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Presence of *Myotis alcathoe* Helversen & Heller, 2001 (Chiroptera: Vespertilionidae) in the Iberian Peninsula

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In a survey of bats from La Rioja (Spain), several specimens of the *mystacinus* group were captured at different mountain localities. Genetic and morphologic analyses have revealed the presence of two lineages within this group in La Rioja. The lineages have been identified as corresponding to two different species: *Myotis mystacinus* sensu stricto and the recently described *M. alcathoe*. Both species were found using the same nocturnal refugia (caves) and the same forest habitats. This study extends the distribution of *M. alcathoe* west and southwards and adds a new mammal species to the Iberian fauna.

Key words: bats, *Myotis mystacinus*, *M. alcathoe*, distribution, Iberia, mtDNA

INTRODUCTION

In the last few years, a surprisingly high number of cryptic species have been found among European bats (Mayer and Helversen, 2001), particularly within the vespertilionid genera *Plecotus* (Kiefer *et al*., 2002; Juste *et al*., 2004) and *Myotis* (Castella *et al*., 2000; Helversen *et al*., 2001). *Myotis mystacinus* (Kuhl, 1817) was considered a single species until recently, but it has turned out to be a species group with as many as four different taxa recognised at present in the Western Palaearctic. *Myotis brandtii* (Eversmann, 1845) was historically the first species to be reinstated based on morphological differences from *M. mystacinus* (Hanák, 1965). Later, Volleth (1987) distinguished two new lineages: *Myotis* sp. A and *Myotis* sp. B, based on the different distribution of active nucleolus organiser regions (NORs). In a thorough morphological revision, Benda and Tsytsulina (2000) assigned the *Myotis* sp. A lineage to the species *Myotis aurascens* Kusjakin, 1935, whereas Helversen *et al*. (2001) almost simultaneously described the lineage *Myotis* sp. B as *Myotis alcathoe* Helversen and Heller, 2001, based on differences in morphology, ecological preferences and mitochondrial DNA sequences (ND1 and 12S rRNA). In a recent phylogenetic study, Ruedi and Mayer (2001) have shown that despite morphological similarity, *M. mystacinus* and *M. brandtii* split a long time ago. The latter species is the only Palaearctic member of an otherwise monophyletic American clade. Similarly, Mayer and

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Helversen (2001) found that *M. mystacinus* and *M. alcathoe* also split long ago and that they are not sister clades. The evolutionary relationships between *M. mystacinus* and *M. aurascens*, remain unclear.

Regarding the distribution of these forms occurring in the West Palaearctic, *M. brandtii* is found mainly across central and northern Europe, being generally absent in the Mediterranean basin, although it has been reported from Italy and the Balkans (Gerell, 1999*a*; Benda and Tsytsulina, 2000). The species *M. alcathoe* was first described as endemic from the Balkans and Hungary (Helversen *et al*., 2001), and later reported from France (Ruedi *et al*., 2002) and Slovakia (Benda *et al*., 2003). *Myotis aurascens* seems to be distributed from the Balkans eastwards to Anatolia, Crimea and the Caucasus until Kazakhstan, whereas *M. mystacinus* is spread across most of Europe except in the east where it does not seem to overlap with *M. aurascens* (Benda and Tsytsulina, 2000). Of the *mystacinus* group only the species *M. mystacinus* has been reported so far from the Iberian Peninsula and shows a fragmented distribution, restricted to mountain ranges in the northern half of the Iberian Peninsula (Fig. 1). Indeed, it is considered among the rarest bats in Iberia (Fernández and Ibáñez, 1989; Ibáñez *et al*., 1992; Agirre-Mendi, 2002). Recently, Benda and Tsytsulina (2000) have suggested that the Iberian *M. mystacinus* is significantly larger than other European bats and have proposed their taxonomic distinction at subspecific level as *M. m. occidentalis* Benda, 2000.

