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ON THE OCCURRENCE OF *MYOTIS ALCATHOE* VON HELVERSEN AND HELLER, 2001 IN AUSTRIA

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ABSTRACT - In 2006, two males and one female of *Myotis alcathoe* were captured by mistnets at two localities in southern Burgenland, Austria. For two individuals the preliminary specific identification based on external measurements was confirmed by sequencing parts of the mitochondrial ND1 gene. Across the sequenced region, the two analysed bats share a 100% identical haplotype that corresponds to the haplotype found in Hungarian bats identified as *M. alcathoe*, and was found also in Spanish, French and Slovakian samples. The three animals from Burgenland constitute the first records of this species in Austria. Age related differences in pelage and membrane coloration and measurements of *M. alcathoe* are described. A comparison of skull length measurements between *M. alcathoe* and *Myotis mystacinus* seems to indicate that the interspecific difference in external dimensions is not reflected in skull dimensions. The Austrian localities of *M. alcathoe* belong to the Pannonian part of the range, as do findings from Slovakia and Hungary.

Key words: M. alcathoe, *Myotis mystacinus*, Austria, species recognition, habitat, abundance

RIASSUNTO – ***Sulla presenza di* Myotis alcathoe *von Helversen and Heller, 2001 in Austria***. Nel 2006, due maschi e una femmina di *Myotis alcathoe* sono stati catturati tramite mist-nets in due località del Burgenland meridionale, Austria. Per due individui, l'identificazione preliminare su basi morfologiche è stata confermata dall'analisi del gene mitocondriale ND1. Nella sequenza analizzata, gli aplotipi dei due individui coincidono al 100% e corrispondono a quelli evidenziati in soggetti spagnoli, francesi e sloveni. I tre esemplari del Burgenland rappresentano la prima segnalazione certa di questa specie per l'Austria. Vengono descritte alcune differenze nella colorazione della pelliccia e del patagio tra adulti e subadulti. Il confronto delle misure del cranio di *M. alcathoe* e *M. mystacinus* sembra non corrispondere alle differenze riscontrate per le dimensioni corporee esterne. Le località di rinvenimento appartengono, insieme a quelle slovene e ungheresi, al sub-areale pannonico della specie.

Parole chiave: M. alcathoe, *Myotis mystacinus*, Austria, identificazione, habitat, abbondanza

INTRODUCTION

The whiskered bat *M. mystacinus* (Kuhl, 1817) is widespread and common in large parts of Europe. It was regarded as one single species until careful analyses of morphology, karyology and mitochondrial DNA sequences revealed that it consisted of four species (Gauckler and Kraus, 1970; Volleth, 1987; Mayer and von Helversen, 2001; Ruedi and Mayer, 2001). A number of molecular studies (Ruedi and Mayer, 2001; Stadelmann *et al.,* 2004, 2007) provided evidence that *M. mystacinus*, *M. brandtii* and *M. alcathoe* belong to highly divergent and not closely related genetic clades and are the products of adaptive convergent evolution. The fourth species, *Myotis aurascens*, can be differentiated from the other European members of the "*Myotis mystacinus*-morphogroup" (sensu Benda and Karataş, 2005) by karyotype, skull size and dental and bacular characters (Benda and Tsytsulina, 2000; Ruedi *et al.,* 2002), but mitochondrial DNA differences are within the range of intraspecific variation (Mayer and von Helversen, 2001). Therefore the phylogenetic position of *M. aurascens* has not been assessed up to now.

Myotis alcathoe von Helversen and Heller, 2001 was the last species of the *M. mystacinus*-morphogroup to be discovered and described. Some differences in the sequence of mitochondrial genes and in the pattern of active nucleolus organiser regions have been reported as unambiguous characters for species recognition (von Helversen *et al.,* 2001). Somatic characters mentioned by the describers of *M. alcathoe* were on average smaller body size than in *M. mystacinus*, and proportions of ear and tragus. According to Benda and Karataş (2005), cranial and dental characters do not allow differentiation of *M. alcathoe* from *M. mystacinus*.

