

# Mitochondrial phylogeography of the long-eared bats (*Plecotus*) in the Mediterranean Palaeartic and Atlantic Islands

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## Abstract

Long-eared bats of the genus *Plecotus* are widespread and common over most of the western Palaeartic. Based on recent molecular evidence, they proved to represent a complex of several cryptic species, with three new species being described from Europe in 2002. Evolutionary relationships among the different lineages are still fragmentary because of the limited geographic coverage of previous studies. Here we analyze *Plecotus* mitochondrial DNA sequences from the entire Mediterranean region and Atlantic Islands. Phylogenetic reconstructions group these western Palaeartic *Plecotus* into two major clades which split at least 5 Myr ago and that are each subdivided into further subgroups. An 'auritus group' includes the traditional *P. auritus* species and its sister taxon *P. macrobullaris* (= *P. alpinus*) plus related specimens from the Middle East. *P. auritus* and *P. macrobullaris* have broadly overlapping distributions in Europe, although the latter is apparently more restricted to mountain ranges. The other major clade, the 'austriacus group,' includes the European species *P. austriacus* and at least two other related taxa from North Africa (including *P. teneriffae* from the Canary Islands), the Balkans and Anatolia (*P. kolombatovici*). The sister species of this 'austriacus group' is *P. balensis*, an Ethiopian endemic. Phylogenetic reconstructions further suggest that *P. austriacus* reached Madeira during its relatively recent westwards expansion through Europe, while the Canary Islands were colonized by a North African ancestor. Although colonization of the two groups of Atlantic Islands by *Plecotus* bats followed very distinct routes, neither involved lineages from the 'auritus group.' Furthermore, the Strait of Gibraltar perfectly segregates the distinct lineages, which confirms its key role as a geographic barrier. This study also stresses the biogeographical importance of the Mediterranean region, and particularly of North Africa, in understanding the evolution of the western Palaeartic biotas.

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**Keywords:** *Plecotus*; Mediterranean basin; Island colonization; Phylogeography; Cytochrome *b*; Control region; Cryptic species; mtDNA

## 1. Introduction

Molecular approaches in evolutionary studies of the Chiroptera have detected a surprising number of cryptic species in the European bat fauna (Mayer and von Helversen, 2001), even within the pipistrelles, which are among the commonest and 'best studied' bat in Europe (Barratt et al., 1997). Long-eared bats of the genus *Plecotus* are essentially restricted to the Palaeartic Re-

gion (Hofer and Van Den Bussche, 2001), but extend to the Ethiopian (Kruskop and Lavrenchenko, 2000), and the Indomalayan Regions (Corbet and Hill, 1992). Historically, a number of taxa have been described in the Palaeartic (see, e.g., Ellerman and Morrison-Scott, 1966). But given the lack of clear diagnostic characters, classic systematic accounts recognized only two (*P. auritus* and *P. austriacus*; see e.g., Corbet, 1978) or three valid species (the former two plus *P. teneriffae* Mitchell-Jones et al., 1999). Recent mitochondrial DNA (mtDNA) studies have revealed a much more complex picture for Europe with three new species being described or recognized in 2002. The first is *P.*

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*macrobullaris* (a senior synonym of the two recently described *P. alpinus* Kiefer and Veith, 2002 and *P. microdontus* Spitzenberger 2002; see Spitzenberger et al., 2003). This species inhabits from the Caucasus and Asia Minor (Spitzenberger et al., 2003), the Alps and the Balkans (Kiefer and Veith, 2002; Spitzenberger et al., 2002), to the Pyrenees (Garin et al., in press). The second new species is *P. kolombatovici* Dulic, 1980 (sensu Kiefer et al., 2002) from the Balkans (Kiefer et al., 2002; Mayer and von Helversen, 2001; Spitzenberger et al., 2002), a taxon which was described originally as a subspecies of *P. austriacus*. Finally, *P. sardus*, an endemic taxon from Sardinia, in the Mediterranean Sea (Mucedda et al., 2002). All of these new species show broad overlap in most external measurements and are therefore hard to identify using morphological characters alone (Mucedda et al., 2002; Spitzenberger et al., 2002).

In addition, *Plecotus* has proved to be good at colonizing distant islands, as they occur on several Mediterranean and Atlantic Islands (Dorst and de Naurois, 1966; Mathias, 1988; Trujillo, 1991). But again, the taxonomy and relationships of these insular long-eared bats are confused, particularly in relation to the new evolutionary picture that is emerging within the genus *Plecotus*.

The study of geographic distribution of evolutionary lineages, known as phylogeography (Avice, 2000), has had an enormous impact on the understanding of relationships among present lineages as well as in identifying potential origins of insular species. But the main prerequisite for a successful phylogeographic approach is to analyse a comprehensive sampling covering most of the current geographic distribution of taxa (Avice, 2000). Although instrumental in the discovery of cryptic diversity in this group, previous attempts to understand the evolutionary relationships among *Plecotus* lineages were limited to continental Europe and Sardinia or to the Canary Islands in relation only to Iberia (Pestano et al., 2003), and hence ignored substantial parts of the taxonomic diversity found elsewhere. In the present study, we extend these previous studies and analyzed *Plecotus* from Europe, North Africa, the Middle East, and from several Atlantic Islands. Using a phylogeographic approach we address the following questions: (1) Which are the evolutionary relationships among the main western Palaearctic lineages of long-eared bats? (2) Is their current taxonomy adequate? (3) Which are the source areas of the long-eared bats found on the Canary and Madeira Islands? (4) Is the Gibraltar Strait acting as a barrier between North African and South West European populations of *Plecotus*?

## 2. Material and methods

A total of 78 *Plecotus* spp. were sampled from 14 countries surrounding the Mediterranean basin, Central

Europe, and the Macaronesian Islands. Sampling locations are listed in Table 1 and mapped in Fig. 4. The only known specimen of *Plecotus* from Cape Verde is held in the Natural History Museum of Paris but we were unable to amplify DNA fragments from it. Similarly, no sample from *P. sardus* could be obtained and comparisons will only rely on the study by Mucedda et al. (2002). To ease comparisons with previous studies based on other genes, we included the homologous sequence of a *P. kolombatovici* obtained by Spitzenberger et al. (2002) and deposited in GenBank (Accession numbers in Table 1). In addition, as comparative taxa from outside the Palaearctic region, we also sampled *P. balensis* from Ethiopia, and *Corynorhinus townsendii* from Mexico (see Table 1). DNA was extracted either from wing biopsies following Higuchi et al. (1988) or from tissue samples preserved in ethanol following standard phenol/chloroform protocols (Maniatis et al., 1989). The primer pair L14724 (Irwin et al., 1991) and MVZ-16 (Smith and Patton, 1993) was used to amplify over 800 bp of the cytochrome *b* gene (*cyt\_b*). Primers L-15975 (Wilkinson and Chapman, 1991) and H-16498 (Fumagalli et al., 1996) were used to amplify a fragment of the Domain I of the control region (CR). This non-coding segment includes the first hypervariable region and a stretch of variable tandem R1 repeats (Fumagalli et al., 1996; Wilkinson et al., 1997). Thermocycling consisted in 4 min initial denaturation at 94 °C followed by 39 cycles of 60 s at 94 °C, 30 s at 45–50 °C (for the *cyt\_b*), and 2 min at 72 °C and a final extension of 10 min at 72 °C. The annealing temperature for the CR was 55 °C. The PCR products were sequenced using ABI 377 and ABI 3100 automated sequencers (PE Biosystems, Warrington, UK) and following the manufacturer's protocols.