In an ongoing faunal study of the 'La Rioja' autonomic region (Northern Spain), several specimens of bats of the *mystacinus* group were captured. The results of their morphological and genetic examination are described here and show the presence of *M. alcathoe* in Iberia, extending considerably the known distribution of this recently described species.

Material and Methods

Study area

The 'La Rioja' region is a small area (5,045 km^2) located in central northern Iberia (42º39'–41º55'N;

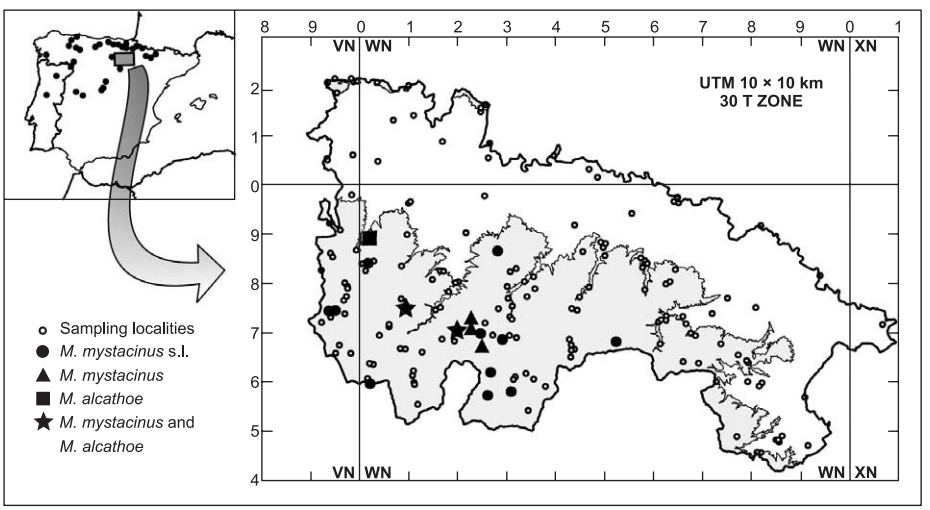


Fig. 1. Left: distribution of bats of the *mystacinus* group in Iberia (Aihartza, 2001; Fernández-Gutiérrez, 2002; Gerell, 1999*b*; Woutersen and Bafaluy-Zoriguel, 2001). Right: sampling localities in La Rioja (Spain). Shade area > 800 m a.s.l.

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3º08'–1º41'W) (Fig. 1). Despite its small size, La Rioja shows a diverse mosaic of landscapes and habitats. Climate and vegetation types are strongly correlated with altitude, which ranges from 260 m at the Ebro river in the north of the region to over 2,200 m in the mountains of the 'Sistema Ibérico' range in the south-western corner. In turns the lowlands, which are mainly under agricultural use, show strong Mediterranean influences. The mountain slopes have high humidity (rainfall over 1,000 mm) and were originally covered by a mixture of broad-leaved (*Fagus sylvatica* and *Quercus* sp.) and pine (*Pinus sylvestris*) forests (Fernández *et al*., 1989; Martínez-Abaigar *et al*., 1994).

Sampling

From 1984 to 2003 a total of 197 localities were sampled for bats in La Rioja (Fig. 1). The sampling was carried out by setting up mist-nets over or near water surfaces (on 195 nights) or at the entrance of nocturnal refugia, caves and mines (on 174 nights).

A total of 25 bats of the *mystacinus* group were captured during the survey. Two older additional bibliographic records from the area (Ibáñez *et al*., 1992) were also included in the study. Five museum specimens (preserved at the Estación Biológica de Doñana' scientific collections — EBD) were used for morphological and molecular comparisons and five additional samples of DNA of released bats were analysed (Appendix). Bats in the field were studied externally, measured (forearm length), identified and released. Wing punches were taken from some specimens, following procedures set out in Worthington Wilmer and Barratt (1996), to permit genetic study.