The assessment of the range of *M. alcathoe*, recently summarised by Niermann *et al.* (2007), was based entirely on captured bats whose specific identity was determined either by using external characters in the field only or by an additional genetic analysis. Until now, no specimen of *M. alcathoe* has been found in museum collections. Nevertheless, information on range and ecology of this recently described species is growing fast. The present paper reports on the first records of *M. alcathoe* in Austria, including information on species recognition and a hypothesis concerning its phylogeography.

STUDY AREA

The study area, "Hills and terraces of Southern Burgenland", is situated in the easternmost province of Austria (Burgenland) near the Hungarian border (Fig. 1). It is an area of approximately 142 km^2 lying between the valleys Pinka and Strem (47° 04' - 47° 13' N; 16° 16' - 16° 27' E). The Tertiary hill country is of moderate elevation descending gradually from 415 m in the north, to 230 m a.s.l. in the southern part, which forms the transition to the Lesser Hungarian Basin. Two short tributaries drain southwards to the river Strem. The climate is characterised by hot summers and cold winters, typical for the Pannonian lowland. The hills and gravel terraces are covered by a large coherent seminatural forest consisting mainly of oak

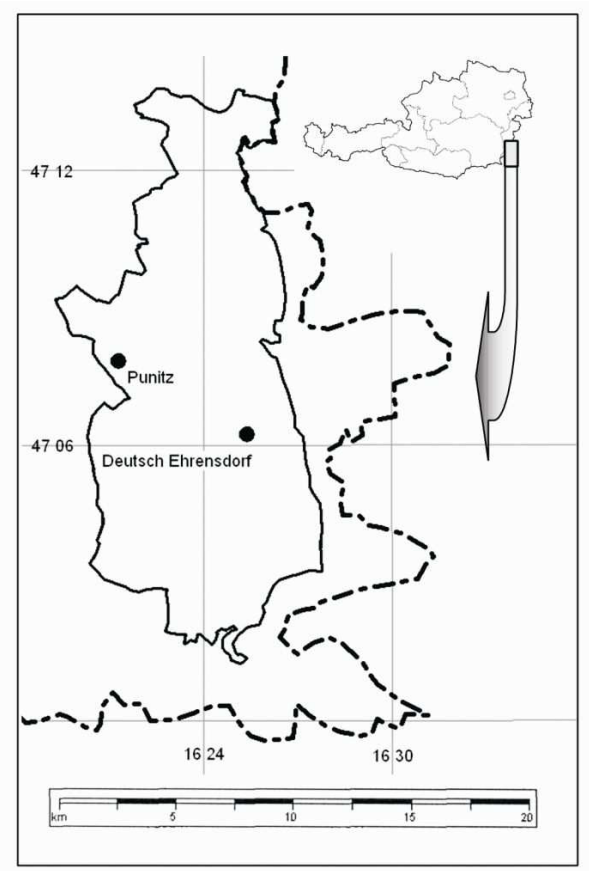

Figure 1 - Study area with the two sampling sites (dots).

(*Quercus petraea*) and hornbeam (*Carpinus betulus*). Deciduous forests of the type Tilio-Acerion grow on the slopes of the hills. In valley bottoms, riverine forests (Alno-Padion, Alnion incanae and Salicion albae) alternate with wet meadows. The study area is regionally protected and is also a site of community interest ("SCI") under the EU habitats directive.

