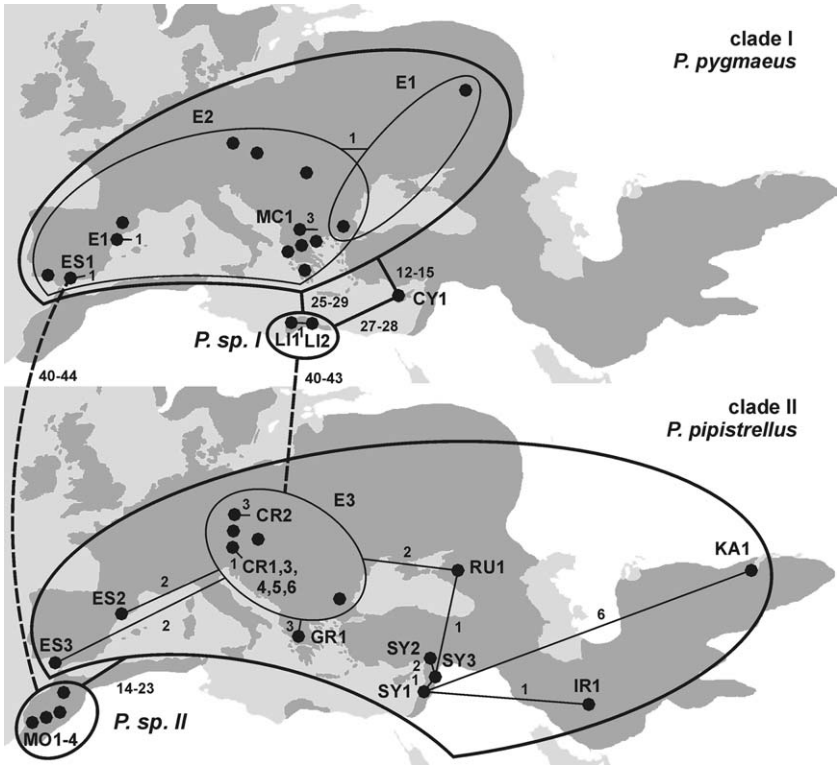

Fig. 3. Geographic arrangement of parsimony networks connecting 27 mtDNA haplotypes of W-palearctic bats from *P. pipistrellus*/*P. pygmaeus* complex. Numbers at network branches reflect numbers of mutational steps along a pathway. Heavier lines designate distinctive clades. Dashed lines—the shortest connections between clades I and II. The shaded area represents current distribution of *P. pipistrellus*/*pygmaeus*. Country codes: CY, Cyprus; CR, Czech Republic; E, Europe; ES, Spain; GR, Greece; IR, Iran; KA, Kazakhstan; LI, Libya; MC, Macedonia; MO, Morocco; RU, Russia; and SY, Syria. The network for Moroccan haplotypes is not displayed due to space limitations.

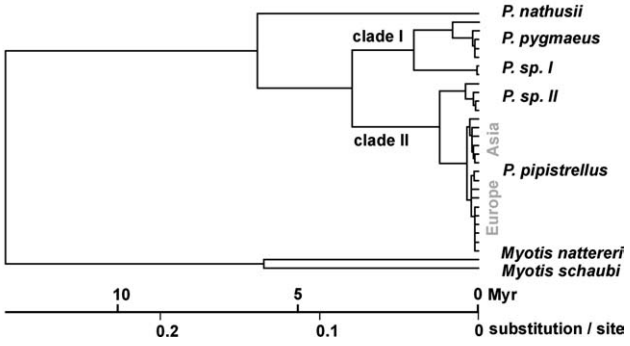

Fig. 4. Clock-like maximum likelihood tree constructed under HKY + G model of DNA substitution. The divergence rate was calibrated with *Myotis nattereri–Myotis schaubi* split (6 Mya).

variation in the most southerly regions of the west-Palearctic range of both clades. While *P. pipistrellus* s. str. and *P. pygmaeus* s. str. occur in sympatry throughout most of Europe, the two African forms are apparently allopatric and apart from them, no other form of the *P. pipistrellus*/*pygmaeus* group was found in northern Africa. As demonstrated elsewhere, in morphometric characters, the African forms differ only slightly from the European populations to a degree that corresponds with differences in other bat sibling species. In contrast to the European species which clearly differ in echolocation calls, viz. 45 and 55 kHz (Jones and van Parijs, 1993; Mayer and von Helversen, 2001a), both of the African forms, however, emit calls with a frequency of maximum energy of approx. 45 kHz (own unpublished data).

