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A new species of the *Miniopterus schreibersii* species complex (Chiroptera: Miniopteridae) from the Maghreb Region, North Africa

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Abstract (English)

We used an integrative approach combining cranio-dental characters, mitochondrial and nuclear data and acoustic data to show the presence in the genus *Miniopterus* of a cryptic species from the Maghreb region. This species was previously recognised as *Miniopterus schreibersii* (Kuhl, 1817). *Miniopterus maghrebensis* **sp. nov.** can be differentiated from *M. schreibersii* sensu stricto on the basis of cranial characters and from mitochondrial DNA and microsatellite evidence. Although slight external morphological and acoustic differences were noted between the two species, these criteria alone did not allow reliable species identification from live animals. Based on the specimens identified morphologically and/or genetically, the distribution range of *M. maghrebensis* **sp. nov.** extends from northern Morocco to south of the High Atlas Mountains and northern Tunisia. The new cryptic species is found in sympatry with *M. schreibersii* s.str. near coastal regions of North Africa.

Key words: Bats, cryptic species, echolocation, Mammalia, Morocco

Abstract (French)

Nous avons utilisé une approche intégrative combinant des analyses cranio-dentaires, des marqueurs moléculaires mitochondriaux et nucléaires ainsi que des données acoustiques pour montrer la présence dans le genre *Miniopterus* d'une espèce cryptique en provenance du Maghreb. Cette espèce était auparavant reconnue en tant que *Miniopterus schreibersii* (Kuhl, 1817). *Miniopterus maghrebensis* **sp. nov.** est différencié de *M. schreibersii* sensu stricto sur la base de caractères crâniens ainsi que des marqueurs moléculaires mitochondriaux et des microsatellites. Bien que de petites différences morphologiques externes et acoustiques aient été notées entre les deux espèces, ces critères à eux seuls ne permettent pas d'identifier de manière fiable les animaux sur le terrain. Sur la base d'identifications morphologiques et/ou génétiques de spécimens, *M. maghrebensis* **sp. nov.** s'étend du nord du Maroc jusqu'au sud des montagnes du Haut Atlas et au nord de la Tunisie. Cette nouvelle espèce cryptique est trouvée en sympatrie avec *M. schreibersii* s.str. près des régions côtières d'Afrique du Nord.

Introduction

Species discoveries and descriptions have gone through different phases and since the 1960's the number of mammal species described per year has continually increased, and the 'species accumulation' curve shows no indication of reaching an asymptote (Reeder *et al.*, 2007). The order Chiroptera follows this trend with, for example, 78 species formally described between July 1992 and June 2006 (Reeder *et al.*, 2007). Within the order Chiroptera, the number of species per family varies greatly, from a single recognised species in the Craseonycteridae family (Puechmaille *et al.*, 2011) to over 400 species in the Vespertilionidae family (Simmons, 2005). The latter have recently been split into three families: Miniopteridae (Mein & Tupinier, 1977; Miller-Butterworth *et al.*, 2007), Cistugidae (Lack *et al.*, 2010) and Vespertilionidae s.str. *Miniopterus* is the only genus within the family Miniopteridae, which has been cited for decades as containing the mammal species with the widest distribution on earth *Miniopterus schreibersii* (Kuhl, 1817). Nevertheless, current taxonomic revisions suggest that *M. schreibersii* sensu lato represents a large complex from which several species have been recognized in the last decade. The latest change being the elevation of *M. s. pallidus* Thomas, 1907, as a full species (*M. pallidus*) following morphometric, mitochondrial and nuclear data (Furman *et al.*, 2010; Bilgin *et al.*, 2012). The type locality of *Vespertilio schreibersii* (= *M. schreibersii*) is Colombäzer Höhle (=Kolumbács Cave), on the left bank of the Danube river, near the village of Coronini, Romania (Ansell & Topál, 1976). The latest taxonomic revision restricts the range of *M. schreibersii* to Europe, coastal Anatolia, the Levant, Cyprus, western Transcaucasia and North Africa (Šrámek *et al.*, 2013). In Europe and North Africa (north of the Sahara), there is no other extant *Miniopterus* species known but *M. schreibersii* (Šrámek *et al.*, 2013). It is however important to note that the morphological features in *M. schreibersii* and *M. pallidus* as well as across most members of this genus are strikingly similar; hence, it can be challenging to reliably identify cryptic species based on morphological characteristics only (e.g. Monadjem *et al.*, 2010). As in other bat genera (e.g. *Hipposideros*; Thong *et al.*, 2012a; 2012b), the implementation of molecular techniques in combination with morphological analyses has greatly helped the recognition and description of many cryptic species within *Miniopterus* (e.g. Goodman, 2009; Furman *et al.*, 2010; Goodman *et al.*, 2010; Bilgin *et al.*, 2012; Monadjem *et al.*, 2013; Šrámek *et al.*, 2013).

Our study aims at describing a new species of *Miniopterus* from the Maghreb Region of northern Africa, providing further evidence that *M. schreibersii* s.str. has a narrower distribution range than previously thought.

Material and methods

Morphology. Data and analyses presented in the current study are based on the work and specimens published by Šrámek *et al.* (2013) and further investigations on the same specimens. All the measurements presented here followed the definition reported by Šrámek *et al.* (2013). The 282 examined specimens were collected from throughout the species range, including near the type locality, Kolumbács Cave (Fig. 1). From now on (unless otherwise stated), we refer to *M. schreibersii* s.str. as defined by Šrámek *et al.* (2013), but with the exclusion of *Miniopterus* **sp. nov.** described herein. Museum abbreviations used are: NMP = National Museum in Prague, Czech Republic; EBD = Estación Biológica de Doñana, Sevilla, Spain. Specimen collection for the species description complied with the national laws of Morocco, from where the specimens originated.

Genetic data. Data presented in the current study are based on the work by Bilgin *et al.* (submitted) and further analyses of those data. Mitochondrial DNA sequences used in the present study originated from 12 countries: Russia (n=8), Lebanon (n=7), Cyprus (n=6), Albania (n=6), Romania (n=20; 16 of which from the type locality of *M. schreibersii*), Slovakia (n=9), Slovenia (n=3), Croatia (n=4), France (n=10), Spain (n=5), Morocco (n=22) and Tunisia (n=2) (Fig. 1). A total of 101 sequences of the tRNA Threonine, tRNA Proline and hyper-variable region I of the Control Region (called HV1 hereafter) and 101 sequences of partial Cytochrome *b* (positions 201–734) were obtained as detailed in Bilgin *et al.* (submitted). The HV1 region was amplified and sequenced using primers C and E as described in Wilkinson & Chapman (1991). The partial Cytochrome *b* was amplified using primers Molcit-F (Ibáñez *et al.* 2006) and MVZ-16 (Smith & Patton 1993) as described in Ibáñez *et al.* (2006). For each individual, sequences of the partial Cytochrome *b* fragment and the HV1 were concatenated (total length of 787 bp) and only unique haplotypes were used for further analyses (GenBank accession numbers KJ535784–KJ535823 [Cytochrome *b*] and KJ535824–KJ535861 [HV1]). A phylogenetic reconstruction was undertaken in BEAST v1.8.0 (Drummond & Rambaut, 2007) with strict molecular clock, Yule process as tree

prior, and UPGMA tree as starting tree. The HKY+I model of sequence evolution was used as selected by the Akaike Information Criterion in jModeltest2 (Darriba *et al.*, 2012). The program was run for 10 million generations and sampled every 500, of which 10% were discarded as burn-in. Effective sample sizes values were all > 4000 for all parameters, suggesting the MCMC run was sufficient to obtain valid estimates of the parameters. Four replicate analyses were carried with a different random seed number to ensure convergence on a similar tree topology and the results were then pooled. Sequence divergence was estimated between groups of interest using the K2P distance as calculated in MEGA 5.2 (Tamura *et al.*, 2011).