Genetic Analysis

DNA was extracted from tissue samples following standard phenol/chloroform protocols (Maniatis *et al*., 1989) or from wing biopsies preserved in ethanol following Higuchi *et al*. (1988). A fragment of subunit 1 of the mitochondrial NADH dehydrogenase (ND1) gene was amplified using primers ER65 and ER66 (Mayer and Helversen, 2001). The PCR cocktail (20 µl final reaction volume) included 2 µl of DNA extract, 1 µl of each primer (10 µM), 0.8 µl of $MgCl_2$ (50 mM), 0.16 µl dNTP (25 mM), 0.5 unit of taq-polymerase with appropriate buffer and H_2O. Thermocycling consisted of 5' initial denaturation at 94°C, followed by 40 cycles at 94°C (30''), 63°C (30'') and 72°C (1'30''), with a final extension at 72°C (5'). The fragment was sequenced directly from purified PCR product using an ABI 3100 automated sequencer (Applied Biosystems, Warrington, UK) following manufacturer's protocols. The partial sequences were aligned and edited using the program Sequencher 4.1 (Gene Codes Corp.) and inspected by eye. For species identification purposes, orthologous sequences of the European species of the *mystacinus* group (*Myotis mystacinus*, *M. alcathoe*, *M. aurascens* and *M. brandtii*) were obtained from GenBank (Helversen *et al*., 2001; Mayer and Helversen, 2001) and included in the alignment. The best fitting substitution model was selected with hierarchical likelihood ratio tests (LRT — Modeltest, Posada and Crandall, 1998). Then, absolute differences and corrected distances between sequences were estimated using PAUP* 4.0b10 software (Swofford, 2000) and MEGA v. 2.1 (Kumar *et al*., 2001).

Results

Genetic Analysis

A 565 bp fragment of the mitochondrial ND1 gene was sequenced from eight specimens from La Rioja. Sequences showed no evidence of heteroplasmy and the alignment, including the reference sequences from GenBank, showed 124 variable positions (Table 1), most of them being transitions and located in third positions. The sample consisted of four different haplotypes, two of them were new haplotypes and are deposited in the GenBank (accession numbers are given in Appendix).

Two clearly distinct lineages were found in the Iberian ND1 sequences that showed 16.3% corrected distance between them and over 16% with respect to *M. brandtii.* One of the lineages (Mmy 936, 937, 945, and 946) showed corrected distances < 0.3% and 0.8%, respectively, compared to the sequences of *M. mystacinus* and *M. aurascens* from the GenBank (Tables 1 and 2). Moreover, two individuals of this lineage shared the same haplotype with specimens of *M. mystacinus* from Germany (AY027848) and Spain (AY027847). Two other sequences (Mmy 104 and 968) clearly belonged to this lineage, but did not reach the 565 bp length in their sequences and were not included in further analyses.

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TABLE 1. Polymorphic sites identified in the alignment of mitochondrial ND1 sequences from 8 bats of the *M. mystacinus* group from La Rioja, Spain (see Appendix) and orthologous sequences of *M. mystacinus* (AY027848Mmy), *M. alcathoe* (AY027836Mal), *M. aurascens* (AY027844Mau), and *M. brandtii* (AY027851Mbr) deposited in the GenBank by Helversen et al. (2001) and Mayer and Helversen (2001)