The phylogeographic motifs resulting from our analyses were further categorized according to Avise (2000, pp. 135–147). In clade I, we have concluded that there is a relatively deep gene tree (European, Cypriot, and Libyan lineage) with allopatric distribution. The considerable distance of the Libyan population from all remaining haplotypes suggests its separate species status (designated *Pipistrellus* sp. throughout this paper). Within Europe, the genetic diversity of *P. pygmaeus* s. str. is quite low, however, except for Greece (Mayer and von Helversen, 2001a). The genetic uniformity in non-Mediterranean Europe can be explained either by: (a) a high gene flow due to a panmictic pattern of population dynamics or by (b) quite recent colonization of the range, providing no time for evolution of a detectable phylogeographic signal. The former explanation is inconsistent with the considerably high genetic diversity in continental Greece (Mayer and von Helversen, 2001a). Thus, we favor the latter explanation. In support of this,

it should also be mentioned that the phylogeographic structure, although weak, is present. Two widespread haplotypes, E1 and E2, although sympatric in the European part of Turkey, are localized geographically (Fig. 3). The occurrence of haplotype E1 in Spain is surprising, because the relatively dense sampling in Europe did not reveal a continuum for its distribution. This haplotype is perhaps rare and fixed to marginal parts of the range. Similar discontinuous occurrence of a rare haplotype has been described, e.g., in the snapping turtle (*Chelydra serpentina*; Walker, 1998). Of course, as the haplotypes E1 and E2 differ in one substitution only, a parallel rise of haplotype E1 cannot be excluded either. Such a possibility is supported also by the fact that the substitutions that define haplotypes E1 and ES1 appear at nearly the same nucleotide position (243 in ES1 and 244 in E1, respectively), which may therefore represent a mutational hotspot in the cytochrome *b* molecule. In addition, despite the still incomplete information on the biology of the pipistrelle sibling species, it seems quite significant that no fundamental differences between *P. pygmaeus* and *P. pipistrellus* have been reported either in vagility, population structure, mating system, and/or other aspects of the natural history that could play a role in this case. However, the actual velocity of gene flow may depend on many factors, and it is quite a hard task to estimate it without a very detailed supplementary sampling.

Concerning the current data, it seems reasonable to conclude that: (a) *P. pygmaeus* s. str. colonized Europe (including the Iberian Peninsula) quite recently, and supposedly from one source area, (b) the corresponding source area was most probably located in the eastern Mediterranean, and (c) according to genetic distances among the local haplotypes of clade I in the eastern Mediterranean, the local distributional dynamics characterizing the colonization of this region proceeded at least throughout the last 2.1–1.8 Myr.

In clade II, we have described two long branches conforming to the Eurasian and Moroccan samples, respectively. With respect to depth of the branching, the species status of Moroccan population should be considered. The Bayesian method, which is considered to be a very powerful tool for resolution of phylogenies (see, e.g., Murphy et al., 2001), puts the Moroccan haplotypes as ancestral lineages of clade II. From this point of view, the Moroccan species would be a paraphyletic taxon. Further research will be necessary to resolve this question. Within the remaining samples of the clade II, i.e., in *P. pipistrellus* s. str., we found a shallow gene tree over the entire range, with mostly allopatric lineage distribution. Consequently, the level of gene flow was relatively low to enable genetic diversification (caused by lineage sorting, random drift or diversifying selection) of lineages, which have nevertheless been in contact during recent history. The more detailed sampling in the Czech Republic revealed: (a) a common haplotype, E3, which is widespread, and (b) a mosaic of several haplotypes, closely related to E3, that are specific to certain sites without following marked geographic gradient. This star phylogeny reflects the ancestral state in haplotype E3 and relatively recent expansion of its range, all of which indicates a low or moderate gene flow. The core position of mid-European haplotypes is apparent also, with regard to further genotypic diversity in other parts of the range, north of Mediterranean (Fig. 3). This suggests that central Europe may have had an important position as a possible crossroads in the respective colonization routes.