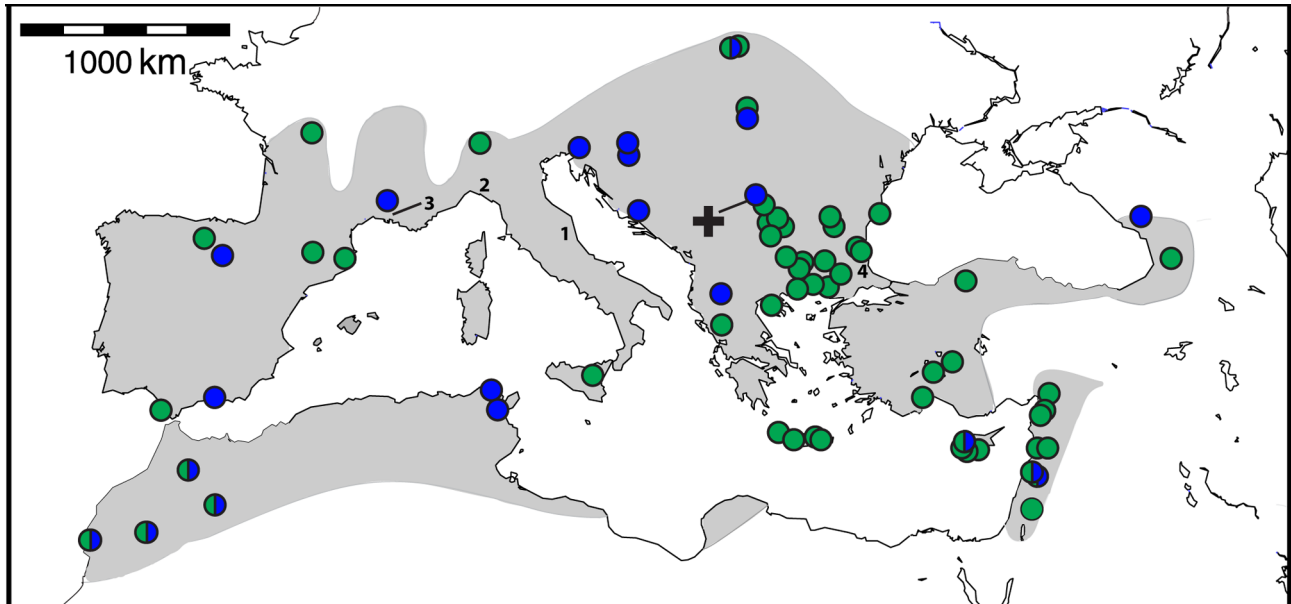


FIGURE 1. Map showing the origin of specimens and samples investigated in this study with coloured shading delimiting the approximate distribution of *M. schreibersii* s.str. as recognised prior to our work (cf. Šrámek *et al.*, 2013). Coloured dots represent localities from which we analysed morphological (green dot) or genetic data (blue dot), or both data types combined (dots half green and half blue). The type locality of *Vespertilio schreibersii* (= *M. schreibersii* s.str.) in Romania is indicated by a cross. The type locality of other previously described species/subspecies of *Minioproterus* from Europe and North Africa are represented by numbers: 1: *Vespertilio ursinii* Bonaparte, 1837; 2: *M. s. italicus* Dal Piaz, 1926; 3: *M. s. baussencis* Laurent, 1944; 4: *M. s. inexpectatus* Heinrich, 1936 (cf. main text for further details).

A set of six microsatellite loci was genotyped for 58 individuals from Iberia and North Africa (26 belonging to the putative new species). Hardy-Weinberg and linkage equilibrium were checked in Genepop 4.0.10 (Rousset, 2008) and a sequential Bonferroni correction was applied to take into consideration multiple testing (Rice, 1989). Differentiation (F_{st}) between sampled colonies (*M. schreibersii*: Spain, Win-Timdouine; *Minioproterus* sp. nov.: Win-Timdouine [+Talmat], Kef Azigza Cave) was calculated and its significance tested in Genepop. As mitochondrial DNA results and F_{st} calculations suggested the presence of two taxa (cf. Results), we further investigated and characterised the extent of genetic differentiation between these two taxa by performing multivariate and Bayesian analyses. Principal component analysis (PCA) is a powerful tool to summarize multivariate (multiple loci and multiple alleles) genetic information into a few synthetic variables also called principal components, with the first components explaining the highest proportion of variance. These methods do not require assumptions about an underlying genetic model, such as the Hardy-Weinberg or linkage equilibriums (Jombart *et al.*, 2009). Only individuals with data for at least five loci ($n=56$) were used to run a principal component analysis using the adegenet package v1.3-9.2 (Jombart, 2008) in R v3.0.2 (R Development Core Team, 2013). For the PCA, our data set was combined with a data set of eight individuals of *M. schreibersii* s.str. from Turkey (Bilgin *et al.*, 2012). To investigate whether hybridisation is occurring between the two taxa, we used a model-based Bayesian statistical technique implemented in STRUCTURE v 2.3.4 (Pritchard *et al.*, 2000). With K set to two, individuals were probabilistically assigned to taxa, or in the case of admixed ancestry, to both parental taxa. We ran the model with the length of the burn-in and MCMC (Markov chain Monte Carlo) chains of 50000 each. We used the admixture model and the correlated allele frequency between populations (Falush *et al.*, 2003). Results presented are based on the average of ten runs.

Acoustic data. Calls were recorded from hand released individuals genetically identified in Morocco (*Miniopterus sp. nov.*; n = 2; Win-Timdouine [30°37' N, 9°28' E, 756 m a.s.l.] and Oued Emi Oggoug [30°40' N, 9°20' E, 1348 m a.s.l.]) and from non-genetically identified individuals in France (n=13; Grotte de Saint-Joseph [43°55' N, 4°20' E, 136 m a.s.l.]) where only *M. schreibersii* s.str. is present (Šrámek *et al.*, 2013; this study). Multiple calls per individual were processed through a Fast Fourier Transformation (1024 points, Hamming window, fast Fourier transformations calculated with 98.43% time overlap; software Avisoft-SASLab Pro version 5.2.07, Avisoft Bioacoustics, Germany). The detection of calls in the recordings, the time at the start and the end of the call were determined via the 'three thresholds' algorithm with thresholds set to -24 dB, -20 dB and -10 dB respectively. Peak frequency was characterized as the frequency at the location of the pulse with maximum amplitude. Start frequency was characterised as the frequency where the amplitude goes first below -5 dB from the peak frequency at the start of the call (e.g. when moving in reverse from the maximum up to higher frequencies). Similarly, end frequency was characterised as the frequency where the amplitude goes first below -5 dB from the peak frequency at the end of the call (e.g. when moving from the maximum down to lower frequencies). The interval between pulses was measured as the time elapsed from the start of the preceding to the start of the current pulse. All calculations were processed in R 3.0.2 (R Development Core Team, 2013).

Results

Morphology. *M. schreibersii* s.str. and *Miniopterus sp. nov.* are generally similar for the measurements investigated (cf. Šrámek *et al.*, 2013) (Figs. 2, 3, 4, 5). The main differences observed are described hereafter. The rostrum of *Miniopterus sp. nov.* is wide, the width across the infraorbital foramen is large, 4.0–4.4 mm; the relative width of rostrum (infraorbital width of rostrum / largest length of skull) is larger in comparison to *M. schreibersii* s.str.: 0.27–0.28 in *Miniopterus sp. nov.* versus 0.24–0.27 in *M. schreibersii* s.str. from Europe and the Levant. In *Miniopterus sp. nov.*, the relative height of the braincase (largest height of braincase / largest length of skull) is large in comparison to *M. schreibersii* s.str.: 0.51–0.53 in *Miniopterus sp. nov.* versus 0.45–0.52 in *M. schreibersii* s.str. from Europe and the Levant. Mandibular molar-row is relatively short in *Miniopterus sp. nov.*: the relative length (lower molar-row / mandible length) is 0.326–0.331 (while 0.336–0.359 in *M. schreibersii* s.str.). *Miniopterus sp. nov.* has very distinct tubercles on the dorsal surface of the ramus mandibulae between the third molar and the coronoid process and on the zygomatic arches (cf. Fig. 3). Canines in *Miniopterus sp. nov.* are relatively wide (canine width / canine length) compared to *M. schreibersii* s.str.: upper canines 0.790–0.902 (mean = 0.867) and lower canines 1.049–1.214 (mean = 1.142) in *Miniopterus sp. nov.* versus upper canines 0.685–1.070 (mean = 0.796) and lower canines 0.871–1.320 (mean = 1.101) in *M. schreibersii* s.str. from Europe and the Levant. Premolars are more robust in *Miniopterus sp. nov.* than in *M. schreibersii* s.str. (Fig. 5). Third lower molars are relatively short in *Miniopterus sp. nov.*, their relative length (third lower molar length / third lower molar width) is 1.643–2.041 (mean = 1.834) (while 1.457–2.250 [mean = 1.955] in *M. schreibersii* s.str. from Europe and the Levant). The cingulum on the palatal side of the first upper molar has a very distinctive concave fold between the protoconus and the hypoconus.