Alignments were obtained with Sequencher 4.1 and inspected by eye. We detected no double sequences that might indicate heteroplasmy. Alignment was unambiguous for the *cyt\_b*, whereas indels were corrected manually in the CR fragment to minimize alignment gaps. Because the number of R1 repeats was species specific, they were excluded from the analyses of CR fragments.

As a first approach to study broad relationships among the different groups within *Plecotus*, phylogenetic analyses using Maximum Likelihood (ML), Parsimony (MP), and Minimum Evolution (ME) were performed on a subset of *cyt\_b* sequences. These sequences represented individuals of all major taxonomic units recognized at present plus some unnamed North African populations. The genus *Corynorhinus* is a closely related taxon to *Plecotus* (Hofer and Van Den Bussche, 2001) and was therefore used to root the trees. To study phylogeographic relationships in more detail, Neighbor-joining (NJ) trees were obtained separately for the *cyt\_b* and the CR fragments for all available

Table 1  
List of specimens, localities, and accession numbers of the samples used of *Plecotus*

Species	Specimen	Locality	Latitude, longitude	GenBank Accession No.		Voucher
				Cyt_b	CR	
<i>C. townsendii</i>	Ctow185.MX	Durango, Mexico	2548N, 10547W	AF513755	AF515151	CRD 3081
<i>P. auritus</i>	Pauri40.IB	Villoslada, La Rioja, Spain	4204N, 0240W	—	AF515173	Biopsy
<i>P. auritus</i>	Pauri74.IB	El Rasillo, La Rioja, Spain	4212N, 0241W	AF513764	AF515174	Biopsy
<i>P. auritus</i>	Pauri75.IB	Lantz, Navarra, Spain	4300N, 0137W	AF513765	AF515175	Biopsy
<i>P. auritus</i>	Pauri90.IB	Condemios de Arriba, Guadalajara, Spain	4113N, 0307W	AF513762	AF515176	Biopsy
<i>P. auritus</i>	Pauri91.IB	Condemios de Arriba, Guadalajara, Spain	4113N, 0307W	AF513762	AF515176	Biopsy
<i>P. auritus</i>	Pauri92.IB	Palomera, Cuenca, Spain	4003N, 0203W	AF513767	AF515177	Biopsy
<i>P. auritus</i>	Pauri160.DK	Jutland, Denmark	5600N, 0915E	AF513756	AF515166	ZMUK (H. Baagoe)
<i>P. auritus</i>	Pauri161.DK	Jutland, Denmark	5600N, 0915E	AF513756	AF515167	ZMUK (H. Baagoe)
<i>P. auritus</i>	Pauri162.DK	Jutland, Denmark	5600N, 0915E	AF513757	AF515168	ZMUK (H. Baagoe)
<i>P. auritus</i>	Pauri166.SW	Vallorbe, Vaud, Switzerland	4643N, 0622E	AF513758	AF515169	MHNG 1806.44
<i>P. auritus</i>	Pauri167.SW	Verbier, Valais, Switzerland	4606N, 0713E	AF513759	AF515170	MHNG 1806.47
<i>P. auritus</i>	Pauri186.IB	Itxina, Vizcaya, Spain	4304N, 0247W	AF513760	AF515171	Biopsy
<i>P. auritus</i>	Pauri187.IB	Vistabella del Maestrazgo, Castellón, Spain	4018N, 0017W	AF513761	AF515172	Biopsy
<i>P. auritus</i>	Pauri193.IB	Condemios de Arriba, Guadalajara, Spain	4113N, 0307W	AF513762	—	Biopsy
<i>P. auritus</i>	Pauri194.IB	Condemios de Arriba, Guadalajara, Spain	4113N, 0307W	AF513762	—	Biopsy
<i>P. auritus</i>	Pauri195.IB	Condemios de Arriba, Guadalajara, Spain	4113N, 0307W	AF513762	—	Biopsy
<i>P. auritus</i>	Pauri196.IB	Condemios de Arriba, Guadalajara, Spain	4113N, 0307W	AF513762	—	Biopsy
<i>P. auritus</i>	Pauri390.GE	Lennbergwald, Mainz, Germany	5000N, 0815E	AF513756	AF515178	(D12) Kiefer and Veith
<i>P. auritus</i>	Pauri391.SW	Curaglia, Grisons, Switzerland	4641N, 0851E	AF513768	—	(H6) Kiefer and Veith
<i>P. auritus</i>	Pauri392.SW	Rifferswill, Zürich, Switzerland	4716N, 0845E	AF513756	—	(H16) Kiefer and Veith
<i>P. auritus</i>	Pauri393.AU	Hall, Graz, Austria	4734N, 1427E	AF513769	AF515168	(O12) Kiefer and Veith
<i>P. auritus</i>	Pauri803.IB	Ordesa, Huesca, Spain	4240N 0000W	AY306211	—	Biopsy
<i>P. austriacus</i>	Paust37.MA	Madeira, Portugal	3244N, 1658W	AF513786	AF515192	D. Trujillo private col.
<i>P. austriacus</i>	Paust76.IB	El Rasillo, La Rioja, Spain	4212N, 0241W	—	AF515179	Biopsy
<i>P. austriacus</i>	Paust77.IB	El Rasillo, La Rioja, Spain	4212N, 0241W	AF513787	AF515179	Biopsy
<i>P. austriacus</i>	Paust78.IB	Calañas, Huelva, Spain	3740N, 0652W	AF513788	AF515193	Biopsy
<i>P. austriacus</i>	Paust79.BA	Minorca, Balearic IIs., Spain	3957N, 0351E	—	AF515181	Biopsy
<i>P. austriacus</i>	Paust80.BA	Minorca, Balearic IIs., Spain	3957N, 0351E	AF513772	AF515181	Biopsy
<i>P. austriacus</i>	Paust94.IB	La Peza, Granada, Spain	3716N, 0316W	AF513792	AF515197	Biopsy
<i>P. austriacus</i>	Paust95.IB	Huélogo, Granada, Spain	3725N, 0315W	AF513793	AF515198	Biopsy
<i>P. austriacus</i>	Paust151.IB	Autol, La Rioja, Spain	4213N, 0200W	AF513770	AF515179	Biopsy
<i>P. austriacus</i>	Paust152.IB	Villoslada, La Rioja, Spain	4204N, 0240W	AF513771	AF515180	Biopsy
<i>P. austriacus</i>	Paust154.BA	Minorca, Balearic IIs., Spain	3957N, 0351E	AF513772	AF515181	Biopsy
<i>P. austriacus</i>	Paust155.BA	Minorca, Balearic IIs., Spain	3957N, 0351E	AF513772	AF515181	Biopsy
<i>P. austriacus</i>	Paust168.SW	Lausanne, Vaud, Switzerland	4631N, 0638E	—	AF515182	MHNG 1806.42
<i>P. austriacus</i>	Paust169.SW	Saillon, Valais, Switzerland	4610N, 0711E	AF513774	AF515182	MHNG 1806.50
<i>P. austriacus</i>	Paust171.GR	Pili, Macedonia, Greece	3651N, 2710E	AF513774	AF515182	MHNG 1807.29
<i>P. austriacus</i>	Paust175.IB	Albuñol, Granada, Spain	3647N, 0312W	AF513776	AF515184	Biopsy