In conclusion, the weak inter-regional differences within *P. pipistrellus* s. str. and a cline pattern of geographic variation throughout the longitudinal span of its recent range suggest that: (a) except for the northward spread (which may be of quite a recent date), the longitudinal extent of the range, from Iberia to Central Asia, had been established at least since the Early Pleistocene (cf. 3% genetic divergence between marginal populations of *P. pipistrellus* s. str.). The deep divergence between the Moroccan and European clades, as well as the particularly high genotypic diversity both in Morocco and Spain, suggests that (b) the source area of the divergence was located in the western Mediterranean, and that (c) it has been continuously colonized for at least 1.6–0.9 Myr by clade II. In this connection, it is also significant that Spain is the only region of Europe from which Early Pleistocene fossil records of pipistrelles are available (Sevilla, 1998).

The above results suggest a scenario in which the two major clades (I and II: *pygmaeus* and *pipistrellus*) were mutually isolated for a long time in the eastern and the western Mediterranean, respectively, and, at the same time, neglecting the differences between them in further dispersal history, their dispersal northward into central and northern Europe was relatively recent. In this respect, it is worth mentioning that the pipistrelles are completely absent from quite a rich fossil record in central and western Europe prior to the Holocene (Horáček et al., 2000). This fact largely contrasts with numerous records of mass Holocene thanatocenoses of these bats in many caves of central Europe (Horáček, 1984), and repeated records of this species in various Holocene sedimentary series (e.g., 51 records in the Czech Republic and Slovakia; Horáček, 1995). All these convincingly suggest that mass spread of pipistrelle bats into central and northern Europe happened only in the Holocene.

4.2. P. pipistrellus/P. pygmaeus speciation: character displacement or range rearrangements, selection or sorting?

The major phylogeographic pattern of the group is a deep divergence of clades I and II, with two most widespread lineages (*P. pipistrellus* s. str. and *P. pyg-*

maeus s. str.) sympatric across most of the European range (Mayer and von Helversen, 2001a). A combination of a clear bimodality in call design and a remarkable degree of morphological uniformity (Barlow and Jones, 1999; Barlow et al., 1997; Jones and van Parijs, 1993), the pattern quite characteristic for the European pipistrelles, evokes questions on the factors involved in the speciation process. In other words, the phylogenetic pattern of the group is a case that calls for a detailed explanation of speciation history.

Jones and van Parijs (1993) have already addressed this issue and proposed a theoretical model suggesting a prevailing role for sympatric speciation, followed by subsequent stabilization of the two lineages under parapatric fragmentation of the distribution range. The scenario of sympatric speciation, further discussed by Barrat et al. (1997) and Jones (1997), predicts that a bimodality of echolocation calls would result from the character displacement within an ancestral population and that just this was the first step in the speciation story. Apparent relations between different frequencies of echolocation calls and specialization for a prey of different size (Barclay, 1986) would explain the ecological advantages of call bimodality: enlarging the spectrum of food resources and reducing competition at the center of the niche. Then, an increased foraging efficiency in the individuals operating at the most distant poles of the niches, i.e., those most distant in their call design, could operate further in the form of a strong disruptive selection on echolocation call design. It could appear under enlarged inter-individual competition for food resources due to: (1) group foraging (quite typical for bats of the subgenus *Pipistrellus*) and (2) strong temporal fluctuations in resource capacity (which undoubtedly occurred after the late Neogene due to increasing climatic seasonality). Last but not least, the model predicts a fixation of the extreme states by sexual selection of the fittest males, i.e., those bearing capacities for the most distant shifts from the plesiomorphic constitution. The model explains quite well the proximate factors of the differences actually observed between the European species in call design and niche partitioning, and answers well why both species can share such a broad zone of sympatry. Nevertheless, it has failed to explain the way in which the RIM was produced—which was capable of keeping the two sonic forms separate throughout some 5 Myr of expected sympatry.

The lack of an answer to that explicit question is perhaps the weakest point of the sympatric hypothesis. The deep-rooted allopatric situations we discovered in the southern Mediterranean support a scenario conforming to a standard speciation model, i.e., geographic (allopatric) speciation with secondary range expansion of the allopatrically diversified clades.

