

FIRST RECORD OF THE ALPINE LONG-EARED BAT (*PLECOTUS MACROBULLARIS* KUZJAKIN, 1965) IN LOMBARDY (NORTHERN ITALY) REVEALED BY DNA ANALYSIS

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Riassunto – Prima segnalazione in Lombardia (Nord-Italia) di *Orecchione alpino* *Plecotus macrobullaris*, KUZJAKIN, 1965 determinata mediante analisi genetica. Viene riportata la prima segnalazione per la Lombardia della specie *Plecotus macrobullaris* recentemente descritta, mediante l'impiego di tecniche genetiche, utilizzando anche campioni di animali provenienti dalla provincia di Trento, che rappresentano la prima segnalazione della specie per l'Italia. Il genere *Plecotus* include, per l'Italia, quattro specie sorelle: oltre a *P. macrobullaris* sono contemplate anche *P. austriacus*, *P. auritus*, *P. sardus*, la sola specie di chiroterro endemica dell'Italia. Per l'esatta determinazione delle specie è necessario quindi ricorrere a tecniche genetiche in quanto non sono state ancora messe a punto funzioni discriminanti basate su parametri biometrici, sebbene siano state identificate alcune caratteristiche morfologiche utili per la distinzione. Nel presente lavoro sono riportate le biometrie di base della specie. Per la determinazione della specie, nel presente lavoro è stato utilizzato il metodo del sequenziamento di una regione del DNA mitocondriale, analizzando 50 campioni di tessuto di animali provenienti da Lombardia e Trentino Alto Adige. Sul totale dei campioni analizzati il 55% è risultato appartenere alla specie *P. macrobullaris*. Sono stati individuati, inoltre, 4 rifugi riproduttivi della specie che, in relazione ai dati attualmente a disposizione, sembra preferire rifugi presso edifici mentre non ne è mai stata segnalata la presenza in grotta, a differenza di *P. auritus*.

La presenza di questa nuova specie in Lombardia dovrebbe indurre ad un monitoraggio a larga scala, per definire in dettaglio la distribuzione della specie e le preferenze di habitat, finalizzate anche alla definizione dello *status* delle popolazioni presenti.

Abstract – Mitochondrial DNA sequencing of 63 samples of bats from the genus *Plecotus*, from Lombardy and Trentino Alto Adige, allowed us to determine for the first time the presence of the new species *Plecotus macrobullaris* Kuzjakin, 1965, in Lombardy. This *taxon* has been recently described, using genetic techniques, as a new species, also using tissue samples from individuals captured in Trento Province, that represented the first record in Italy. Recent taxonomic studies on the genus *Plecotus* have revealed the presence of four cryptic species in Italy: in addition to *P. macrobullaris* there are *P. austriacus*, *P. auritus*, *P. sardus*, the only endemic Italian bat species. Since no discriminant function based on morphometric parameters is currently available, only genetic techniques allow to discriminate exactly the different species. Mitochondrial DNA sequencing allowed us to assign 18 out of 33 bats (55%) of the genus *Plecotus* caught in Lombardy to *P. macrobullaris*, the others to *P. auritus*. Moreover, four reproductive roosts of *P. alpinus* were identified, all in buildings. The species was never found in caves, in contrast with *P. auritus*.

The presence of this new species in Lombardy should activate large-scale monitoring to define species distribution and habitat preferences, finalised to define population status and conservation strategies.

INTRODUCTION

There has recently been growing interest in the taxonomy of European bats, thanks to the development of molecular genetic techniques, in particular mitochondrial DNA sequencing. As a consequence, several new species have been described or proposed: e.g. for the genus *Myotis*, with *Myotis punicus* (CASTELLA *et*

al., 2000; RUEDI and ARLETTAZ, in press) and *M. al-cathoe* (HELVERSEN *et al.*, 2001), and, particularly, in the genus *Plecotus*, where two new species have been described: *P. macrobullaris* and *P. sardus* (KIEFER and VEITH, 2001; MUCEDDA *et al.*, 2002; SPITZENBERGER *et al.*, 2003). Hence, the geographic distribution of the two "historic" sibling long-eared bat species in Europe, *P. auritus* and *P. austriacus*, needs to be re-

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sed. Moreover the distribution, habitat preferences and potential niche segregation of four of these species, which seem to occur sympatrically over large areas in Europe, is still controversial.