<i>P. austriacus</i>	Paust188.IB	Villahermosa del Río, Castellón, Spain	4012N, 0025W	—	AF515185	Biopsy
<i>P. austriacus</i>	Paust201.IB	Villahermosa del Río, Castellón, Spain	4012N, 0025W	AF513778	AF515189	Biopsy
<i>P. austriacus</i>	Paust202.IB	Villahermosa del Río, Castellón, Spain	4012N, 0025W	AF513779	—	Biopsy
<i>P. austriacus</i>	Paust203.IB	Villahermosa del Río, Castellón, Spain	4012N, 0025W	AF513780	—	Biopsy
<i>P. austriacus</i>	Paust221.IB	Baells, Huesca, Spain	4157N, 0027E	AF513781	—	Biopsy
<i>P. austriacus</i>	Paust386.GE	Stromberg, Mainz, Germany	4957N, 0745E	AF513774	AF515182	(St1) Kiefer and Veith
<i>P. austriacus</i>	Paust387.AU	Unterlamm, Graz, Austria	4659N, 1603E	AF513774	AF515182	(Oe1) Kiefer and Veith
<i>P. austriacus</i>	Paust389.FR	Atlantic Coast, France		AF513774	—	ZFMK95.128
<i>P. balensis</i>	Pbale43.ET	Abune Yusef, Ethiopia	1207N, 3909E	AF513798	AF515199	EBD25842
<i>P. balensis</i>	Pbale44.ET	Abune Yusef, Ethiopia	1207N, 3909E	AF513799	AF515200	EBD25844
<i>P. kolombatovici</i>	Pkol-AF498260	Dalmatian Isl., Lastovo, Croatia	4245N 1653E	—	AF498260	GenBank
<i>P. kolombatovici</i>	Pkol36.TK	Ermenek, Karaman, Turkey	3637N, 3255E	AF513785	AF515204	(1999/86) A. Karatash
<i>P. kolombatovici</i>	Pkol42.TK	Ermenek, Karaman, Turkey	3637N, 3255E	AF513785	AF515204	(1999/87) A. Karatash
<i>P. teneriffae</i>	Ptener83.CAT	Santa Úrsula, Tenerife, Canary Isl., Spain	2824N, 1628W	AF513810	AF515207	Biopsy
<i>P. teneriffae</i>	Ptener84.CAT	Santa Úrsula, Tenerife, Canary Isl., Spain	2824N, 1628W	AF513810	AF515207	Biopsy
<i>P. teneriffae</i>	Ptener85.CAH	El Hierro, Canary Isl., Spain	2444N, 1800W	AF513811	AF515208	Biopsy
<i>P. teneriffae</i>	Ptener86.CAH	El Hierro, Canary Isl., Spain	2444N, 1800W	AF513811	AF515208	Biopsy
<i>P. teneriffae</i>	Ptener156.CAT	Santa Úrsula, Tenerife, Canary Isl., Spain	2824N, 1628W	AF513810	AF515207	Biopsy
<i>P. teneriffae</i>	Ptener157.CAH	El Hierro, Canary Isl., Spain	2444N, 1800W	AF513811	AF515208	Biopsy
<i>P. teneriffae</i>	Ptener158.CAH	El Hierro, Canary Isl., Spain	2444N, 1800W	AF513811	AF515208	Biopsy
<i>P. cf. kolombatovici</i>	P.cf.kol285.LI	Shahhat, Cyrenaica, Libya	3249N, 2152E	AF513782	—	NMP48330
<i>P. cf. kolombatovici</i>	P.cf.kol286.LI	Shahhat, Cyrenaica, Libya	3249N, 2152E	AF513783	AF515190	NMP48331
<i>P. macrobullaris</i>	Pmacr279.SY	Yabroud, Dimasq, Syria	3359N, 3638E	AF513802	—	NMP48052
<i>P. macrobullaris</i>	Pmacr280.SY	Yabroud, Dimasq, Syria	3359N, 3638E	AF513803	—	NMP48053
<i>P. macrobullaris</i>	Pmacr281.IR	Rud-e Saruq River, West Azerbaijan, Iran	3424N, 4929E	AF513804	—	NMP48123
<i>P. macrobullaris</i>	Pmacr282.IR	Rud-e Saruq River, West Azerbaijan, Iran	3424N, 4929E	AF513805	—	NMP48124
<i>P. macrobullaris</i>	Pmacr283.IR	Rud-e Saruq River, West Azerbaijan, Iran	3424N, 4929E	AF513806	—	NMP48125
<i>P. macrobullaris</i>	Pmacr284.IR	Tahkt-e Suleyman, West Azerbaijan, Iran	3618N, 4713E	AF513804	AF515203	NMP48138
<i>P. macrobullaris</i>	Pmacr804.IB	Ordesa, Huesca, Spain	4240N 0000W	AY306213	AY306215	Biopsy
<i>P. macrobullaris</i>	Pmacr805.IB	Ordesa, Huesca, Spain	4240N 0000W	AY306213	AY306215	Biopsy
<i>P. macrobullaris</i>	Pmacr806.IB	Ordesa, Huesca, Spain	4240N 0000W	AY306214	—	Biopsy
<i>P. macrobullaris</i>	Pmacr287.CR	Roufas, Iraklio, Crete, Greece	4607N, 0705E	AF513801	AF515201	Biopsy
<i>P. macrobullaris</i>	Pmacr288.SW	Fully, Valais, Switzerland	3504N, 2455E	AF513800	AF515202	MHNG 1806.53
<i>Plecotus</i> sp.	P.sp33.MO	Tazzouguert, Errachidia, Morocco	3204N, 0346W	AF513784	AF515191	EBD25781
<i>Plecotus</i> sp.	P.sp81.MO	Azrou, Morocco	3324N, 0513W	AF513790	AF515195	Biopsy
<i>Plecotus</i> sp.	P.sp82.MO	Azrou, Morocco	3324N, 0513W	AF513791	AF515196	Biopsy
<i>Plecotus</i> sp.	P.sp170.MO	Tazzouguert, Errachidia, Morocco	3204N, 0346W	AF513775	AF515183	MHNG 1806.51
<i>Plecotus</i> sp.	P.sp189.MO	Tetouan, Morocco	3535N, 0520W	AF513777	AF515186	Biopsy
<i>Plecotus</i> sp.	P.sp190.MO	Tetouan, Morocco	3535N, 0520W	AF513777	AF515186	Biopsy
<i>Plecotus</i> sp.	P.sp191.MO	Tetouan, Morocco	3535N, 0520W	AF513777	AF515188	Biopsy
<i>Plecotus</i> sp.	P.sp192.MO	Tetouan, Morocco	3535N, 0520W	AF513777	AF515186	Biopsy
<i>Plecotus</i> sp.	P.sp881.MO	Bou Maaden, Agadir, Morocco	3015N, 0906W	—	AY306212	Biopsy
<i>Plecotus</i> sp.	P.sp882.MO	Bou Maaden, Agadir, Morocco	3015N, 0906W	—	AY306212	Biopsy

samples within each of the main groups defined in the previous analyses and using *P. balensis* as a functional outgroup.

The best fitting substitution models were selected for each analysis using the hierarchical likelihood ratio tests (LRT) implemented in Modeltest (Posada and Crandall, 1998). LRT tests were used to test for homogeneity of rates between lineages (molecular clock hypothesis) for the *cyt\_b* sequences. Robustness of topologies was assessed through bootstrapping (Felsenstein, 1985) and after Quartet puzzling (100,000 puzzling steps) for ML. All phylogenetic reconstructions were performed using PAUP\* 4.0b10 software (Swofford, 2000). Genetic distances (levels of genetic differentiation) between groups were calculated according to each evolutionary model using MEGA v. 2.1 (Kumar et al., 2001).