Genetic data. At the mitochondrial DNA level, all samples (n=80 sequences) from Russia, Lebanon, Cyprus, Albania, Romania, Slovakia, Slovenia, Croatia, France, Spain plus a subset of samples from Morocco and Tunisia (n=11 sequences) formed a well supported monophyletic clade (n=30 haplotypes) (Fig. 6). These include 10 sequences from the type locality of *M. schreibersii* in southeast Romania and are therefore referred to as *M. schreibersii* s.str. The Moroccan and Tunisian samples grouping in the *M. schreibersii* s.str. clade originated from sites within ca. 40 km from the coastal line. A second monophyletic and strongly supported clade (Bayesian posterior probability=1) was composed of 10 sequences representing four haplotypes. These 10 sequences all originated from individuals from Moroccan and Tunisian sites situated further than ca. 40 km from the coastal line. This second clade corresponds to *Miniopterus sp. nov.* The average K2P distance between *M. schreibersii* s.str. and *Miniopterus sp. nov.* sequences was 1.2%.

Nuclear DNA. All colonies were in Hardy-Weinberg equilibrium and no linkage was detected between any locus at any colony (Fisher's method; P>0.05). The Fst values between the four colonies investigated were all significantly different from zero (Fst range: 0.1–0.49, Exact G test, P<0.001) except between the two *Miniopterus sp. nov.* colonies (Win-Timdouine [+Talmat] versus Kef Azigza Cave, Fst=0.001, P=0.67). The Fst between the

Miniopterus **sp. nov.** and *M. schreibersii* s.str. colonies were all highly significant (Exact G test, $P < 0.001$) and ranged between 0.44 and 0.49, including when both species were found in syntopy at Win-Timdouine (Fst=0.44, Exact G test, $P < 0.001$).

The principal component analysis clearly separated all genotyped individuals belonging to *Miniopterus* **sp. nov.** from those belonging to *M. schreibersii* s.str. along axis 1 (explaining 36.3% of the variance; Fig. 7). *Miniopterus schreibersii* s.str. from Morocco and Iberia are closer to Turkish *M. schreibersii* s.str. (mostly differentiated along axis 2, explaining 10.7% of the variance) than they are to *Miniopterus* **sp. nov.** from Morocco. *Miniopterus schreibersii* s.str. individuals from Win-Timdouine (light blue triangles in Fig. 7) group with *M. schreibersii* s.str. individuals from Iberia (red triangles in Fig. 7) and were very clearly differentiated from syntopic *Miniopterus* **sp. nov.** (light blue dots in Fig. 7), further demonstrating the presence of two species. The absence of intermediate individuals in Fig. 7 and complete agreement between mitochondrial and nuclear DNA suggest that there were no hybrids between the two species in our data set.

The results of the model-based Bayesian analysis were in full agreement with the results of the PCA and showed consistently across the five runs (data not shown) that the two species are clearly differentiated, with individuals of both species *M. schreibersii* s.str. and *Miniopterus* **sp. nov.** having a high median posterior probability (0.9947 and 0.995 respectively) to belong to their respective species (Fig. 8). Three individuals of *M. schreibersii* s.str. (two from Win-Timdouine and one from Spain) had a posterior probability to belong to *M. schreibersii* s.str., which was slightly lower than others (range: 0.89–0.96); these could be signs of past hybridisation or more likely of a recent common ancestry. Hence all the genetic analyses are congruent with each other and provide strong evidence of two isolated and differentiated gene pools which are represented by *Miniopterus schreibersii* s.str. and *Miniopterus* **sp. nov.**

Acoustic data. The calls ($n=43$) of the hand released *Miniopterus* **sp. nov.** individuals ($n=2$; Win-Timdouine & Oued Emi Oggoug) were typical frequency modulated *Miniopterus* calls with low duty cycle (Fig. 9). The peak frequency globally varied from 51 to 55 kHz with call duration from 3.2 to 5.6 ms while start and end frequency averaged 74.69 and 50.36 kHz respectively (Table 1). Although the data set of *Miniopterus* **sp. nov.** from Morocco contained recordings from only two individuals, the temporal and frequency parameters of the calls largely overlapped with call parameters from 13 individuals of *M. schreibersii* s.str. recorded in southern France. Although the number of calls and recordings analysed here was limited, both in terms of numbers and geographic coverage, we did not detect any obvious differences between the calls of *Miniopterus* **sp. nov.** and those of *M. schreibersii* s.str.

Systematics

Miniopterus maghrebensis Puechmaille, Allegrini, Benda, Bilgin, Ibañez & Juste **nov. sp.**

Holotype. Adult ♂ (NMP 94426, field number pb3907 [specimen in alcohol with skull extracted], Kef Azigza Cave, Ksar Tazougart, Morocco, 26 April 2008, leg. P. Benda, J. Červený, A. Konečný and P. Vallo.

Paratypes. 1 ♂ (EBD 25780 [specimen in alcohol]), Kef Azigza Cave, 30 April 2000, leg. C. Ibañez, J. Juste, J.A. Garrido and J. Quetglas;—4 ♂♂, 7 ♀♀ (NMP 94506–94512, 94514, 94515 [specimens in alcohol with skulls extracted], 94505, 94513 [specimens in alcohol]), Kef Azigza Cave, 26 April 2008, leg. P. Benda, J. Červený, A. Konečný and P. Vallo;—1 ♀ (NMP 90103 [specimen in alcohol with skull extracted]), Oued El Ammar, Sebtes-Âit-Serhrouchen, Morocco, 9 September 2003, leg. P. Benda;—2 ♂♂ (NMP 90047 [specimens in alcohol], 90051 [specimen in alcohol with skull extracted]), Oued Tessaoud, Talknout, Morocco, 30 August 2003, leg. P. Benda.

Type Locality. Kef Azigza Cave (or Tazzouguert Cave), 5.7 km S of Ksar Tazougart, 19 km W-NW of Boudenib, Er Rachidiyah Province, Morocco (32° 01' 46.6" N, 03° 47' 16.7" W, 1060 m a. s. l.); for a detailed description of the site see Aulagnier and Destre (1985).

Description and Diagnosis. *Miniopterus maghrebensis* **sp. nov.** is a medium-sized member of the genus *Miniopterus* Bonaparte, 1837 in most respects similar to *M. schreibersii* (Kuhl, 1817) s.str. (Figs. 2, 3, 4, 5). Forearm length 45–48 mm, condylobasal length of skull 14.7–15.3 mm, zygomatic width of skull 8.4–9.1 mm, length of upper tooth-row (canine to third molar, incl.) 5.7–6.2 mm (for all measurements, $n = 25$).

The dorsal pelage in *M. maghrebensis* **sp. nov.** is chestnut brown to dark greyish-brown, the ventral pelage is pale brown (Fig. 2). Ventral hairs are bicoloured, dark brown on the proximal parts, pale brown to buffy in the

distal parts. The dorsal skin of ears and the naked parts of face are pale greyish-brown, the ventral skin of ears is almost without pigments, fleshy pale to pinkish (Fig. 2). Wing membranes are dark brown to dark greyish-brown. Based on the skull size (cf. Fig. 3), *M. maghrebensis* **sp. nov.** conforms to the Central European representatives of *M. schreibersii* s.str., however, is slightly larger than most of the south-European and Levantine specimens, exceeding them on average by ca. 0.3–0.5 mm in largest skull measurements. The rostrum of *M. maghrebensis* **sp. nov.** is wide, the width across the infraorbital foramina is large, 4.0–4.4 mm; the relative width of rostrum (infraorbital width of rostrum / largest length of skull) is very large in comparison to *M. schreibersii* s.str.: 0.27–0.28 in *M. maghrebensis* **sp. nov.** versus 0.24–0.27 in *M. schreibersii* s.str. from Europe and the Levant. The braincase in *M. maghrebensis* **sp. nov.** is high, the height of braincase (incl. the tympanic bullae) 7.7–8.4 mm; the relative height of braincase (largest height of braincase / largest length of skull) is large in comparison to *M. schreibersii* s.str.: 0.51–0.53 in *M. maghrebensis* **sp. nov.** versus 0.45–0.52 in *M. schreibersii* s.str. from Europe and the Levant. Mandibular molar-row is relatively short in *M. maghrebensis* **sp. nov.**, its relative length (lower molar-row / mandible length) is 0.326–0.331 (while 0.336–0.359 in *M. schreibersii* s.str. from Europe and the Levant). *M. maghrebensis* **sp. nov.** has very distinct tubercle on the dorsal surface of the ramus mandibulae between the third molar and the coronoid process and on the zygomatic arches (cf. Fig. 3 & Šrámek *et al.*, 2013).