The *Plecotus* species can be considered sibling species, a phenomenon well known among other bat genera, particularly, among European *Myotis* species: e.g. *M. mystacinus* and *M. brandtii*, *M. myotis* and *M. blythii* (ARLETTAZ, 1996; ARLETTAZ *et al.*, 1997). Sibling species are in general difficult to discriminate based on traditional morphometric parameters or bioacoustic analysis, and this also holds for the genus *Plecotus*. In 2001 SPITZENBERGER *et al.* described *P. kolombatovici* Dulic (1980) as a third species of *Plecotus*, different from *P. auritus* (although originally described as a subspecies of *P. austriacus*). In the same year, KIEFER and VEITH (2001) described another new species of *Plecotus* allied to *P. auritus*: *P. alpinus* (KIEFER and VEITH, 2001). Shortly afterwards, SPITZENBERGER *et al.* (2002) described a new species of *Plecotus* allied to *P. auritus*: *P. microdontus* SPITZENBERGER *et al.* 2002. When MAYER and VON HELVERSEN (2001) demonstrated a sibling relationship between *P. kolombatovici* and *P. austriacus*, SPITZENBERGER *et al.*, (2002) also revised their former opinion and agreed that the name *kolombatovici* should be used for the species sibling to *P. austriacus*, and described the sibling species of *P. auritus* as a new species, *P. microdontus*, a synonym of *P. alpinus*, named in 2003 (SPITZENBERGER *et al.*) *P. macrobullaris*.

In a wider context addressing ecological preferences and distribution of the new species *P. macrobullaris* in Northern Italy, we here report the first record of *P. macrobullaris* in Lombardy, verified using genetic analysis. Its relative abundance with respect to *P. auritus* is estimated, and basic biometric parameters are presented.

STUDY AREA

The study area includes the alpine and prealpine areas of the provinces of Como, Lecco, Sondrio, Varese (Lombardy) and Trento (Trentino Alto Adige) (Fig. 1). Due to the wide extension of the area, bat habitats varied from highly urbanised lowlands and lower hills, typical of the southern parts of most provinces in Lombardy, to the sparsely inhabited mountains, in the provinces of Sondrio and Trento, and in the northern and central parts of the provinces of Varese, Como and Lecco. The study area as a whole

can be subdivided into three major landscape classes: alpine (76.9% of total area), hilly (17.6%) and southern plains (5.5%), the latter coinciding with the northern areas of the upper Po Plain. Bats were predominantly monitored in the alpine and hilly parts of the study area. *Plecotus* spp. were monitored at 23 sampling sites, 14 in Lombardy and 9 in Trentino Alto Adige.



Fig. 1 – The five provinces included in the study area (thin line). Thick line represents boundary between Italy and Switzerland.

MATERIAL AND METHODS

Trapping and handling bats

Mistnet censuses were carried out during the summers of 2000 to 2002, using nylon twisted-thread mistnets (TUTTLE, 1976), with a 36 mm wide square mesh. Each net was composed of five pockets, 60 cm high each, for a total length of 3 to 10 metres.

Mistnetting sites were chosen on the basis of a homogeneous sampling scheme, covering different habitat types and aimed at an exhaustive coverage both of number of species and number of animals. Fixed mistnets were placed in sites where obligatory or preferential fly lanes were identified, in particular near or across streams, or at the entrance of caves, by hooking each net to a pair of aluminium poles. Nets were opened at dusk, just before bats left their roosting sites, and closed and dismantled after a clear decrease in bat activity, identified both by the absence of catches for at least an hour and by the absence (or high decrease) of ultrasonic signals, monitored using an ultrasound detector in frequency division mode. After opening the nets, they were checked continuously. Trapped individuals were removed immediately and held in cotton sacks until the nets were closed. After that, animals were measured and tissue samples were taken, then releasing each animal at the point of capture.

Other catches were made during surveys in potential roost sites, using both mistnets and hand nets with 1 cm mesh, in the latter case when animals were hiding in crevices or suspended from the ceiling. Surveys were conducted in old buildings, where it is easiest to find bat roosts. Visits to roost sites were conducted according to LUTZ *et al.* (1986). Preliminary inspections were done by visiting as many buildings as possible, at all elevations. A survey consisted of a thorough inspection of the building, checking for animals in flight, or droppings, or food remains such as moth wings. Dead animals, when found, were collected, and date and collection site were noted. For each visited site a survey form was filled out, and site characteristics, location, elevation, survey date and time were noted.