### 3. Results

#### 3.1. Sequence analyses

A total of 75 specimens were sequenced for 680 bp of the *cyt\_b* and 61 specimens for the 383 bp of the CR. A total of 49 and 41 different haplotypes were found for the *cyt\_b* and for the CR fragments, respectively. All these unique haplotypes are deposited in the GenBank (accession numbers in Table 1).

#### 3.2. Phylogenetic divergence among main lineages

For the general phylogeny of *Plecotus*, only 15 *cyt\_b* sequences were used, resulting in a total of 244 variable characters, of which 157 were parsimony-informative.

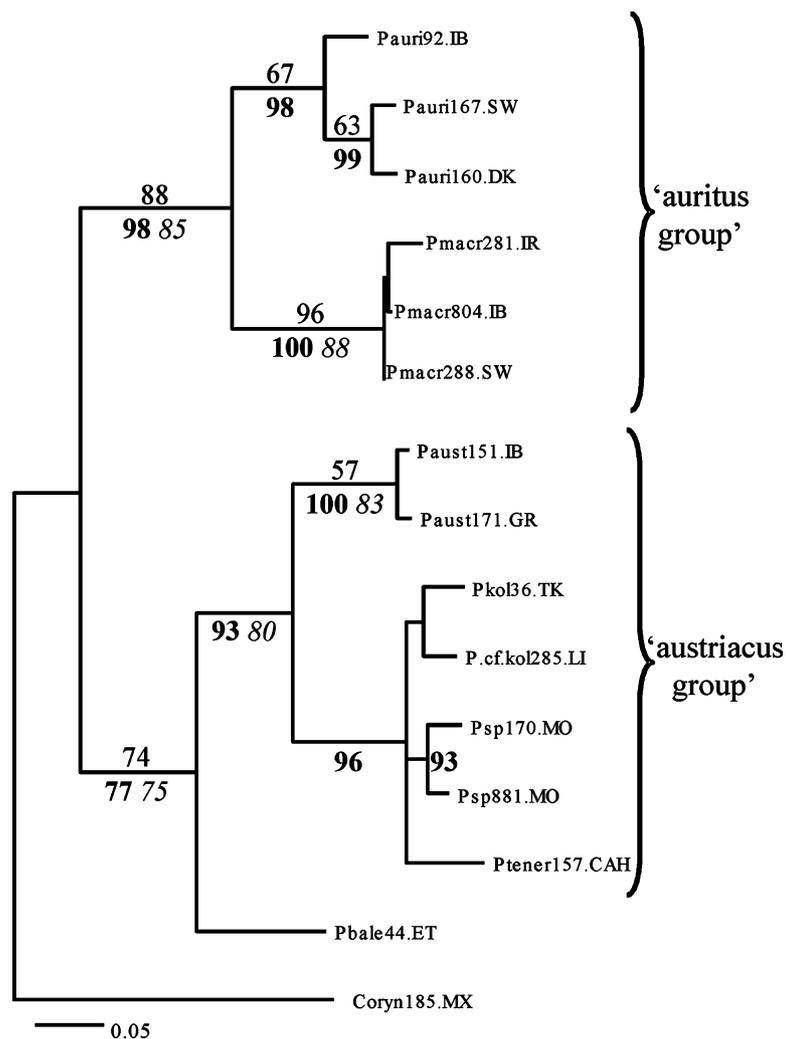


Fig. 1. Maximum Likelihood reconstruction (HKY85 model, see text for details) of relationships among the traditional and recently described species of *Plecotus*, based on a 680 bp cytochrome *b* fragment and using *Corynorhinus townsendii* as outgroup. Bootstrap values (above nodes) after Quartet puzzling (100,000 puzzling steps). Maximum Parsimony (unweighed heuristic search, TBR branch swapping) and Minimum Evolution (Neighbor-joining, HKY85 model) yielded the same topology. Bootstrap values of Parsimony (in bold below nodes) and Minimum Evolution (in italics below nodes) after 5000 replicates. Species codes: Coryn, *Corynorhinus townsendii*; Pauri, *P. auritus*; Pmacr, *P. macrobullaris*; Paus, *P. austriacus*; Ptener, *P. teneriffae*; Pbale, *P. balensis*. Location codes: CAH, El Hierro (Canary Islands); DK, Denmark; GR, Greece; IB, Iberia; IR, Iran; LI, Libya; MO, Morocco; MX, Mexico; SW, Switzerland; TK, Turkey.

The selection included all the genetic variation found in our samples and the main structure of the resulting topologies was not altered after changing the selection of samples. On the basis of the LTR tests, we assumed a HKY85 model (Hasegawa et al., 1985) with site heterogeneity (gamma shape parameter = 0.18) and Ts:Tv ratio = 15.86 for the ML and ME analyses. As ML, MP, and ME reconstructions yielded identical topologies, only the ML tree is presented (Fig. 1). The reconstructions strongly supported the existence of two major western Palaearctic clusters of *Plecotus* (80–98% bootstrap values): an ‘auritus group’ and an ‘austriacus group.’ The Ethiopian *P. balensis* is the sister species to the latter clade (Fig. 1).

Corrected sequence divergence (HKY85 model) ranged over 20% between the ‘auritus’ and ‘austriacus’ groups, to 16% between *P. balensis* and the ‘austriacus group.’ Within the “auritus group,” sequences of *P. auritus* (s. str.) were about 14.7% distinct from those from *P. macrobullaris* (= *P. alpinus*). Likewise, *P. aus-*

*triacus* (s. str.) was about 14.1% distinct from other lineages within the “austriacus group” (Fig. 1).

No statistically significant differences in log-likelihood were found between the reconstructions enforcing ( $-\ln L = 3041.413$ ) or not enforcing ( $-\ln L = 3036.144$ ) a constant rate of evolution in the topology of Fig. 1. The divergence between the genera *Corynorhinus* and *Plecotus* has been dated at about 6 Myr ago from the fossil record in Hungary (Topál, 1989). As they differ by a mean of 21% corrected sequence divergence, we calibrated the molecular clock for the *cyt\_b* as 3.5% Myr<sup>-1</sup>. This divergence rate is slightly slower than the value (4.8% Myr<sup>-1</sup>) reported for mtDNA in other vespertilionid bats (Ruedi and Mayer, 2001) and suggests that the two main ‘auritus’ and ‘austriacus’ groups split around 5 Myr ago.