TABLE 1. Temporal and frequency parameters of *Miniopterus maghrebensis* **sp. nov.** (Win-Timdouine and Oued Emi Oggoug, Morocco) and *M. schreibersii* s.str. (Grotte de Saint-Joseph, France) echolocation calls recorded upon release of bats in an open habitat. Mean, median, minimum, maximum and standard deviation (s.d.) of each parameter are presented.

Species		<i>M. sp. nov.</i>	<i>M. sch</i>
No. calls (recordings)		42 (2)	118(13)
Peak frequency	Mean	52.89	53.29
	Median	52.77	53.02
	Minimum	51.00	50.41
	Maximum	54.78	58.48
	s.d.	0.74	1.33
Start frequency	Mean	74.69	77.99
	Median	74.12	78.75
	Minimum	63.57	60.00
	Maximum	101.07	95.25
	s.d.	7.66	7.12
End frequency	Mean	50.36	51.62
	Median	50.39	51.75
	Minimum	48.92	50.25
	Maximum	51.85	52.88
	s.d.	0.81	0.65
Call duration	Mean	4.48	6.13
	Median	4.72	6.04
	Minimum	3.20	3.20
	Maximum	5.60	9.70
	s.d.	0.67	1.21
IBP	Mean	107.14	97.75
	Median	108.96	93.29
	Minimum	67.20	58.20
	Maximum	239.20	192.41
	s.d.	27.35	25.93

*IBP: Interval Between Pulses.



FIGURE 2. Photographs of *Miniopterus maghrebensis* **sp. nov.** individuals from the type locality Kef Azigza Cave, showing the typical appearance of the species. Note that a) was photographed with day light and a flash while b) was photographed during night time with a flash, hence the apparent pelage colour differences (photographs by Jaroslav Červený & Antonín Reiter respectively).

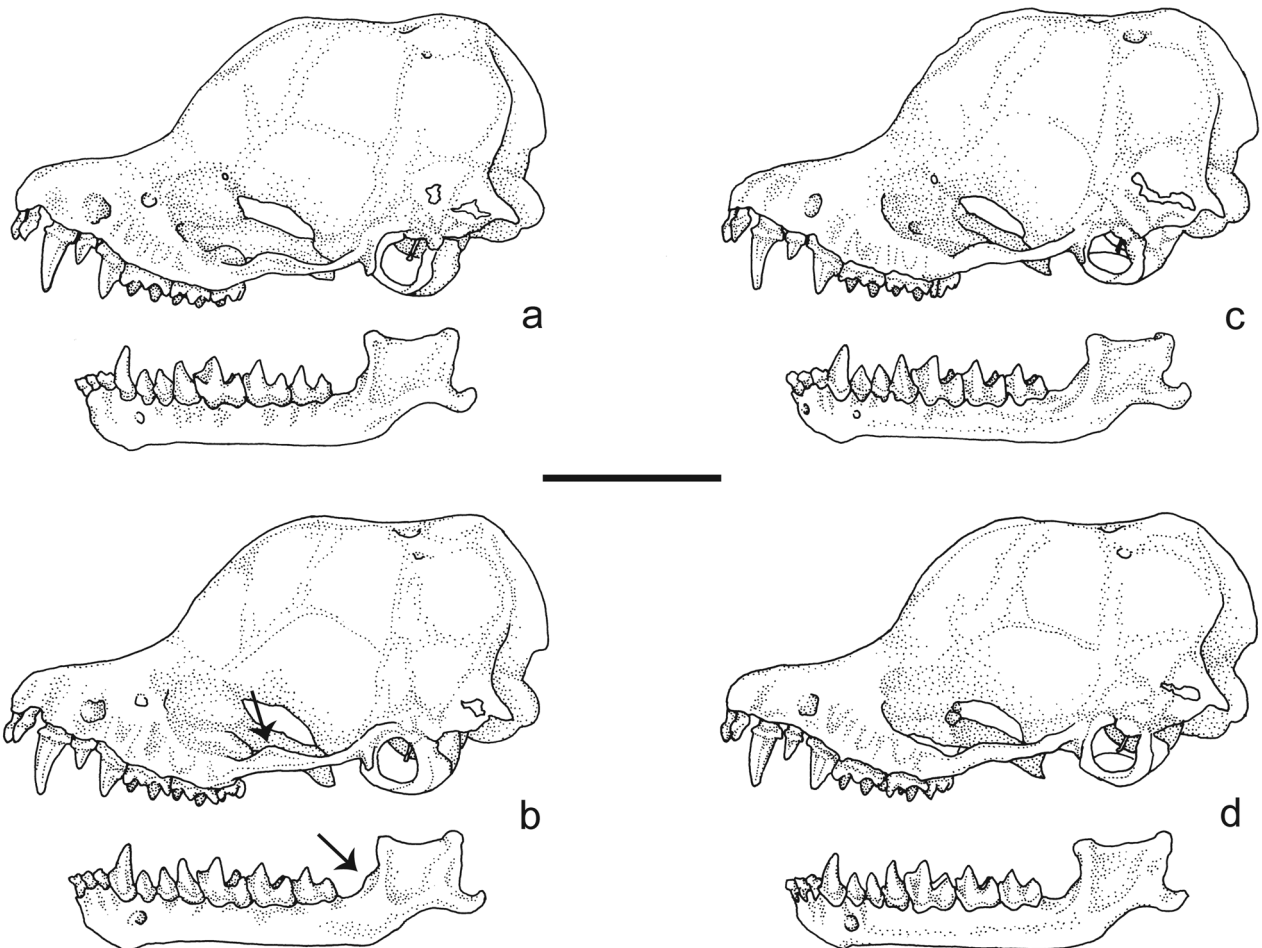


FIGURE 3. Lateral views on skulls and mandibles of *Miniopterus maghrebensis* **sp. nov.** and *Miniopterus schreibersii* (Kuhl, 1817). Legend: a—*Miniopterus maghrebensis* **sp. nov.**, holotype, NMP 94426, Kef Azigza Cave, Morocco; b—*Miniopterus maghrebensis* **sp. nov.**, paratype, NMP 94506, Kef Azigza Cave, Morocco; c—*M. schreibersii*, NMP 94558, Betfia Cave, Romania; d—*M. schreibersii*, NMP 94560, Betfia Cave, Romania. The arrows point to the very distinct tubercles on the zygomatic arch (top arrow) and on the dorsal surface of the ramus mandibulae of *Miniopterus maghrebensis* **sp. nov.** (bottom arrow). Scale bar—5 mm.