Each year, during the months of June and September, animals were caught in buildings where the presence of bats had been previously ascertained. During the winter season, a critical period for bats from an energetic point of view, catches and surveys were suspended in order to avoid a negative impact on animal survival, due to excessive consumption of water and fat tissue depletion (TUTTLE, 1976; 1979).

Species identification of netted animals was made with the help of several biometric parameters. A precision calliper (± 0.1 mm) was used to measure: (1) forearm length, from wrist joint to elbow joint; (2) third finger length, from fingertip to wrist joint; (3) fifth finger length, from fingertip to wrist joint; (4) thumb (1st finger) length, from nail insertion to posterior thumb articulation; (5) thumb claw length, from nail insertion to nail tip; (6) hind foot length; (7) tibia length, from ankle joint to knee joint; (8) tragus length, from tragus tip to tragus basal insertion; (9) tragus maximum width; (10) tail length; and (11) wing length, from the tip of third finger to armpit. Body mass was taken with a precision dynamometer (*Pesola*) with a maximum capacity of 50 g (± 1 g). Three age classes were considered: juvenile, sub-adult and adult, and individuals were assigned to these classes by observing the closure of epiphyseal growth plates in the metacarpal-phalangeal joint of the fourth finger against a bright light source (KUNZ, 1988; STEBBINGS, 1988).

Preliminary species determination in the field was carried out according to the existing literature (LANZA, 1959; VON HELVERSEN, 1989; SCHÖBER and GRIMMBERGER, 1997) and specific identification keys (ROESLI and MORETTI, 2000).

Two tissue disks of 4 mm diameter were taken

from the tail membrane (*uropatagium*) of each skin-sampled bat using a sterile biopsy punch, and placed in a 0.2 ml Eppendorf vial filled with 70% ethanol and stored at -20 °C until the moment of genetic analysis. The patagium has a high regenerating capacity and lesion were completely regenerated in less than a month.

DNA analysis

DNA was extracted from tissue samples using traditional phenol:chloroform method. Genomic DNA was used to amplify a ca. 550 bp mitochondrial DNA fragment using following primers: 16SA (5' – CGC CTG TTT ATC AAA AAC AT – 3') and 16SB (5' – CCG GTC TGA ACT CAG ATC ACG T – 3'). PCR cycling procedure was set up as follows: initial denaturation step 90 s at 94° C, 33 cycles; denaturation for 45 s at 94° C; primer annealing for 45 s at 55° C; extension for 90 s at 72° C, and a final extension for 7 min at 72° C. PCR products were purified using the Qiaquick purification kit (Qiagen) and sequenced on an ABI 377 automatic sequencer using standard protocols. The obtained sequences were aligned to previously published sequences of all European *Plecotus* species (GenBank Accession Nos. AY134012-134026, AF529229-529230 – KIEFER and VEITH, 2001; KIEFER *et al.*, 2002) using Clustal X software (THOMSON *et al.*, 1997).

The phylogenetic relationships were inferred both by distance methods, applying the neighbor-joining algorithm (SAITOU and NEI, 1987) and by maximum parsimony (MP). Other methods did not produce significant different tree topologies. All calculations were carried using PHYLIP software package (FELSENSTEIN, 1989).

RESULTS AND DISCUSSION

Mitochondrial DNA sequencing was carried out on a total of 59 individuals of the genus *Plecotus*, 33 from Lombardy and 26 from Trentino Alto Adige. The obtained sequences were comparable to those published by Kiefer and Veith (2001):

GTATTAGAGGCATTGCCTGCCAGTGACTC-TAGTTAAACGGCCGCGGTATCCTGACCGTG-CAAAGGTAGCATAATCATTTGTTCTCTAAA-TAGGGACTTGTATGAATGGCCTCAC-GAGGGTTAACTGTCTCTTACTTTTAATCAGT-