### 3.3. Divergence within the ‘auritus group’

For the ‘auritus group,’ a total of 22 and 17 different haplotypes were found for the *cyt\_b* and CR fragments,

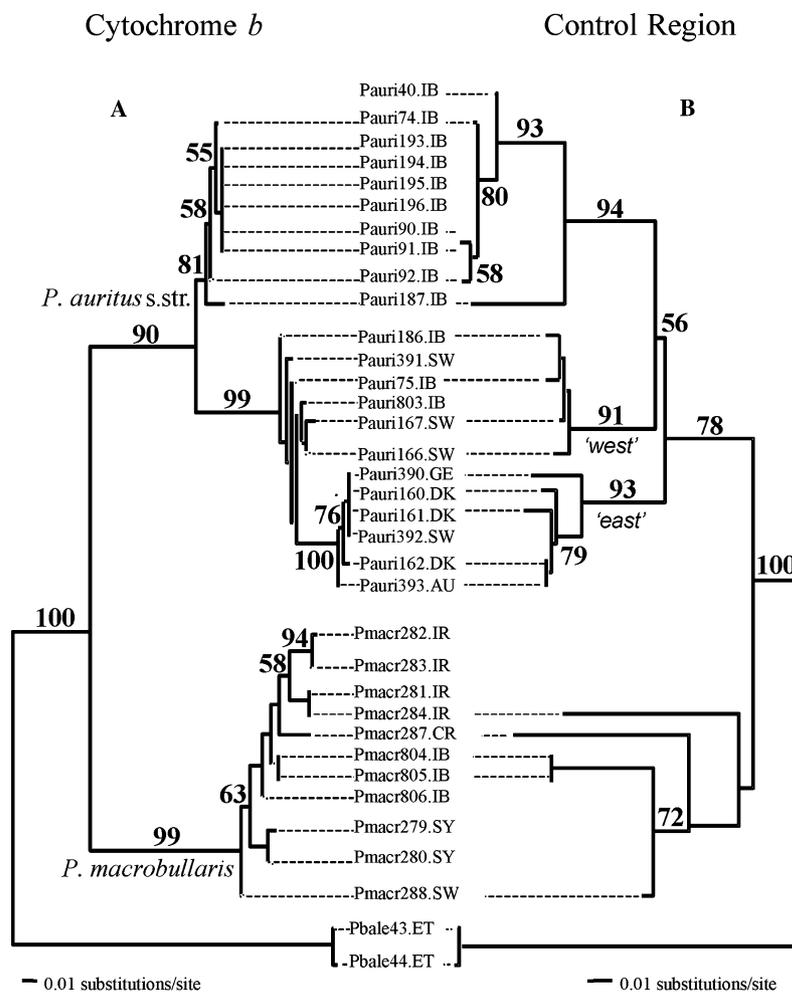


Fig. 2. Neighbor-joining (based on HKY85 model) trees of: cytochrome *b* (A) and control region (B) haplotypes for western Palaearctic *Plecotus* bats within the ‘auritus group’ and using *P. balensis* as outgroup. Bootstrap values after 5000 replicates. Species codes: Pauri, *P. auritus* (s. str.); Pmacr, *P. macrobullaris*; Pbale, *P. balensis*. Location codes: AU, Austria; CR, Creta Island; DK, Denmark; ET, Ethiopia; GE, Germany; IB, Iberia; IR, Iran; SY, Syria; SW, Switzerland.

respectively (Fig. 2). The models selected for the *cyt\_b* and CR were HKY85, with Ts:Tv ratio = 11.06 plus gamma shape parameter = 0.26 and Ts:Tv ratio = 7.16 plus gamma shape parameter = 0.25, respectively, for each fragment. Differences in log-likelihood were significant ( $P < 0.01$ ) between the reconstructions enforcing ( $-\ln L = 2190.105$ ) and not enforcing ( $-\ln L = 2164.133$ ) a constant rate of evolution. Therefore, a molecular clock could not be assumed within this group.

The NJ reconstructions based on *cyt\_b* and CR sequences are largely concordant although, as expected, the CR tends to provide more resolution at terminal nodes and less support at the more basal ones (Fig. 2). Both fragments agree in separating the ‘auritus’ group into two major clades. One corresponds to all sequences of European *P. auritus*, while the other includes all *P. macrobullaris* intermixed with sequences from Middle Eastern long eared bats. There is a strong geographic concordance in a further subdivision within *P. auritus*,

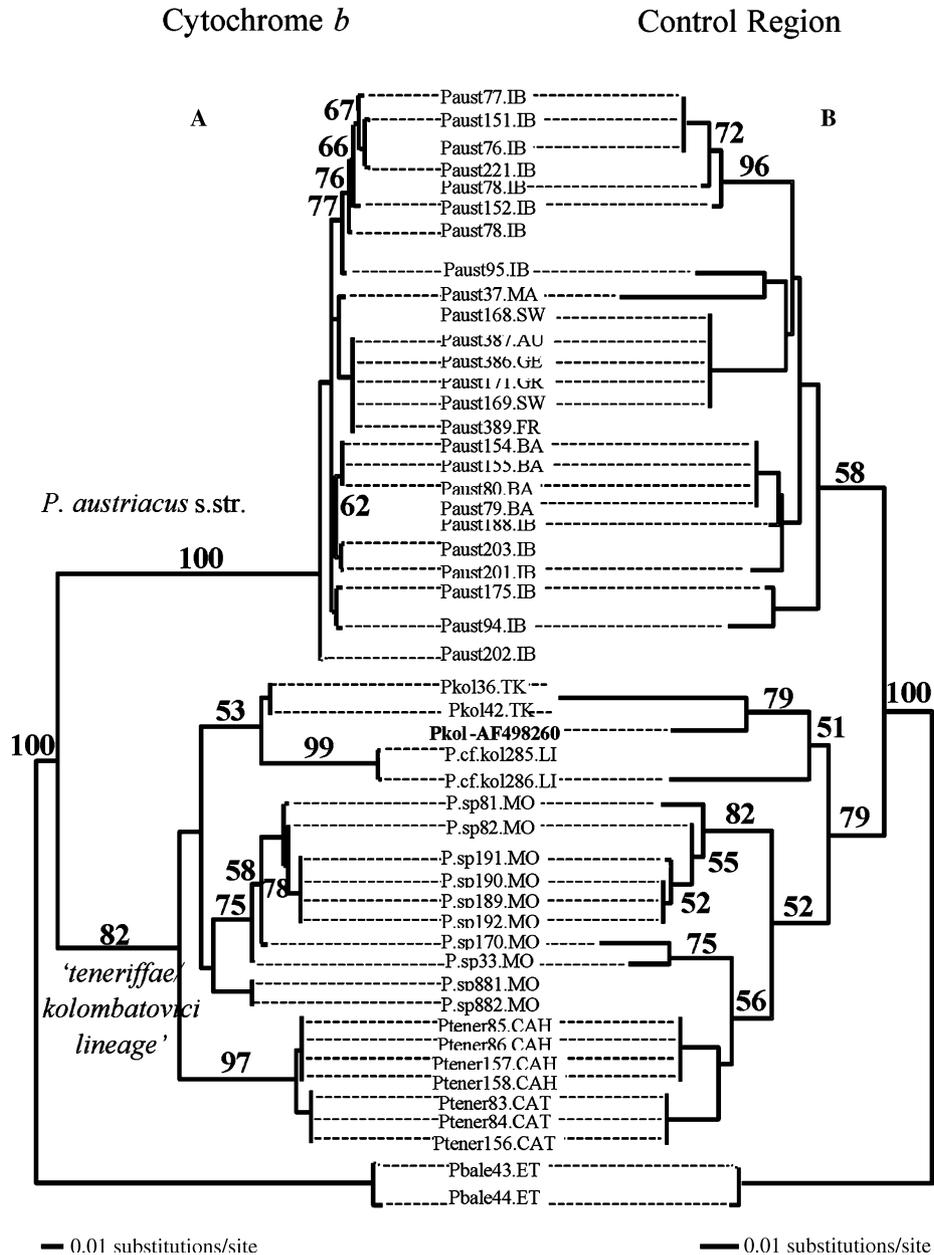


Fig. 3. Neighbor-joining (based on HKY85 model) trees of: cytochrome *b* (A) and control region (B) haplotypes for western Palearctic *Plecotus* bats within the ‘austriacus group’ and using *P. balensis* as outgroup. Bootstrap values after 5000 replicates. A sequence of *P. kolombatovici* from GenBank is given in bold. Species codes: Paust, *P. austriacus* (s. str.); Pkol, *P. kolombatovici*; Ptener, *P. teneriffae*; Pbale, *P. balensis*. Location codes: AU, Austria; BA, Balearic Islands; CAH, El Hierro (Canary Islands); CAT, Tenerife (Canary Islands); ET, Ethiopia; FR, France; GE, Germany; GR, Greece; IB, Iberia; IR, Iran; LI, Libya; MA, Madeira Island; MO, Morocco; SW, Switzerland; TK, Turkey.

as all Central Iberian samples group together in a highly supported cluster (81% and 94% bootstrap values for *cyt\_b* and CR respectively, see Fig. 2). In turn, sequences of European *P. macrobullaris* and those from the Middle East form a single cluster without clear geographic correlation. For instance, the Swiss sample of *P. macrobullaris*, which has been caught close to the type locality of *P. alpinus* in the Alps, is basal to the whole group (Fig. 2A) or more closely related to the Pyrenean samples (Fig. 2B).