METHODS

1. Sampling

On 19 August 2006, we set mistnets next to two large fishponds east of Deutsch Ehrensdorf (district Güssing, province Burgenland - 47° 06'N/16° 25' E, altitude 233 m). Being unused, the ponds were almost completely covered by reed and surrounded by riverine and deciduous forest vegetation. Apart from one *Pipistrellus pygmaeus* and nine *Myotis daubentonii* we captured one male of the *M. mystacinus*-morphogroup which we preliminarily identified as *M. alcathoe* and collected for further examination (permit nr. 5-N-A1007/270-2006, 07.02.2006, Amt der Burgenländischen Landesregierung). It is now deposited in the Mammal Collection of the Natural History Museum Vienna (NMW 66186).

In mistnets set around a small fishpond located 1.2 km NE of Punitz (district Güssing, province Burgenland, 47 07' N/16° 21' E, altitude 248 m) we captured three specimens belonging to the *M. mystacinus*-morphogroup on 22 August 2006. While one of two females belonged clearly to *M. mystacinus* (forearm 34.5 mm), the other female and the male had shorter forearms (31.8 and 32.0 mm respectively) and distinctly smaller thumbs and feet. The female was sampled (NMW 66185) to confirm its supposed identification as *M. alcathoe*. This fishpond is located in a mown meadow near to a road and is surrounded by a young forest plantation consisting mainly of spruce (*Picea abies*) and pine (*Pinus sylvestris*) which is intersected by no less than five forest roads. Nearby is a small dry valley lined by a narrow row of *Alnus incana* and *Salix* sp.

2. Genetic analysis

Total genomic DNA was extracted using DNeasy Tissue Kit by QIAgen GmbH from muscle tissue of the two ethanol preserved museum specimens. The part of the mitochondrial NADH dehydrogenase subunit 1 gene (*ND1*) was amplified via polymerase chain reaction (PCR) using specific primer pairs ER65 (Petit *et al.,* 1999, Mayer and von Helversen 2001) and HMyot-637ND1 (5' - GGTCCTCCTGCATATTCTAC - 3') and then re-amplified using ER70 (Petit *et al.,* 1999; Mayer and von Helversen 2001) and HMyot-637ND1 primers. Amplifications were performed in the 50 µl reaction mixtures on a Perkin Elmer GeneAmp 2400 Thermocycler using the MasterTaq Kit (Eppendorf) according to the instructions of the manufacturer. Primers concentrations were 0.4 µM and 1µl of total DNA obtained from approximately 1 mg of tissue used as a template. Amplification conditions involved an initial denaturation step of 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 50°C and 90 s at 72°C and a final extension step of 7 min at 72°C.
For reamplification reactions the annealing temperature was increased to 51°C.
Sequencing was carried out by Sequencing Service Ruđer Bošković Institute (Zagreb) using oligonucleotides ER70 for the specimen NMW 66185 and H-Myot-637ND1 for NMW 66186 as sequencing primers. A BLAST search (Altschul *et al.,* 1990) was performed in order to determine the most similar sequences available in the GenBank.

3. Morphological analysis

With the exception of the forearm length, which was measured in the field, external measurements (length of head and body, tail, hind foot and ear) were taken from the collected animals preserved in ethanol. Sixteen skull measurements were taken with dial callipers accurate to 0.01 mm. For comparison we used 180 subadult (young of the year) and adult specimens of the *M. mystacinus*-morphogroup from Austria housed in the mammal collection of the Natural History Museum in Vienna (NMW) which were determined as *M. mystacinus*.
All skull measurements were taken by the same person, Edmund Weiss. The abbreviations used are:
Bb – braincase breadth; Bh – braincase height; Cbl – condylobasal length; C-C – anterior palatal breadth; Ccl – condylocanine length; C-M_3 – length of lower toothrow between canine and third molar; C-M^3 – length of upper toothrow from canine to third molar; Corh – height of coronoid process; Ear – length of ear; FA – length of forearm; GrSl – greatest skull length; HBl – head and body length; HF – length of hind foot; I-M_3 – length of lower toothrow from first incisor to third molar; I-M^3 – length of upper toothrow from first incisor to third molar; IO – breadth of interorbital constriction; M – length of mandible from symphysis to condylar process;

M^3-M^3 – maxillarly breadth between upper molars; Mb – mastoid breadth; Tl – tail length; Zb – zygomatic breadth.