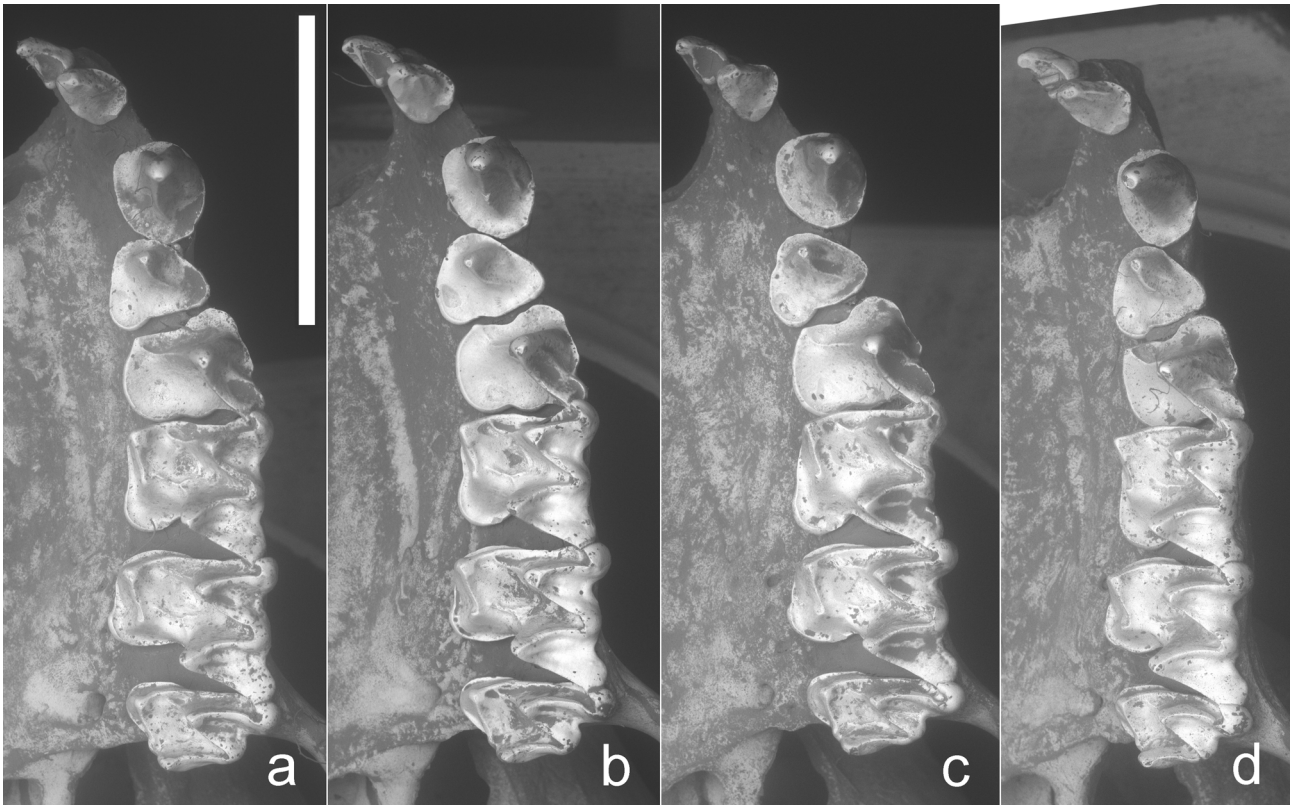


FIGURE 4. Occlusal views on the upper tooth-rows of *Miniopterus maghrebensis* **sp. nov.** and *M. schreibersii* (Kuhl, 1817). For legend see Fig. 3. Scale bar—3 mm.

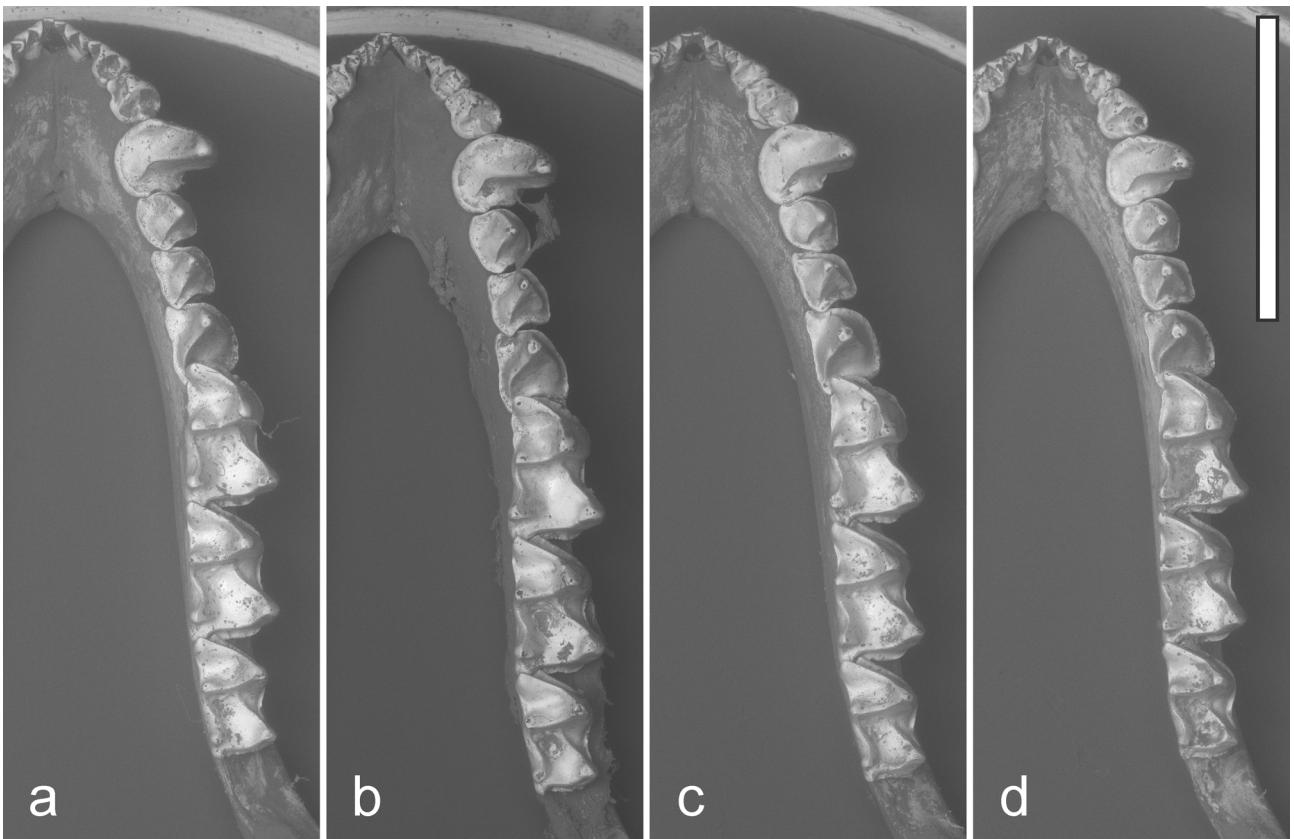


FIGURE 5. Occlusal views on the lower tooth-rows of *Miniopterus maghrebensis* **sp. nov.** and *M. schreibersii* (Kuhl, 1817). For legend see Fig. 3. Scale bar—3 mm.

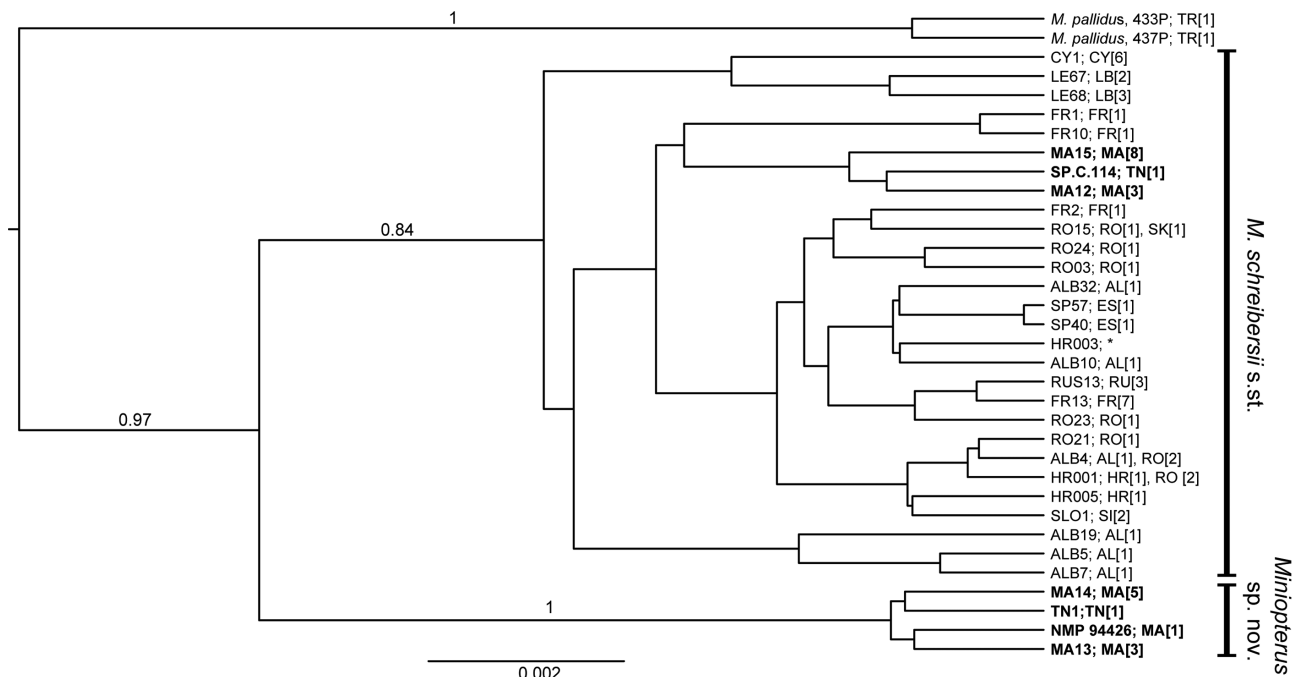


FIGURE 6. Bayesian consensus tree of mitochondrial DNA showing the phylogenetic relationship between individuals of *Miniopterus maghrebensis* sp. nov. and *M. schreibersii* (Kuhl, 1817). Tip of labels are composed of haplotype name followed by the abbreviated two letter country name with the number of samples having this haplotype in square brackets. * HR[2], RO[12], RU[5], SK[8], SI[1], ES[3]. NMP 94426 is the type specimen of *Miniopterus maghrebensis* sp. nov. Haplotypes from North Africa are in bold face, irrespective of species identity. Abbreviated country names are as follows: AL=Albania, CY=Cyprus, HR=Croatia, ES=Spain, FR=France, LE=Lebanon, MA=Morocco, RO=Romania, RU=Russia, SI=Slovenia, SK=Slovakia, TN=Tunisia, TR=Turkey.