#### 3.4. Divergence within the 'austriacus group'

For the 'austriacus group,' a total of 25 and 22 different haplotypes were found for the *cyt\_b* and CR fragments, respectively (Fig. 2). The models selected were again HKY85 with Ts:Tv ratio = 32.15 plus gamma shape parameter = 0.21, and Ts:Tv ratio = 8.36 plus gamma shape parameter = 0.14, respectively, for the *cyt\_b* and CR fragments. In this group, differences in log-likelihood were not significant ( $P > 0.05$ ) between the reconstructions enforcing ( $-\ln L = 2168.201$ ) and not enforcing ( $-\ln L = 2158.021$ ) a constant rate of evolution in the *cyt\_b* based analysis. Therefore, a molecular clock was assumed.

The topologies of the 'austriacus group' based on *cyt\_b* and CR data set again broadly agree, although *cyt\_b* provides more resolution at deeper nodes and levels of bootstrap support are variable (Fig. 3). As in the previous case, phylogenetic relationships show a strong dichotomy between the 'true' *P. austriacus* from the northern Mediterranean basin, and those from the Canary Islands, Northern Africa, and Anatolia. This last clade also includes *P. kolombatovici* from the Balkans (Fig. 3). Within the *P. austriacus* clade, there is a shallow subdivision between lineages within Spain, but most other relationships are only weakly supported and largely independent of geography. Interestingly, samples from Madeira Island fall within this large clade, without strong affinity to a particular continental sample. Nevertheless, the Atlantic coast of Iberia was not included in the sampling, and it could there be an un-sampled lineage more closely related to the Madeiran *P. austriacus*. In fact, the Balearic Islands tend to cluster in the *cyt\_b* close to samples from the Iberian Mediterranean coast (Fig. 3 and Table 1). The maximal divergence within the *P. austriacus* clade measured at the *cyt\_b* is between samples from the Balearic Islands and Europe (1.7%).

Contrary to the *P. austriacus* clade, sequences from the Canary Islands, North African, and Near Eastern *Plecotus* form a rather heterogeneous clade, with strong subdivisions. For instance, all Canary Islands sequences form a monophyletic group (97% bootstrap support for the *cyt\_b* data set). Haplotypes of *P. kolombatovici* from the type locality in the Balkans are closely related to

samples from Turkey and Libya, but precise relationships within this group are somewhat ambiguous due to differences in sampling and support to the topologies by the fragments used (Fig. 3).

## 4. Discussion

Controversies about the systematic and taxonomic subdivision of *Plecotus* bats have persisted during the last century and resulted into the description of several poorly defined forms (see review in Strelkov, 1988). The discovery of two distinctive baculum morphologies (Topál, 1958) and concordant differences in the tympanic bullae (Bauer, 1960) led to the conclusion that two sibling species coexisted in Europe. However, the occurrence of intermediate specimens living in Central Europe (Aellen, 1971), the Balkans (Dulic, 1980), the Middle East (e.g., Benda and Horáček, 1998), and North Africa (Hanák and Elgadi, 1984; Qumsiyeh, 1985) arose again doubts about this taxonomic arrangement and the usefulness of these characters. It is only with the recent use of molecular characters that the evolutionary picture of the Palearctic *Plecotus* has begun to be unveiled. Indeed, different genetic studies have shown that as many as five distinct species can be distinguished in Europe; most of them showing broadly overlapping distributions (Kiefer et al., 2002; Mayer and von Helversen, 2001; Mucedda et al., 2002; Spitzenberger et al., 2001, 2002). However these genetic studies did not examine the *Plecotus* from North Africa and the Atlantic Islands and a recent study of the Canarian *Plecotus* (Pestano et al., 2003) has only compared these among islands and with specimens from Iberia. The molecular data presented here largely fill these geographic gaps and contribute not only to a better understanding of the systematics of the whole group, but provide explicit biogeographic scenarios to the evolution of the main *Plecotus* lineages in the West Palearctic.

#### 4.1. Evolutionary relationships and systematic implications

All phylogenetic reconstructions (Figs. 1–3) confirm that the western Palearctic *Plecotus* can be grouped into two major lineages, the 'austriacus' and 'auritus' groups. The species *P. balensis* from Ethiopia is clearly more related to the 'austriacus group' (Fig. 1), although it shares some skull features with *P. auritus* (Kruskop and Lavrenchenko, 2000). This isolated, African species could have resulted from the arrival of a Palearctic ancestor to the Ethiopian massifs using the Nile as a corridor, similarly to other Palearctic taxa (Yalden and Largen, 1992).

*Cyt\_b* and CR sequences suggest that the 'auritus' and 'austriacus' groups are each subdivided into two

further, strongly supported, subgroups (Figs. 1–3). These results are broadly concordant with previous data collected on other coding (ND1) and non-coding (16S, CR) mitochondrial genes (Kiefer et al., 2002; Mayer and von Helversen, 2001; Mucedda et al., 2002; Spitzenberger et al., 2001, 2002). The magnitude of genetic differentiation between each of these major clades (11–20%) corresponds to values found at homologous sequences in other congeneric species of bats (Barratt et al., 1997; Bradley and Baker, 2001; Ruedi and Mayer, 2001). Representatives from three of the four main clades co-exist in a small area of the Swiss Alps (the Valais; see Fig. 4 and Table 1), which further supports the interpretation that these main clades really correspond to different species. Since these evidences are currently

based on a single maternally transmitted molecule (mtDNA), the absence of hybridisation among these species has still to be demonstrated with nuclear markers.