RESULTS AND DISCUSSION

1. Genetic analysis

The 561 bp and 618 bp long section of the *ND1* gene was sequenced from the samples NMW 66185 and NMW 66186, respectively. BLAST search revealed that, across the sequenced region, the two analysed bats share a 100% identical haplotype corresponding to the haplotypes of Hungarian bats identified as *M. alcathoe* -GenBank accession numbers AYO27835 and AY027836 (von Helversen *et al.,* 2001).

The very small number of available *M. alcathoe* mtDNA sequences does not entirely allow conclusions on the phylogeography of the species. Nonetheless, covering a large geographic range they already seem to indicate a phylogeographic pattern showing many similarities with that of another European *Myotis* species, *M. myotis* (Ruedi and Castella, 2003). All currently analysed *M. alcathoe* belonged to one of two haplogroups described by von Helversen *et al.* (2001) from Hungary and Greece. While the "Greek" haplogroup encompasses three different mt haplotypes that differ from each other only by a single base substitution, the "Hungarian" haplogroup is represented by a single mt haplotype. The sequence divergence between those two haplogroups (1.3-1.4 %) indicates that they probably diverged in isolation in two separate glacial refugia. The "Greek" haplotypes were found only in the samples originating from different parts of Greece (von Helversen *et al*., 2001; Mayer and von Helversen 2001) and in one of the two analysed samples from Slovakia (Benda *et al.,* 2003). The "Hungarian" haplotype is widespread and was recorded from Hungary (von Helversen *et al.,* 2001), Austria (this study), France (Ruedi *et al.,* 2002), Spain (Agirre-Mendi *et al.,* 2004) and the second Slovakian sample (Benda *et al.,* 2003). This leads us to assume a recent and rapid range expansion of bats carrying the "Hungarian" haplogroup from a glacial refugium in Western Europe (probably in Iberia) and the existence of at least one further refugium (probably in the Balkan peninsula) where bats carrying the "Greek" haplogroup survived the last ice age.

2. External characters

The specimens NMW 66185 and 66186 and a third individual were recognised as *M. alcathoe* in the field by their small and delicate feet, short forearms and thumbs. With the exception of the feet, the external and cranial measurements of the male *M. alcathoe* NMW 66186 were slightly larger than the female (Tab. 1 and Fig. 2). This unexpected ratio can most probably be explained by a difference in age of these two individuals. In the male, the joint between the metacarpal and the 1st phalanx of the 3rd finger was hard when touched between two fingers and the epiphyses were completely ossified. In the female this joint felt softer and the metacarpal showed a light line of epi-

Table 1 - External and cranial measurements (mm) of two *M. alcathoe* captured in Burgenland, Austria (for abbreviations see Methods - Morphological analysis).

Number	NMW 66185	NMW 66186
Sex	Female	Male
HBl	39.0	40.5
Tl	34.5	33.5
Ear	11.4	13.2
HF	6.2	5.8
FA	32.4	32.6
GrSI	13.17	13.46
Ccl	11.52	11.90
Cbl	12.19	12.64
Bh	5.71	5.84
Bb	6.16	6.30
Mb	6.96	6.98
Zb	-	8.21
IO	3.19	3.41
C-C	3.29	3.35
M^3-M^3	5.22	5.31
I-M^3	5.88	6.20
C-M^3	4.78	5.13
M	9.79	10.09
I-M_3	5.97	6.52
C-M_3	5.19	5.39
Corh	2.78	2.89

physeal cartilage (Morris 1972). Judging from the results of an investigation of a large series of *M. daubentonii* (Baagøe 1977), it can be concluded that the female NMW 66185 was born in summer 2006 whereas the male was older.