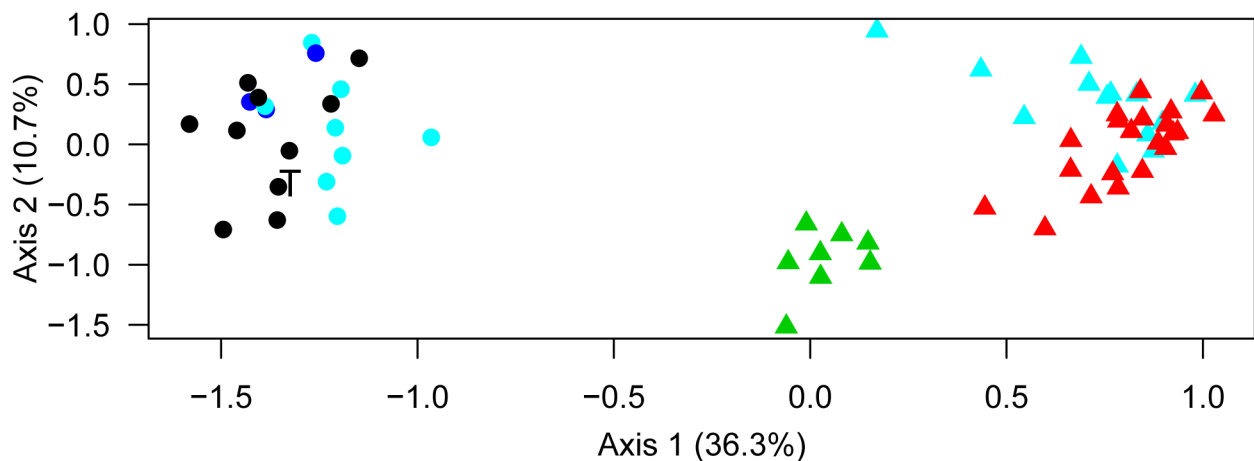


FIGURE 7. Principal component analysis (PCA) showing the clear separation of *Miniopterus maghrebensis* sp. nov. (filled circles; type specimen NMP 94426 denoted by a "T") and *M. schreibersii* (Kuhl, 1817) (triangles) based on individuals genotyped at six microsatellite loci. Marks of the same colour indicate individuals sampled at the same location (irrespective of species); Red: Iberia, light blue: Win-Timdouine, dark blue: Talmat, black: Kef Azigza Cave, green: Turkey.

Compared to *M. schreibersii* s.str. from Europe and the Levant (see Šrámek *et al.*, 2013) unicuspidal teeth are more robust in *M. maghrebensis* sp. nov. The cingulum on the palatal side of the first upper molar has a very distinctive concave fold between the protoconus and the hypoconus in *M. maghrebensis* sp. nov.

Measurements of the Holotype (in mm). **External:** Forearm length 45.8; head and body length 60; tail length 63; ear length 13.4; tragus length 6.3. **Cranial:** greatest length 15.15; condylobasal length 15.12; zygomatic width 8.59; width of interorbital constriction 3.58; neurocranium width 8.17; neurocranium height 6.31; largest horizontal diameter of tympanic bulla 2.97; rostrum width across upper canines 4.53; rostral width across third

upper molars 6.27; length of upper tooth-row (canine to third molar, incl.) 5.93; condylar length of mandible 10.74; coronoid height of mandible 2.57; length of lower tooth-row (canine to third molar, incl.) 6.26.

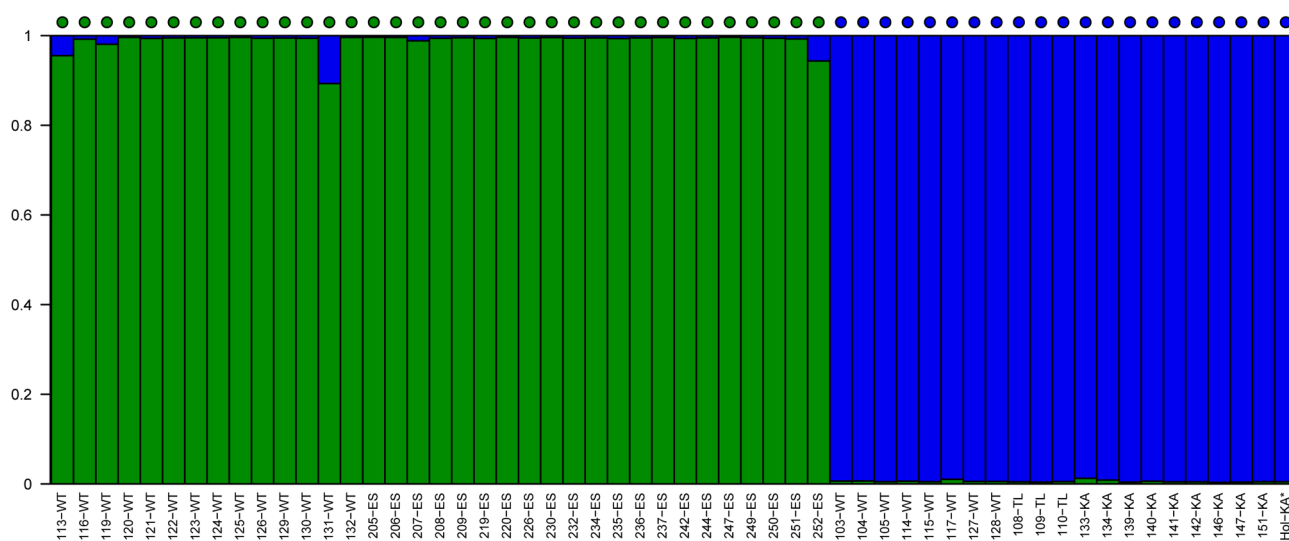


FIGURE 8. Bar plot depicting the posterior probability of assignment of individuals (vertical bars), based on a model-based Bayesian analysis (STRUCTURE), to either *M. schreibersii* s.str. (green) or *Miniopterus maghrebensis* sp. nov. (blue). The circle above each bar represents the mitochondrial DNA sequence of the individual grouping in the *M. schreibersii* s.str. clade (green) or *Miniopterus maghrebensis* sp. nov. (blue) clade. The individual names followed by the site of origin are presented on the x-axis. WT=Win-Timdouine, ES=Spain, TL=Talimat, KA= Kef Azigza Cave. * type specimen of *Miniopterus maghrebensis* sp. nov.