Within the ‘auritus group’ one of the main clade clearly corresponds to *P. auritus* (s. str.). According to the current molecular evidences, it is distributed from western Europe eastwards at least to Russia. It is also found on the island of Sardinia (Mucedda et al., 2002), but not on the Balearics, the Atlantic Islands or Crete. Further sampling in the Eastern Palaearctic is still needed to assess the systematic position of other taxa associated with this nominal form (e.g., Corbet, 1978 or Corbet and Hill, 1985). Based on morphological characters, De Paz (1994) described a distinct subspecies,

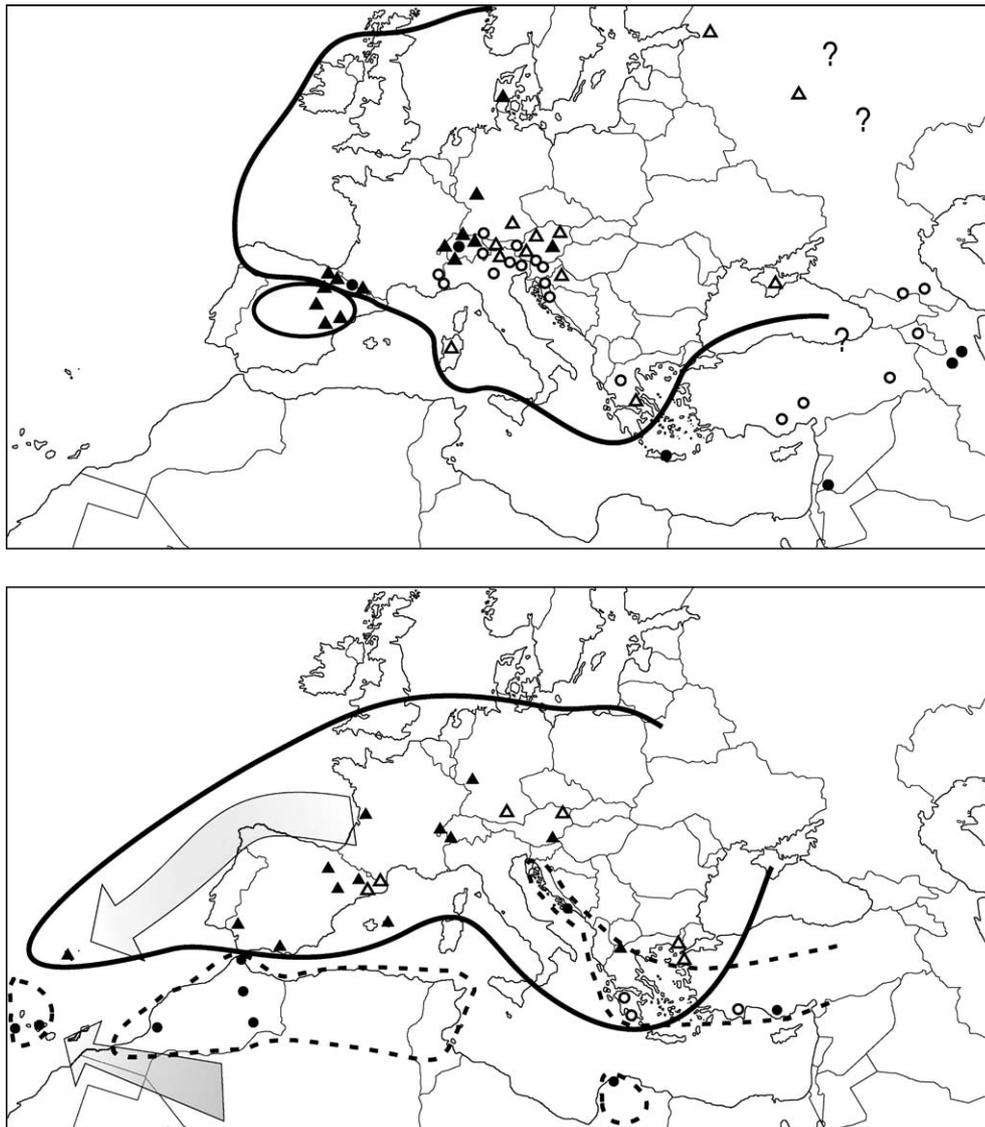


Fig. 4. Approximate distribution maps and location of the samples used in molecular studies of *Plecotus* bats. The ‘auritus group’ (above): *P. auritus* (s. str.) (triangles) and *P. macrobullaris* (circles). The ‘austriacus group’ (below): *P. austriacus* (s. str.) (triangles) and *P. teneriffaelkolombatovici* (circles). Full black symbols refer to samples from this study and empty symbols refer to samples from Kiefer et al. (2002), Irwin et al. (1991), Spitzenberger et al. (2001), and Spitzenberger et al. (2003).

*P. a. begognae*, from montane areas of Central Iberia. Interestingly, all *P. auritus* haplotypes south of the Ebro river in Spain pertain to a distinct, highly supported monophyletic clade (Fig. 2) with no apparent geographic overlap with *P. auritus* (s. str.). Thus, the recognition of *begognae* is confirmed and the subspecific level maintained according to the genetic distances between both clades (7% for *cyt\_b*).

The other major clade within the ‘auritus group’ includes all haplotypes of specimens attributed to *P. macrobullaris* (Fig. 1). We confirm its presence in the Pyrenees, the Swiss Alps, and on Crete (see Table 1) and stress the apparent preference of this lineage for mountain habitat (Kiefer et al., 2002). Haplotypes of *Plecotus* from mountains of Syria and Iran were, however, also included in this strongly supported clade and that they are genetically hardly distinct from the European samples (Fig. 2). Spitzenberger et al. (2003) have recently shown from molecular grounds that the new western European lineage described as *P. alpinus* Kiefer and Veith, 2002 or *P. microdontus* Spitzenberger, 2002, should be considered as junior synonym of *P. macrobullaris* Kuzjakin, 1965, described from the Great Caucasus. As this taxonomic treatment corresponds perfectly to the new molecular and biogeographic evidences presented here, we follow Spitzenberger et al. (2003) in calling *P. macrobullaris* the representatives of this clade.

Within the ‘austriacus group,’ the branch containing all haplotypes from Central Europe corresponds to the nominal *P. austriacus* (s. str.). It indeed includes samples close to its type locality in Austria. According to our molecular evidence, this species extends its distribution throughout Southern Europe, on Madeira and the Balearic Islands (Fig. 4), and on Sardinia (Mucedda et al., 2002), but it does not occur in North Africa where it is replaced by another clade (Fig. 3). The genetic homogeneity displayed by *P. austriacus* in Europe, and the absence of geographic concordance of haplotypes within the Iberian Peninsula contradicts the suggestion made by Kiefer et al. (2002) to recognize *hispanicus* as a distinct subspecies for the Iberian representatives of *austriacus*.

The other main branch within the ‘austriacus group’ is genetically far more heterogeneous (Fig. 3), and is patchily distributed from the Canary Islands and North Africa, to the Balkans and Anatolia (Fig. 4). The inclusion of the long-eared bats from the Canary Islands within the ‘austriacus’ group has been shown recently by Pestano et al. (2003). Here we show, the Canarian haplotypes form a highly supported, monophyletic clade (Fig. 3A), which is well differentiated from related lineages from the continent (8.2% divergence in the *cyt\_b* from the closest, Moroccan *Plecotus*). Interestingly, CR sequences are less conclusive and rather suggest a paraphyletic position of Canarian long-eared bats, com-

pared to the desertic forms from Morocco (Er-rachidia). To be conservative with the current taxonomy, we propose that *P. teneriffae* Barret-Hamilton, 1907, be maintained as a valid species endemic to the Canary Islands (Ibáñez and Fernández, 1985). On the other hand, the sequences from coastal Anatolia are clearly related to the topotypic material of *P. kolombatovici* from the Adriatic coast (Kiefer et al., 2002; Spitzenberger et al., 2002), but are also closely related to long-eared bats from the Cyrenaica (Fig. 3). This new molecular evidence thus extends the known distribution of *P. kolombatovici* to Anatolia and the Cyrenaica (Fig. 4). In turn, Morocco samples cluster in a loose group that may extend from Morocco to Algeria and Tunisia. *Plecotus* bats from this area are usually referred as *Plecotus austriacus christii* Gray, 1838, but the type locality of this North African taxon was restricted to the lower Nile by Qumsiyeh (1985). Furthermore, and according to Hanák and Elgadi (1984) and Qumsiyeh (1985), *christii* is morphologically different (pale color, different baculum and bullae tympanica) from other *Plecotus* from both the Cyrenaica and the Maghreb. This view was also supported by comparative material from Arabia, which proved to be morphologically similar to the pale *christii* (Harrison and Bates, 1991). Therefore, our samples from Maghreb could represent an undescribed taxon. Alternatively, if topotypic material from southern Egypt could be compared, all these sequences of *Plecotus* from North Africa may represent a single species, whose name should be *P. christii*.