Specimen	Haplo type	11222233 6928124767	3444456778 9023880252	11111 8999901122 4036914703	1111111111 2234555667 6937039381	1111112222 7888890000 7012381568	2222222222 1122444455 3959136919
AY027836Mal Hungary	1	TTTGGAGGCA	TCACCTAAAT	ACAACGCTGC	CCTCTCTTCC	ACATAATACC	CTCCTGTCTA
Mal935 La Rioja	1	....	....	....	....	....	....
Mal944 La Rioja	1	....	....	....	....	....	....
Mal947 La Rioja	1	....	....	....	....	....	....
Mal105 La Rioja	1	....	....	....	....	....	....
AY027832Mal Greece	2	.......A..	....	...G....AT	.....T....	....	....
AY027851Mbr Germany	4	C.CAAGAAAG	C.TT.C..GC	......TCA.	TT.TC.CC..	..C.C.CGTT	TCTT..CA.G
AY027844Mau Greece	3	CCCAA.AAA.	C...T.GG.C	.TG.T...TT	ATC.CTC.TT	GT.C.GC..T	T.T.CACAC.
AY027848Mmy Germany	5	CCCAA.AAA.	C...T.GG.C	GTG.T...TT	ATC.CT..TT	GT.C.G...T	T.T.CACAC.
Mmy945 La Rioja	5	CCCAA.AAA.	C...T.GG.C	GTG.T...TT	ATC.CT..TT	GT.C.G...T	T.T.CACAC.
Mmy946 La Rioja	5	CCCAA.AAA.	C...T.GG.C	GTG.T...TT	ATC.CT..TT	GT.C.G...T	T.T.CACAC.
Mmy936 La Rioja	6	CCCAA.AAA.	C...T.GG.C	.TG.T...TT	ATC.CT..TT	GT.C.G...T	T.T.CACAC.
Mmy937 La Rioja	7	CCCAA.AAA.	C...T.GG.C	GTG.T...TT	ATC.CT..TT	GT.C.G...T	T.T.CACAC.

	Haplo type	2222222222 6667788899 1470323817	2333333333 9000133334 8346203692	3333333333 4456789999 8946210367	3444444444 9112223344 9140362814	4444444444 4555567789 7013901793	4555555555 9112223334 8360581230	5555 4445 1368
AY027836Mal	1	TCGCAATCTC	TCAACTACAC	CTGCCTCGCC	ATGCCTAGTT	TCTATTTCCC	CTTCTTTTCT	CATT
Mal935 La Rioja	1	....	....	....	....	....	....	....
Mal944 La Rioja	1	....	....	....	....	....	....	....
Mal947 La Rioja	1	....	....	....	....	....	....	....
Mal105 La Rioja	1	....	....	....	....	....	....	....
AY027832Mal Greece	2	....	....	....	.......A..	....	....	....
AY027851Mbr Germany	4	CAAA.CC...	C..GTC.TCT	.CATTCTA.T	.CATTC..CC	C.CC.CC.T.	.CATACC..C	.G.C
AY027844Mau Greece	3	.A.AG..TCT	CTG.TCGTC.	TCA.TCTAT.	GCA..CGA..	CT..C.CATT	T.....CCTC	T.CC
AY027848Mmy Germany	5	.A.AG..TCT	CTG.TCGTC.	TCA.TCTAT.	GCA..CGA..	CT..C.CATT	T.....CCTC	T.C.
Mmy945 La Rioja	5	.A.AG..TCT	CTG.TCGTC.	TCA.TCTAT.	GCA..CGA..	CT..C.CATT	T.....CCTC	T.C.
Mmy946 La Rioja	5	.A.AG..TCT	CTG.TCGTC.	TCA.TCTAT.	GCA..CGA..	CT..C.CATT	T.....CCTC	T.C.
Mmy936 La Rioja	6	.A.AG..TCT	CTG.TCGTC.	TCA.TCTA..	GCA..CGA..	CT..C.CATT	T.....CCTC	T.C.
Mmy937 La Rioja	7	.A.AG..TCT	CTG.TCGTC.	TCA.TCTA..	GCA..CGA..	CT..C.CATT	T.....CCTC	T.C.