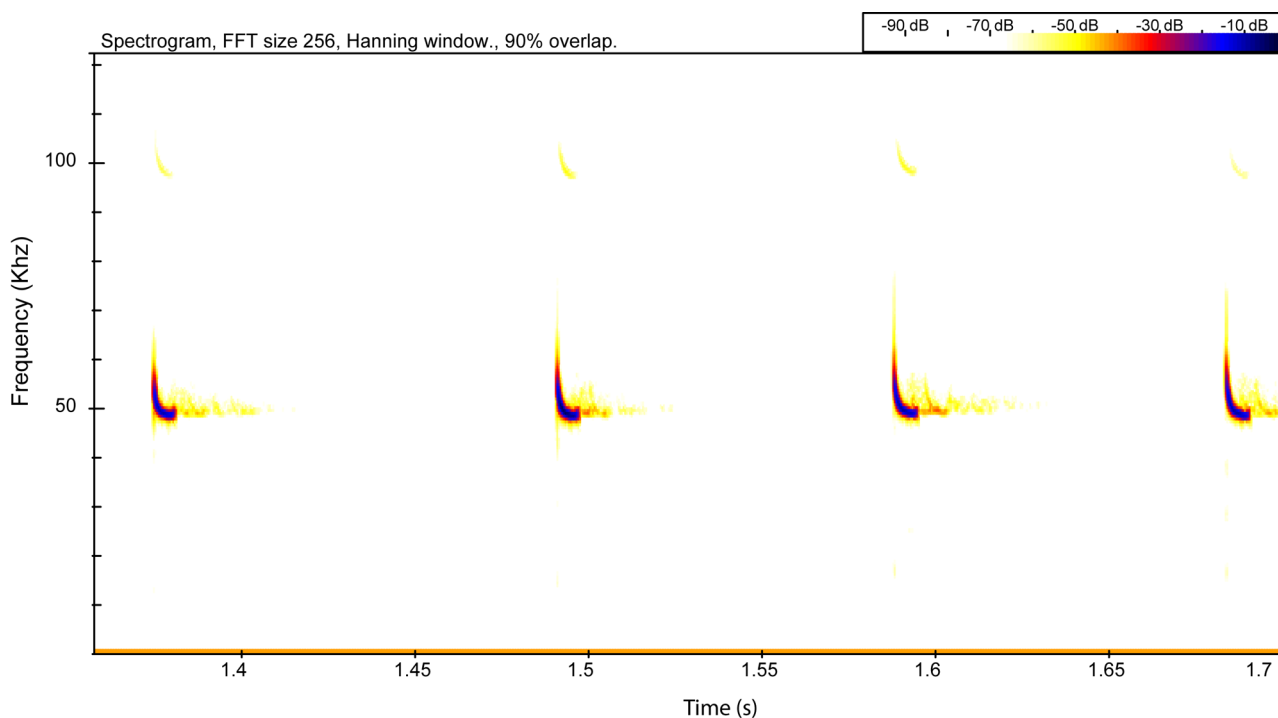


FIGURE 9. Sequence of frequency modulated calls typical of *Miniopterus maghrebensis* sp. nov. (recorded at Win-Timdouine, Morocco).

Genetic Characters. Mitochondrial DNA. The GenBank sequence (partial Cytochrome *b*, tRNA-Threonine, tRNA-Proline, HV1) of the holotype corresponds to the accession numbers KJ535784 & KJ535824. *M. maghrebensis* sp. nov. and *M. schreibersii* s.str. are sister groups, each species forming a strongly supported monophyletic clade (Fig. 6). The average K2P distance between *M. schreibersii* s.str. and *Miniopterus maghrebensis* sp. nov. sequences was 1.2%. **Nuclear DNA.** The microsatellite data are congruent with the

mitochondrial DNA signal and provide clear evidence of two strongly isolated and differentiated gene pools which are represented by *Miniopterus schreibersii* s.str. and *M. maghrebensis* sp. nov. (Figs. 7 & 8). The *F_{st}* between the *Miniopterus maghrebensis* sp. nov. and *M. schreibersii* s.str. colonies ranged between 0.44 and 0.49 (significant values).

Etymology. The name *maghrebensis* refers to the region (the Maghreb; the region of northern Africa located between the Atlantic Ocean, the Mediterranean Sea, and the Sahara) where the new species was discovered. The proposed vernacular names are Maghrebian bent-wing bat (English), Minioptère du Maghreb (French), Murciélago de cueva magrebí (Spanish), létavec maghrebský (Czech), لخباشا المغاربي ذو لأجنحة المطوية (Arabic) ⵜ ⵓ ⴱ ⵏ ⵍⴰⵎⵖⵔⵉ ⴰⵎⵖⵔⵉⴱⵉ ⵏ ⵓⵏ ⵔⵉⵎⴰⵏⵏⵉⵙⴰⵏⵉ (Berber).

Distribution. Genetic analyses confirmed its presence from northern Morocco to south of the High Atlas Mountains and northern Tunisia (Fig. 10) (Šrámek *et al.*, 2013; Puechmaille *et al.*, 2012b; this study); however, these individuals were originally referred to as *M. schreibersii*. Based on these data and the current extent of *Miniopterus* distribution in the region (Aulagnier & Destre, 1986; Kowalski & RzebiK-Kowalska, 1991; Dieuleveut *et al.*, 2010; Puechmaille *et al.*, 2012b), we present a hypothetical distribution map of both species. Nevertheless, all previous records of *M. schreibersii* from the Maghreb need to be re-evaluated as they could potentially belong to *M. maghrebensis* sp. nov. since both species are found in the region (Bilgin *et al.*, submitted).

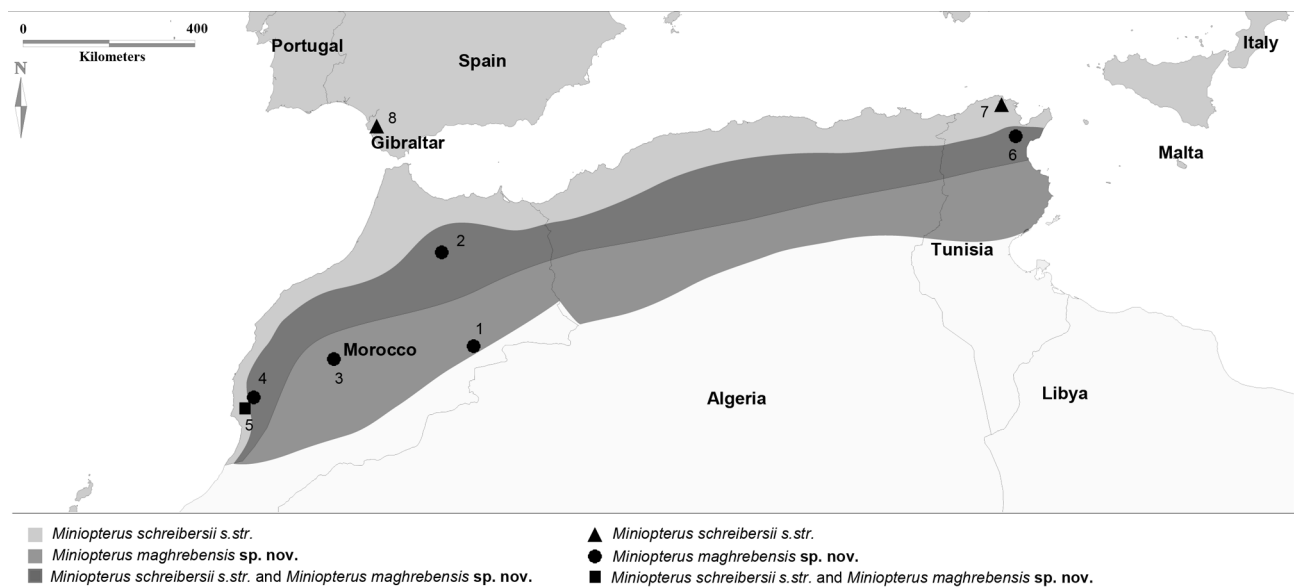


FIGURE 10. Hypothetical distribution maps of *Miniopterus maghrebensis* sp. nov. and *M. schreibersii* s.str. (Kuhl, 1817). Sites with confirmed species identifications are: 1: Kef Azigza Cave (or Tazzouguert Cave); 2: Oued El Ammar, Sebts-es-Âit-Serhrouchèn; 3: Oued Tessaoud, Talknout; 4: Talmat; 5: Win-Timdouine & Oued Emi Oggoug; 6: Zaghouan mine; 7: Ichkeul National Park; 8: Iberia.

Discussion

The type locality of *M. s. schreibersii* is Kolumbács Cave, left bank of the River Danube, near Coronini, Romania; sensu Ansell & Topál (1976). All the *Miniopterus* specimens genetically characterised from the type locality and the surrounding region (South-eastern Europe) fall within the same clade. Additionally, all the specimens morphologically investigated from near the type locality in Romania/Bulgaria and from South-eastern Europe are morphologically similar, hence we consider these specimens as representing *M. schreibersii* s.str. The species is distributed in Europe, coastal Anatolia, the Levant, Cyprus, western Transcaucasia, and coastal North Africa. We reviewed the literature to identify earlier possible descriptions for a new *Miniopterus* species from the Maghreb. However, as all previous authors had agreed that the Maghrebian populations belong to the nominotypical form, a previously proposed species name does not appear to be available. The only subspecies described that are somewhat geographically close to the Maghreb are *Vespertilio ursinii* Bonaparte, 1837 (type locality [t.l.] Monte Corno, Ascoli, Italy), *Miniopterus schreibersii italicus* Dal Piaz, 1926 (t.l. Arma del Frate, Liguria, Italy), *M. schreibersii baussencis* Laurent, 1944 (t.l. Grotte des Fées, Beaux de Provence, France), and *Miniopterus*

schreibersii inexpectatus Heinrich, 1936 (t.l. Stranja Mts., SE Bulgaria), all of the above being considered synonyms of *Vespertilio schreibersii* Kuhl, 1817 (t.l. Kolumbács Cave, SW Romania [Ansell & Topál 1976]) = *M. schreibersii schreibersii* (Simmons, 2005). Gunnell *et al.* (2011) described a fossil *M. horaceki* from the late Pliocene (2.5 Ma) from Ahl al Oughlam near Casablanca (Morocco) but this species is distinct from the *M. schreibersii* group based on both skull and tooth size.