The subadult female and the adult male differed conspicuously in the colouration of fur and membranes. Wing and tail membranes as well as ears and feet were light brown in the old male and blackish brown in the subadult female. The colour of the tragi corresponded to the colour of the ears which were transparent in the old male but opaque in the subadult female.

In their field identification key, Dietz and von Helversen (2004) admit that it is not possible to assign all individuals

of the *M. mystacinus* – morphogroup unambiguously to one species. Our results seem to confirm that adult *M. alcathoe* can be identified by their brown, *Myotis daubentonii*-like pelage, brown membranes and transparent ears in combination with small feet and thumbs and relatively short tragus, as was previously suggested by other authors (von Helversen *et al.,* 2001; Agirre-Mendi, 2004; Dietz and von Helversen, 2004). As the fur and membrane colour of subadult individuals of both species are very similar, future records of *M. alcathoe* based solely on morphological field identification should be restricted to adult individuals.

3. Cranial characters

The shape of the skulls of *M. alcathoe* and *M. mystacinus* is different (Fig. 3). The dorsal profile of *M. alcathoe* is characterised by a distinct concavity in the frontal region, the braincase tends to be higher, and the rostral part longer than in *M. mystacinus.* The upper canine is slightly shorter and weaker than in *M. mystacinus*. According to these characters, all the 180 Austrian skulls in the NMW collection previously identified as *M. mystacinus* belong indeed to this species.

In order to test whether the smaller dimensions of the body (e.g. length of the forearm) of *M. alcathoe* compared to

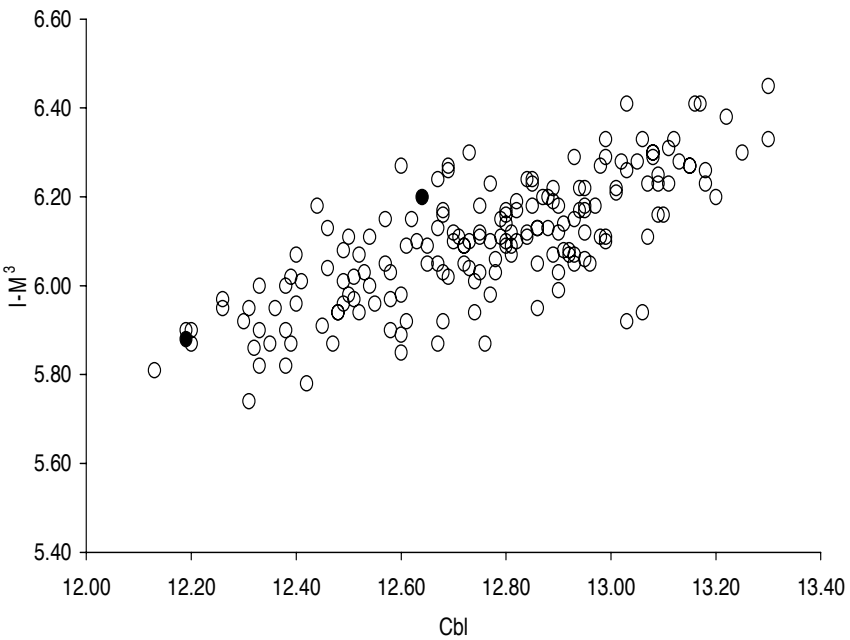

Figure 2 - Bivariate scatter plot of condylobasal length (Cbl) against length of upper toothrow (I-M^3) of 180 skulls of *M. mystacinus* (circles) and *M. alcathoe* (dots) from Austria.