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TABLE 2. Differences among haplotypes within the *mystacinus* group across a 561 bp fragment of the mitochondrial ND1 gene. The lower half of the matrix shows absolute differences and the upper half corrected distances according to a HKY85 model. Haplotypes information in Table 1

Haplotype	1 (Mal)	2 (Mal)	3 (Mau)	4 (Mbr)	5 (Mmy)	6 (Mmy)	7 (Mmy)
1 (Mal)	–	0.011	0.172	0.165	0.167	0.162	0.164
2 (Mal)	6	–	0.165	0.171	0.159	0.155	0.157
3 (Mau)	82	79	–	0.167	0.007	0.007	0.008
4 (Mbr)	80	82	80	–	0.176	0.171	0.174
5 (Mmy)	80	77	4	84	–	0.004	0.002
6 (Mmy)	78	75	4	82	2	–	0.002
7 (Mmy)	79	76	5	83	1	1	–

The other lineage (Mal 135, 935, 944 and 947) shared a unique haplotype that showed a genetic distance at the ND1 fragment of 0 and 1.1% with respect to the two known sequences of *M. alcathoe* (Tables 1 and 2).

Morphological Comparisons

The specimens identified as belonging to the *M. mystacinus/aurascens* lineage, showed dorsally greyish pelage, a relatively broad and rather rounded base of the upper canine. This dental character can tell the two species apart (P. Benda, in litt.). The four specimens identified as belonging to the *M. alcathoe* lineage were clearly smaller in external measurements than the six *M. mystacinus/aurascens* for both males (Table 3) and females (forearm length 32.5 and 33.0 mm in *M. alcathoe* and 34.9 mm in *M. mystacinus/aurascens*). Two specimens identified as *M. alcathoe* showed reddish brown dorsal colouration but the other two *M. alcathoe* showed a greyish coloration similar to the *M. mystacinus/aurascens* specimens. Both greyish and reddish brown forms of *M. alcathoe* had pinkish on the internal basal surface of the ears and hairless areas of the face. The only skull of the Spanish *M. alcathoe* showed the anterior upper premolar located slightly off the tooth-row and displaced internally, which also showed a more developed cingulum cusp than the specimens of the *M. mystacinus/aurascens* lineage.

TABLE 3. Measurements ($\bar{x} \pm$ 1SD) of male specimens of *M. alcathoe* and *M. mystacinus* from La Rioja (Spain) and conspecifics from Greece (Helversen *et al*., 2001) and Slovakia (Benda *et al*., 2003). Acronyms: FA: forearm length; GSL: greatest skull length; CBL: condylobasal length; ZW: zygomatic width; M^3–M^3: width across upper third molars; C–M^3: length of the upper tooth-row; BCW: braincase width; ML: mandibular length; C–M_3: length of the lower tooth-row. Sample size in parentheses

Trait	*M. alcathoe*			*M. mystacinus*
	La Rioja	Greece	Slovakia	La Rioja
FA	31.5 (2)	30.5 (1)	31.3 (1)	33.5 ± 0.19 (6)
GSL	12.9 (1)	–	12.66 (1)	13.25 (2)
CBL	12.3 (1)	11.70 (1)	12.20 (1)	13.15 (2)
ZW	8.0 (1)	7.87 (1)	8.22 (1)	8.30 (1)
M^3–M^3	5.0 (1)	5.15 (1)	5.26 (1)	5.20 (1)
C–M^3	4.8 (1)	4.80 (1)	4.90 (1)	5.35 (2)
BCW	6.4 (1)	–	6.41 (1)	6.75 (2)
ML	9.2 (1)	9.01 (1)	9.31 (1)	9.85 (2)
C–M_3	5.2 (1)	5.05 (1)	5.20 (1)	5.70 (2)

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Habitat Preference

Despite intensive sampling, bats of the *mystacinus* group were found only in the humid mountain areas of the southwest of La Rioja (Fig. 1). Both species were captured in the same habitats and in fact, *M. mystacinus/aurascens* was also caught in two out of the three known localities for *M. alcathoe* (Fig. 1). Altitudinal range varied from 790 to 1,390 m for *M. alcathoe* and from 914 to 1,460 m for *M. mystacinus/M. aurascens*. Both species were captured at the entrance of the same nocturnal refugia, and also using the same beech forest. Besides, *M. alcathoe* was captured in riparian forest and *M. mystacinus* in Scottish pine forests.