Although *M. maghrebensis* **sp. nov.** can be diagnosed based on cranio-dental characters as well as genetic evidence, it may prove difficult to diagnose the species in the field from live animals. Such a situation is rather common in bats as many cryptic species are only diagnosable in the field with subtle qualitative differences in external characters as it is the case in Europe with *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus* or *Plecotus* species (Dietz *et al.*, 2009). Furthermore, the Miniopteridae family is notorious for its morphological stasis and having species with very similar morphology (Yoshiyuki, 1989), hence more research is necessary to study the external morphology of *M. schreibersii* and *M. maghrebensis* **sp. nov.**, especially in areas of sympatry or parapatry to identify reliable diagnostic field characters. It is not unusual to have closely related bat species showing divergence in echolocation calls (Douangboubpha *et al.*, 2010; Ith *et al.*, 2011; Puechmaille *et al.*, 2011; Thong *et al.*, 2012b) and those differences can be used to discriminate species (Walters *et al.*, 2012). Nevertheless, our analyses of echolocation calls from a limited number of individuals tend to suggest that *M. maghrebensis* **sp. nov.** and *M. schreibersii* s.str. have very similar echolocation calls, which would render their acoustic identification to species level difficult. It is however important to mention that the recordings were only from hand released individuals (which was necessary to confirm species identification), hence we only obtained a small portion of both species' vocal repertoire. Upon hand release, bats generally tend to emit short steep frequency modulated (FM) calls that are more typical of animals flying in cluttered habitat. These calls are generally the most difficult to identify to species as they show greater overlap between species than calls emitted when animals are flying in the open and producing shallow FM or quasi-constant frequency calls (Barataud, 2012). More research is needed to characterize the echolocation call repertoire of *M. maghrebensis* **sp. nov.** in greater detail and to search for diagnostic features that would differentiate it from *M. schreibersii* s.str.

Although the percentage of mitochondrial DNA divergence between the two species (K2P distance: 1.2% for Cytochrome *b*) is low, it is within the range of values previously reported for different species of mammals (Hebert *et al.*, 2003), including bats (Clare *et al.*, 2007; Francis *et al.*, 2010). Some bat species distinct at the morphological level show low inter-specific divergence at mitochondrial DNA genes (<1% for COI; e.g. *Myotis annamiticus* and *M. laniger*; Francis *et al.*, 2010), others share the same mitochondrial DNA despite being sympatric and having clear differences in their nuclear genome (e.g. *Myotis myotis* and *M. blythii*; Berthier *et al.*, 2006), while others are paraphyletic based on mitochondrial DNA (e.g. *Hipposideros armiger*; Thong *et al.*, 2012b). Intra-specific sequence divergence values below 2 to 5% have been commonly reported in the literature for mitochondrial DNA genes, but it should be emphasized that these values are only indicative guidelines, as it is clear that there is no biologically meaningful cut-off value separating intra- and inter-specific variability for any taxonomic group (e.g. Baker & Bradley, 2006; Luo *et al.*, 2011). Importantly, and in addition to the mitochondrial DNA divergence between *M. schreibersii* s.str. and *M. maghrebensis* **sp. nov.**, the set of six microsatellite loci provide evidence that the two species have separated and isolated gene pools, even when found in sympatry. North African *M. schreibersii* s.str. are genetically (at mitochondrial and nuclear levels) more closely related to Turkish conspecifics (situated >4000 km away) than they are to syntopic *M. maghrebensis* **sp. nov.** The absence of intermediate individuals between the *M. schreibersii* s.str. from Turkey and conspecifics from Iberia and North Africa in the PCA plot is very likely due to the absence of samples from across Europe in our study, which would be expected to fill this gap. The non-significant *F*_{st} between *M. maghrebensis* **sp. nov.** colonies found ca. 560 km apart (Win-Timdouine [+Talmat] versus Kef Azigza Cave) strongly suggests that the colonies we studied are not isolated from each other but represent a rather homogeneous gene pool. Hence although our sampling might not cover the entire range of *M. maghrebensis* **sp. nov.**, it is likely that the species presents a rather homogeneous gene pool across its range. The absence of detected gene flow between the two species, hence the integrity of their respective gene pools, combined with homogeneous gene pools within species over large distances clearly demonstrate the presence of two separate species according to the biological species definition proposed by Mayr (1996): "*Species are groups of interbreeding natural populations that are reproductively isolated from other such groups*". In our case, the isolating mechanism by which reproductive isolation is effected is unknown, but it must be properties of individuals rather than simply geographic isolation as individuals from both species can be found in sympatry (e.g. at Win-Timdouine).

Although bats in North Africa have not received as much attention as those in Europe, a significant number of new taxa have recently been elevated to species rank or described from this region (Castella *et al.*, 2000; Benda *et al.*, 2004a; Benda *et al.*, 2004b; Ibañez *et al.*, 2006; Garcia-Mударра *et al.*, 2009; Juste *et al.*, 2009; Benda & Vallo, 2012). Some taxa deserving full species rank identified through analyses of genetic data are still awaiting formal description (Garcia-Mударра *et al.*, 2009; Salicini *et al.*, 2011; Puechmaille *et al.*, 2012a), while the status of populations of several species remains unclear but strongly suggests the presence of further cryptic species (Hulva *et al.*, 2010; Puechmaille *et al.*, 2012b). This, in combination with patterns of species diversity recently uncovered in other taxonomic groups (Brito *et al.*, 2014), lead us to believe that the current bat diversity in the Maghreb and more generally in North Africa might be underappreciated. As recently highlighted by Puechmaille *et al.* (2012b), the taxonomic status of bats in North Africa needs to be investigated and reviewed in detail. The combination of morphological, genetic (including nuclear DNA) and acoustic studies in conjunction with re-inspections of type specimens (when relevant) will greatly contribute to the clarification of the taxonomic status of bats species in the region and reveal the region's real diversity. Such studies will also largely improve our understanding of the phylogeography of many European species that so far have not included populations from North Africa (for some exceptions, see Juste *et al.*, 2004; Flanders *et al.*, 2009; Dool *et al.*, 2013), populations which have potentially survived during ice ages and hence were refugial populations (Dool *et al.*, 2013). A better understanding of biodiversity patterns in North Africa and across the Sahara-Sahel will also be of particular importance as the magnitude and velocity of climate change in deserts and xeric shrublands are predicted to be strong and fast (Loarie *et al.*, 2009). Large protected areas may mitigate the problem in desert biomes, but to increase their efficiency in preserving biodiversity, these protected areas need to be designed based on patterns of biodiversity, hence the importance of resolving the taxonomy and phylogeography of organisms in the region.

Studying species ecology can also reveal some important differences between cryptic species, whether this relates to foraging ecology (Arlettaz, 1999; Davidson-Watts *et al.*, 2006; 2006b; Nicholls & Racey, 2006a) or roosting ecology (Ibañez *et al.*, 2006; Puechmaille *et al.*, 2012a). Studies of reproductive phenology and especially the timing of parturition commonly reveal asynchrony between closely related cryptic species as illustrated for *Myotis myotis* and *Myotis blythii* (Arlettaz *et al.*, 2001). Kowalski & Rzebik-Kowalska (1991) report that in Algeria, parturition in *Miniopterus* lasts from mid-April until almost to the end of June, which suggests that the two species might have different breeding periods (with *M. maghrebensis* **sp. nov.** most likely being earlier than *M. schreibersii* s.str., for which birth takes place in Europe between mid-June and early July; Dietz *et al.*, 2009). More research is needed to investigate these ecological and phenological differences between these two cryptic species, differences which might have been involved in the speciation process and that could now represent prezygotic isolating mechanisms.

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