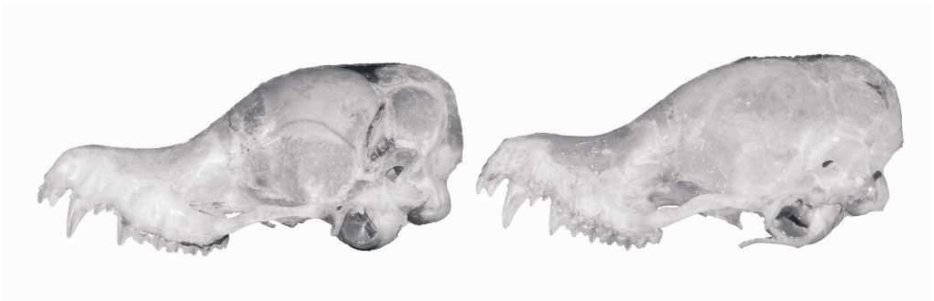

Figure 3 - Lateral aspect of skulls of male *M. alcathoe* (NMW 66186) and *M. mystacinus* (NMW 52285) with the same condylobasal length (12.6 mm) from Austria. *M. alcathoe* on the left side, *M. mystacinus* on the right side.

M. mystacinus are reflected also in their skull size, we performed a bivariate scatter plot of Cbl against the length of the upper toothrow (I-M^3) of 180 skulls of Austrian *M. mystacinus* specimens in the NMW collection and the skulls of the two genetically identified *M. alcathoe* (Fig. 2). The condylobasal length of the adult male *M. alcathoe* occupied a position near the arithmetic mean of this measurement of *M. mystacinus*, the subadult female clustered with subadult specimens of *M. mystacinus*. Craniological analyses of more genetically identified museum material of *M. alcathoe* and *M. mystacinus* will show whether the described differences in skull shape and different ratios of skull/forearm length suggested by our results will provide useful characters for separating these two species.

4. Distribution, habitat and abundance

As we could not find *M. alcathoe* among the previously collected Austrian specimens determined as *M. mystacinus* in the collection of the Natural History Museum in Vienna, the three bats captured in 2006 in the district Güssing in Burgenland represent the first records of *M. alcathoe* in Austria.

M. alcathoe may be missing in medium and high elevations of the Alps. Until now, it was recorded north-west of the Alps for the Swiss Jura mountains (Stadelmann *et al.,* 2004) and east of the Alps at the foot of the mountains surrounding the Pannonian Basin and on hills rising above this plain. The Austrian localities lie on the eastern foothills of the Alps, the only known Slovakian finding (Benda *et al.,* 2003) on the southern slopes of the Carpathian mountains. The Hungarian records (Estók, 2007) come from the Hungarian secondary chain of mountains (Bükk, Mátra and Bakony) which divides the Lesser and Greater Hungarian Basins. The distance between Bakony mountains (highest peak 704 m) and the Austrian localities in the district Güssing is little more than 100 km. Thus, the Austrian records belong to the Pannonian part of the range of *M. alcathoe*.

The typical habitat of *M. alcathoe* in the Pannonian part of the range is hilly

country covered by warm oak-hornbeam forests in elevations between 200 and 600 m a.s.l. where surface water is available also in the summer months (see also Estók, 2007).

There are contradictory statements concerning the abundance of *M. alcathoe* in its European range. Niermann *et al.* (2007) listed 221 records of which almost two thirds lie in France whereas the remaining 65 findings are distributed over 10 other countries. Estók (2007) reported that *M. alcathoe* was mistnetted in Hungary at 30 localities. In other parts of the range, however, *M. alcathoe* seems to be a rare species in most places (Dietz *et al.,* 2007; Niermann *et al.,* 2007 and this paper). As only some of the records listed by Niermann *et al.* (2007) were confirmed by genetic analyses, it is possible that the results of this paper do not depict the actual distribution of abundance of *M. alcathoe* in its range.

The difficulty with identifying cryptic bat species without using genetic markers has probably peaked in the *M. mystacinus*-morphogroup. Only careful morphological comparison of numerous genetically identified museum specimens of *M. mystacinus*, *M. brandtii*, *M. alcathoe* and *M. ausrascens* can provide diagnostic characters for effective species identification in the field.

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