Discussion

The presence of two lineages in the mitochondrial DNA sequences of ten bats from La Rioja indicates that two species within the *mystacinus* group coexist in the Iberian Peninsula. The shape of the base of the upper canine identifies provisionally the specimens within the *M. mystacinus/M. aurascens* lineage as *M. mystacinus*. This was expected based on the exclusively eastern distribution of *M. aurascens* in Europe (Benda and Tsytsulina, 2000). It was not possible to distinguish between *M. aurascens* and *M. mystacinus* using mitochondrial DNA (Mayer and Helversen, 2001). Interestingly, Castella *et al*. (2000) and Mayer and Helversen (2001) found also that mtDNA was unreliable to distinguish *M. myotis* and *M. blythii*. The location of the NORs in the chromosomes remains the main diagnostic characteristic between the former two species and needs to be checked for the Iberian *M. mystacinus*.

The other lineage is identified, without doubt, as *M. alcathoe* since all specimens within the lineage showed the molecular diagnostic sequence of this species (Helversen *et al*., 2001; Ruedi *et al*., 2002; Benda *et al*., 2003). The genetic distance found between the two species in Iberia (> 16%) is similar to the value reported for this mitochondrial fragment in other studies (Helversen *et al*., 2001). The two lineages seem to show important differences in the level of polymorphism in Iberia. The four *M. alcathoe* from Iberia are monomorphic and share a unique haplotype with individuals from Hungary (Helversen *et al*., 2001) and with two other known specimens from France (Ruedi *et al*., 2002) and Slovakia (Benda *et al*., 2003). However three haplotypes are found in the orthologous fragment of ND1 in the four Iberian *M. mystacinus*. One of these haplotypes is shared with specimens from Germany and was already reported for Spain by Helversen *et al*. (2001). Although more detailed analyses with larger sample sizes are needed, this high polymorphism could possibly indicate a role of the Iberian Peninsula as a quaternary refugium for *M. mystacinus* in recent cold periods, as is well known for other species (Hewitt, 1999; Ruedi and Castella, 2003).

At morphological level, the Iberian *M. alcathoe* show two colour phases: the dorsal reddish brown pelage, similar to other European co-specifics and a greyer one, similar to *M. mystacinus* and already reported in the original description of the species by Helversen *et al*. (2001). The range of colour variation within *M. alcathoe* is still unknown but the lack of pigmentation along the base of the ears and hairless areas of the face in *M. alcathoe* appears to be a way of distinguishing between both species from external examination. The length of the forearm also seems to be a useful characteristic for distinguishing *M. alcathoe* from *M. mystacinus*, since their measurements, at least in Iberia, do not overlap (31.4–33.0 mm in *M. alcathoe* and 33.0–34.9 mm in *M. mystacinus*). The validity of other proposed

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diagnostic characters such as relative length of tragus, length of hind feet, length of thumb and length of the claw (Helversen *et al*., 2001; Ruedi *et al*., 2002; Benda *et al*., 2003) could not be checked since most of the bats were captured before the new species was identified.

Helversen *et al*. (2001) suggest habitat segregation between these species in Greece; *M. alcathoe* was more commonly found in small valleys with closed vegetation, whereas *M. mystacinus* was found in forests surrounding large water bodies. Besides, Ruedi *et al*. (2002) found *M. alcathoe* hunting far away from any watercourse. In La Rioja the habitat preferences of both species were found to strongly overlap. In fact, the two species were captured twice in the same net the same night. Sibling species found in Europe are generally sympatric and/or syntopic across large areas (e.g., *Pipistrellus pipistrellus*/*P. pygmaeus*, *M. myotis*/*M. blythii*, etc.). In Iberia, the *M. mystacinus/M. alcathoe* species-complex complies with this pattern even for a very restricted distribution area. In fact, the *mystacinus* group is only found in Iberia in the humid mountain ranges in the northern regions (Fig. 1). *M. mystacinus* sensu stricto is also currently known, apart from La Rioja, from the western mountains of the 'Sistema Central' range. In fact, individuals from these mountains (Gredos and Linares de Riofrío) were molecularly identified by Helversen *et al*. (2001). Nevertheless, more intensive studies are necessary to revise the distribution of this species group in Iberia and to define the ecological requirements of the two species.