#### 4.2. Phylogeography and palaeontology

Our reconstructions confirm the distinction of four western Palaearctic lineages of *Plecotus*, with overlapping distribution in Europe:

##### 4.2.1. *Plecotus auritus sensu stricto*

Fossils attest the continuous presence of *P. auritus* in Europe since at least the lower Pliocene about 5 Myr ago (Topál, 1989). This long history is reflected in both the *cyt\_b* and CR trees by the existence of strong structure among the different populations (Fig. 2). Apart from the endemic subspecies from the Central Iberian Peninsula, two additional groups (‘west’ and ‘east’ clades) are apparent, particularly in the CR based tree (Fig. 2B). According to our molecular clock, these two clades diverged in the Middle Pleistocene around 0.8 Myr ago (3.2% divergence in *cyt\_b* sequences). The reconstructions also shows that the two lineages have later met in Central Europe. A western and an eastern component was also suggested in previous molecular analyses (Kiefer et al., 2002; Spitzenberger et al., 2001). This phylogeographic pattern is observed in other European mammals (Taberlet et al., 1998) and corresponds to a variant of the ‘Hedgehog paradigm’ (Hewitt, 2000),

which attribute actual distributions due to a postglacial recolonization of Europe from two distinct Quaternary refugia, in Iberia and the Balkans, respectively. The presence of the endemic *P. a. begognae* in the Iberian Peninsula also, suggests that more than one major lineage may have survived in Iberia or alternatively, that other unidentified refugia areas existed in southern Europe (i.e., in the Italian Peninsula).

#### 4.2.2. *Plecotus macrobullaris*

The lack of geographic structuring and little genetic differentiation within the *P. macrobullaris* lineage (maximum 2.4% found in the *cyt\_b* sequences between the Pyrenees and Iran) suggest that its distribution corresponds to a recent recolonization from a single refuge. As the western populations of *P. macrobullaris* are known to occur only in montane areas of Europe (Garin et al., in press; Mucedda et al., 2002; Kiefer et al., 2002) which were largely covered by glaciers during the Ice Ages, these current populations must have originated from other, more suitable Quaternary habitats. We suggest that such a potential refuge area could be the Middle East, where closely related haplotypes are still extant. The finding in this lineage of two differentiated ‘western’ and ‘eastern’ clades (Spitzenberger et al., 2003), would indicate the existence of more than one refuge. This clade is not shown in our trees in spite of using larger sequences and a more comprehensive sampling seems necessary to reconstruct the history of this lineage.

#### 4.2.3. *Plecotus austriacus sensu stricto*

The relatively high haplotypic diversity found among the Iberian *P. austriacus* (without any apparent geographic structure) is remarkable compared to the other European samples, which show only one haplotype (at both markers) over a wide area from the French Atlantic coast to Greece. This might indicate a rapid recolonization of Central and Eastern Europe from an Iberian glacial refuge, with most haplotypic diversity lost on route (see Ruedi and Castella, 2003). The fossil record supports this interpretation as the oldest trace of *P. austriacus* are found in middle Pleistocene deposits of Spain (Sevilla, 1988). *P. austriacus* has been fairly successful at colonizing most of the Mediterranean Islands (Bogdanowicz, 1999). Haplotypes from the Balearic Islands show very little difference with those from the mainland, which would also indicate a recent colonization (less than 0.5 Myr ago according to our molecular clock). Again, this is compatible with the fossil remains found on Ibiza, and attributed to Upper Pleistocene *P. austriacus* (Alcover, in press).

#### 4.2.4. *Plecotus ‘teneriffae kolombatovici’*

The strong geographic component but close phylogenetic relationships between Canarian and North African long-eared bats with those representing

*P. kolombatovici* from the Balkans and Anatolia suggest that these bats have an ancient origin south of the Mediterranean Basin. Our current molecular survey is still too sparse to elaborate a more detailed picture of the possible colonization routes in this vast area. Unveiling relationships within this lineage, unnoticed in previous studies, requires further research.

#### 4.3. Colonization of Atlantic Islands and the effect of the Gibraltar Strait

The tree topology and branch support strongly indicate the arrival of two independent lineages of *Plecotus* to the Canaries and Madeira (14.1% divergence in the *cyt\_b* sequences between the *Plecotus* from the two Atlantic archipelagos) (Fig. 4). Indeed, our molecular results indicate that, the ‘austriacus’ group arrived to Madeira around 0.3 Myr ago, while the ‘teneriffae/kolombatovici’ lineage reached the Canary archipelago at least 2.3 Myr ago.

Understanding the relationships of the North African and South European fauna requires the simultaneous examination of the role of the narrow Strait of Gibraltar, and of other possible routes around the Mediterranean Sea (Heim de Balsac, 1936). The simple comparison of geographic distributions of species on either side of the Strait without actually testing their evolutionary relationships may lead to erroneous conclusions. For instance, Dobson and Wright (2000) suggested widespread movements of bats across the Gibraltar Strait to explain the dominant Palaearctic character displayed by the Maghrebian bat fauna. Yet, a recent genetic study of the larger, most vagile species of *Myotis* (Castella et al., 2000) has shown that the Strait represents a complete and ancient barrier to gene flow to these bats. In fact, *Myotis* populations found on either side of the Strait are so divergent (10.6% divergence at the *cyt\_b*) that they certainly represent distinct biological species (Castella et al., 2000).

Our phylogenetic reconstructions also demonstrate that the Gibraltar Strait is a strong barrier to *Plecotus* dispersal between Morocco and Iberia. Indeed, the high divergence (13.9% in the *cyt\_b* sequence) between the *Plecotus* populations on either sides of the Gibraltar Strait is even larger than the divergence found in *Myotis* (Castella et al., 2000). Given the good colonization ability shown by long-eared bats elsewhere (they reached Madeira, the Canary Islands, and many other remote islands), the Gibraltar Strait per se—only 14 km wide in straight line—should not represent an impassable barrier to migration in any direction. Rather, we suggest that occasional migrants across the Strait may be out-competed by ecologically equivalent bats already established. The degree to which the Gibraltar Strait limits the dispersal of bat species seems thus by far more important than assumed previously (Dobson, 1998; Dobson and

Wright, 2000), but more bat species should be analyzed before making generalizations about this geographical barrier on bat assemblages. In this context, it would be interesting to see if other water barriers in the range of *Plecotus*, such as the English Channel and the Red Sea, are also indicative of deep phylogeographic divisions.

In conclusion, the inclusion of North African and Middle Eastern *Plecotus* has allowed us to complete previous studies and unveil unexpected evolutionary connections between newly described and the well-known taxa. It has suggested new taxonomic arrangements, as well identifying needed research towards the full understanding of the evolution of this species complex. This study also confirms the biogeographical importance of the Mediterranean region, and of the ancient isolated populations of North Africa, in understanding the evolutionary paths leading to the contemporary communities of the western Palaearctic region (Benda and Horáček, 1998).

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