As the centers of origin are traditionally coidentified with the areas of highest diversity (Cann, 2001; Darlington, 1957), and the extremely high diversity was discovered in both species in the southern Mediterranean, we place the center of the group just there. The different source areas predicted for ingroups (eastern Mediterranean for clade I and western Mediterranean for clade II) suggests an early split of the ancestral range into eastern and western units, inhabited by the founders of clades I and II, respectively. A remarkable coincidence of the most ancient divergences within both of the clades and the distribution of the ancient allopatric population in northern Africa (western in clade II and eastern in clade I) suggests that the ancient range of the group was in northern Africa and that its early split could be ascribed to the paleogeographic events affecting that particular region.

While in both clades, significant phylogenetic gaps mirroring geographic barriers occur between populations inhabiting particular regions of the Mediterranean basin (comp. Libya, Cyprus, and Greece in clade I, Morocco, Spain, and the Middle East in clade II), no such gaps were discovered in biogeographically continuous continental areas (comp. from western Europe to Central Asia in the case of *P. pipistrellus* s. str.). Also, in this respect, the phylogeographic pattern of the group may include the shallow geographic structures but not deeper local divergences supporting the sympatric speciation model. In this connection, it is worth mentioning that deep phylogenetic structures on continuous areas can be expected and were detected only in strict cave dwellers with a particularly pronounced tradition in the use of particular roosts (typically *Miniopterus*; Miller-Butterworth et al., 2003), not in bat species with opportunistic roosting selection, such as *Nyctalus* (Petit and Mayer, 2000) or pipistrelles.

The dating of the speciation event represents further support for the proposed allopatric model. We conclude that the following scenario may be close to the truth: (1) a split between clades I and II, in the southern Mediterranean, supposedly northern Africa, 3.1–4.1 Mya, (2) a split between Libyan phylogroup and *P. pygmaeus* s. str., eastern Mediterranean, 2.1–1.8 Mya, (3) a split between Moroccan lineages and *P. pipistrellus* s. str., Western Mediterranean, 1.6–0.9 Mya, (4) colonization of Europe, and later, Asia by *P. pipistrellus* s. str. via Iberian route, divergence of shallow genealogical structure, after 0.8 Mya, and (5) colonization of Europe by *P. pygmaeus* s. str. via the eastern Mediterranean route, after 0.25 Mya.

A major pulse of the divergence (clade I vs. clade II) can be correlated with the consequences of an important stage of the Late Neogene environmental changes in the Mediterranean region: the Messinian salinity crisis (MN 13, 5.96–5.33 Mya; see, e.g., Duggen et al., 2003). Extensive aridisation and splitting of many habitats that appeared in consequence of the tectonic rearrangements terminating with the Messinian crisis, dramatically impacted many

clades, including allopatric speciation events such as in Palearctic brown frogs (Amphibia: Ranidae; Veith et al., 2003) or—as discussed here—in pipistrelles.

Phylogeographic patterns with deep trees and sympatry of major lineages are quite common and have been illustrated repeatedly in the phylogeographic literature (e.g., Arctander et al., 1996; Avise et al., 1984, 1992, 1997; Avise, 2000; Baker and Marshall, 1997; Kim et al., 1998; Monehan, 1994; Quinn et al., 1991; Scribner and Avise, 1993; Taberlet et al., 1992). If the explanations involving past population subdivisions and subsequent codistribution were obvious (e.g., due to glacial history of corresponding areas), allopatric diversification and secondary admixture was proposed as a mechanism for evolution of these patterns.

In addition to the above model, the scenario proposed for the speciation of pipistrelles further predicts: (a) the standard allopatric model for the early split of the northern African population into two major clades (with the effects of Messinian salinity crisis as the sorting factor), (b) a split of the western Mediterranean range of clade II into the Morrocan phylogroup and *P. pipistrellus* s. str., at the latest by the beginning of the Pleistocene (cf. its fossil record in Spain; Sevilla, 1998), and (c) subsequent colonization of the whole Mediterranean and its eastern extension into central Asia by the *P. pipistrellus* s. str. phylogroup, which also resulted in sympatry with the clade I resident in the eastern Mediterranean. The sonic divergence of the two European species evolved then in a zone of sympatry due to character displacement. As the 45-kHz call is common to all forms of the group except for the European *P. pygmaeus*, it can be considered a plesiomorphic condition and it seems that it was *P. pygmaeus* s. str. that had to step aside.

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