The description of *M. alcathoe* and its finding in Iberia, might challenge the subspecies *M. m. occidentalis* described for Iberia by Benda and Tsytsulyna (2000) in their revision of the *mystacinus* group. The little genetic differentiation found between the Iberian and German haplotypes of *M. mystacinus* does not give support to such subspecific recognition of the Iberian population. Nevertheless, as discussed above, mitochondrial DNA seems of little use in clarifying systematics and evolutionary relationships within the *M. mystacinus/M. aurascens* lineage. The measurements of the molecular identified *M. mystacinus* from La Rioja are still larger than the values reported for typical specimens from Central Europe (Benda *et al*., 2003) and this would support the differentiation suggested by Benda *et al*. (2003). Given the overlapping distributions of *M. mystacinus* and *M. alcathoe*, more detailed studies on the genetic and morphological variation patterns across the Western Palaearctic seem necessary to reconstruct the systematics and evolutionary relationships within the group.

The finding of *M. alcathoe* in La Rioja implies the first record of the species for the Iberian Peninsula. This record, along with the recent additions of *Pipistrellus pygmaeus* and *Plecotus macrobullaris* (Barratt *et al*., 1997; Garin *et al*., 2003), gives a total of 28 bat species for the Iberian Peninsula. It also extends the southwestern limit of the range of *M. alcathoe*, a species that was originally proposed as endemic to the Balkans (Helversen *et al*., 2001). Finally, this, and the recent records from France and Slovakia, indicate that this species is far more widely spread across southern Europe than previously thought, although most probably in a discontinuous distribution.

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APPENDIX

Bats of the *mystacinus* group from La Rioja (Spain) used in this study. The specimens that could not be molecularly identified are referred as *M. mystacinus* s.l. Haplotypes AY027835 and AY027847 are from Helversen *et al*. (2001)

Taxon	Locality (date of capture or number of individuals)	Accession No.	DNA ID	Voucher
alcathoe	El Rasillo (07.08.87)	AY027835	Mal 105	EBD16000
alcathoe	Anguiano (01.08.03)	AY027835	Mal 947	Biopsy
alcathoe	Ojacastro (19.06.01)	AY027835	Mal 935	Biopsy
alcathoe	Ojacastro (29.07.03)	AY027835	Mal 944	Biopsy
mystacinus	El Rasillo (07.08.87)	AY552330	Mmy 936	EBD16001
mystacinus	El Rasillo (24.08.88)	AY552331	Mmy 937	EBD17801
mystacinus	El Rasillo (22.08.88)	–	Mmy 104	EBD18289
mystacinus	El Rasillo (02.09.88)	–	Mmy 968	EBD18290
mystacinus	Anguiano (01.08.03)	AY027847	Mmy 945	Biopsy
mystacinus	Anguiano (01.08.03)	AY027847	Mmy 946	Biopsy
mystacinus s.l.	Anguiano (1)			
mystacinus s.l.	Castroviejo (1)	–	–	–
mystacinus s.l.	Ezcaray (3)	–	–	–
mystacinus s.l.	Lumbreras (1)	–	–	–
mystacinus s.l.	Ortigosa de C. (2)	–	–	–
mystacinus s.l.	Villanueva de C. (1)	–	–	–
mystacinus s.l.	Villavelayo (5)	–	–	–
mystacinus s.l.	Villoslada de C. (2)	–	–	–
mystacinus s.l.	Zarzosa (1)	–	–	–

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