GAAATTGACACTCCCGTGAAGAGGCGGGAAT-TAAAAATAAGACGAGAAGACCCTATG-GAGCTTCAATTAACCTTATAAGTTATAA-TACTAATCTAAAAGAGACAAATCAAACCTT-GACTAAGTTAACAATTTGGGTTGGGGC-GACCTCGGAATAAAAATCAACTTCCGAGATA-GATCTACTAAGACCTACAAGTCAAAGTTATCT-TACCACACATTGATCCGCAATGACGATCAAC-GAAACAAGTTACCCTAGGGATAACAGCG-CAATCCTATTTAAGAGCCCATATCGACAAT-TAGGGTTTACGACCTCGATGTTGGATCAGGA-CATCCCAATGGTG CAGCAGCTAT-TAATGTGTTTCGTTTGTTCACGATTAAAGTCC-TACGTGATCTGAGT

Eighteen out of 33 animals (55%) found in Lombardy, and ten out of 26 animals (38%) from Trentino were *P. macrobullaris*, the others belonged to the species *P. auritus*. These data represent the first record of *P. macrobullaris* in Lombardy. Differences in the proportion of *P. macrobullaris* between regions were not significant (Fisher's exact test $P = 0.30$). Reproduction of *P. macrobullaris* in our study area was confirmed by the discovery of four nurseries, two in Lombardy, in Varese province, and two in Trento province. Each colony is constituted by a low number of animals (about 2 to 20). These preliminary data suggested that, in the study area, *P. macrobullaris* is as common as *P. auritus*. The exact nursery sites locations are kept confidential, to avoid colonies disturbance, but the authors will consider personnel requests by bat experts for access to more detailed data. Morphologically, some characteristics were noted that might reveal useful to distinguish this new species from other congeners. First, a triangular pad in the central part of the lower lip (SPITZENBERGER *et al.*, 2002; TRIZIO *et al.*, 2003) is present in all indivi-

duals we checked. However, where this pad is clearly evident and black in juveniles, it becomes grey in adults, and in some cases is no longer easily distinguishable. The thumbnail is typical of the “*auritus* group” (composed by *P. auritus* and *P. macrobullaris*), i.e. is longer and sharper than in the species of the “*austriacus* group” (composed by *P. austriacus* and *P. kolombatovici*). The feet are sparsely haired with long vibrissae (SPITZENBERGER *et al.*, 2002). It is still to be ascertained whether these external morphological traits allow exact species determination of *P. macrobullaris*: further testing is needed, on a larger sample of the two sibling species. It should be also preferable, based on the same larger sample, to find quantitative morphometric indicators instead of qualitative morphological traits.

Descriptive biometric data of 25 adult alpine long-eared bats are presented in Table 1.

The two sibling species never occurred together at the same sampling site. There was no significant difference in elevation between sampling sites with *P. macrobullaris* and those with *P. auritus* (mean \pm 693 \pm 341 m a.s.l. and 892 \pm 292 m a.s.l. respectively, Mann-Whitney U-test $U = 36$, $n_1 = 8$, $n_2 = 13$, $p = 0.25$).

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FEMALES	Thumbnail	Thumb	Tibia	Foot	Tail	Wing	Forearm	III finger	V finger	Tragus	Weight
N of cases	23	23	16	23	23	23	23	23	23	23	13
Mean \pm st. dev.	2.6 \pm 0.3	7.0 \pm 0.5	20.6 \pm 0.8	7.7 \pm 0.5	50.3 \pm 3.6	119.0 \pm 3.8	41.2 \pm 1.0	68.7 \pm 2.1	54.1 \pm 1.4	16.6 \pm 1.2	8.5 \pm 0.9
Range	2.1-3.6	5.5-7.9	19.3-22.0	6.8-8.4	40.3-55.6	111.7-125.8	39.2-42.9	64.6-72.5	51.7-56.7	14.2-18.9	6.5-10.0
MALES	Thumbnail	Thumb	Tibia	Foot	Tail	Wing	Forearm	III finger	V finger	Tragus	Weight
N of cases	2	2	.	.	2	2	2	2	2	.	2
Mean \pm st. dev.	2.3 \pm 0.3	7.2 \pm 0.0	.	.	46.7 \pm 1.6	113.3 \pm 0.4	39.9 \pm 0.4	67.4 \pm 1.6	54.2 \pm 1.5	.	7.5 \pm 0.7
Range	2.1-2.5	7.2-7.2	.	.	45.5-47.8	113.0-113.5	39.6-40.2	66.2-68.5	53.1-55.2	.	7.0-8.0

Tab. 1 – Measurements of morphological parameters of adult *Plecotus macrobullaris* from Lombardy and Trentino Alto Adige. Pregnant females were excluded from mean body weight calculations.

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