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Novel molecular tools to identify *Plecotus* bats in sympatry and a review of their distribution in Switzerland

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Abstract: Three species of long-eared bats (*Plecotus auritus*, *P. macrobullaris* and *P. austriacus*) are known to occur in sympatry in Western Europe. They share very similar morphological characters that complicate their reliable discrimination. As a consequence, many records from these areas are reported to the generic level only and the exact distribution of species is uncertain. Even cranial characters are ambiguous since discriminant values traditionally used to identify those species vary with geography. We present a novel use of molecular tools to identify reliably the three cryptic species of long-eared bats. The methods proposed here for time- and cost-effective molecular identification of *Plecotus* species are applied to variously degraded samples such as ancient museum specimens and guano samples collected under roosts. We used this molecular approach to identify over 810 samples and reassess the distribution range of the three species occurring in Switzerland. We further showed that some skull measurements used for species recognition overlap to a greater extent than anticipated and can be misleading for morphologically intermediate individuals.

Keywords: Chiroptera - molecular identification - 16S - DNA barcoding - cryptic species - ancient DNA - forensic mammal identification.

INTRODUCTION

Three *Plecotus* species are present in Switzerland: the brown long-eared bat *Plecotus auritus* (Linnaeus, 1758), the alpine long-eared bat *P. macrobullaris* Kuzjakin, 1965 and the grey long-eared bat *P. austriacus* (J.B. Fischer, 1829). They are all threatened to the national level and listed in the Swiss Red List either as vulnerable, endangered or critically endangered, respectively (Bohnenstengel *et al.*, 2014). Since their densities, altitudinal preferences and habitat requirements vary greatly (Preatoni *et al.*, 2011; Razgour *et al.*, 2011; Ashrafi *et al.*, 2013), they certainly need specific measures of protection based on an exact knowledge of their distributions. Unfortunately, these species exhibit a conservative external morphology (Tvrtković *et al.*, 2005; Spitzenberger *et al.*, 2006; Ashrafi *et al.*, 2010), and highly similar or identical bioacoustic characteristics (Russo & Jones, 2002; Skiba, 2003; Middleton *et al.*, 2014; Barataud, 2015) that render unambiguous species recognition highly problematic. For these reasons, many field observations made in Europe in areas of sympatry are simply reported as "*Plecotus* sp." (Courtois *et al.*, 2011; LPO, 2014b; Gilliéron *et al.*, 2015), thus challenging the development of adequate conservation measures and management strategies to protect populations (Bickford *et al.*, 2007; Rutishauser *et al.*, 2012).

Multivariate analyses of many cranial characters have proven their ability to discriminate efficiently the different species of *Plecotus* (e.g. Spitzenberger *et al.*, 2006; Pavlinić & Đaković, 2015). However, such multivariate approaches require intact skulls to get all measurements and thus limit their use for more fragmentary material such as that recovered from mummies or from pellets of birds of prey. In an attempt to reduce the required measurements to a minimum number of characters, several authors (Bauer, 2001; Benda & Ivanova, 2003; Blant *et al.*, 2008) proposed identification keys concentrating on the best two discriminating variables, the largest diameter of the tympanic bullae (DBT) and maxillary tooth row length CM^3, but no molecular-based validation of the proposed criteria was performed. Such cranial or morphological identification methods in general were questioned. For instance, molecular reassessment of *Plecotus* distribution in Ticino could not confirm the presence of *P. austriacus* in the province (Mattei-Roesli, 2010) whilst the species was previously reported on the basis of morphological identifications (Maddalena & Moretti, 1994; Moretti *et*

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al., 2003) and suitable habitats apparently exist in the province (Rutishauser *et al.*, 2012).
Molecular tools are thus highly recommended to reliably assess species identification independently of morphology and ensure robustness of subsequent conclusions. The barcoding approach, whereby an unknown sample is sequenced for a standard DNA fragment (usually the mitochondrial cytochrome *c* oxidase subunit I, COI) and compared to a reference database (Hebert *et al.*, 2003), has been shown to be efficient for species recognition in European bats (Galimberti *et al.*, 2012). However, this approach requires time-consuming and relatively expensive methods to sequence the standard DNA fragment (657 bp). Furthermore, due to unknown stages of DNA degradation, it is often difficult or impossible to amplify such large fragments in ancient specimens or in guano samples of unknown age. Here, we present an efficient methodology particularly suited for the routine identification of old DNA samples of *Plecotus* bats. We apply this novel molecular approach to identify a large sample of museum specimens from Switzerland and neighbouring countries in order to evaluate the discriminatory power of the two cranial characters (DBT and CM^3) used in previous studies. We also implement this molecular approach to identify guano, tissue or mummy samples from Switzerland and provide a new picture of the distribution of the three species in this country.

MATERIAL AND METHODS

Cranial measurements

The skulls of 194 adult specimens housed in the Natural History Museum of Geneva (MHNG) were examined, representing the three species *P. auritus* (n = 109), *P. austriacus* (n = 50) and *P. macrobullaris* (n = 40) (Appendix 1; DOI: 10.5281/zenodo.1041631). Specimens originated from Western Europe, primarily from Switzerland (n = 166) and France (n = 24). These skulls were measured with a digital calliper with accuracy of 0.01 mm for the following two measurements: DBT – maximum diameter of tympanic bulla, and CM^3 – mandibular tooth row length, measured from the canine to the third molar, without cingulum (Fig. 1). Statistical analyses were all carried out with standard functions implemented in R (R Core Team, 2017), and plot figures were produced using the ggplot2 package (Wickham, 2016).

Sampling and genomic DNA extraction

Our primary aim was to identify molecularly the three cryptic species of *Plecotus* living in sympatry in Switzerland issued from a variety of sources, such as faecal samples, old mummies, or ethanol-preserved museum materials. Guano samples were collected non-

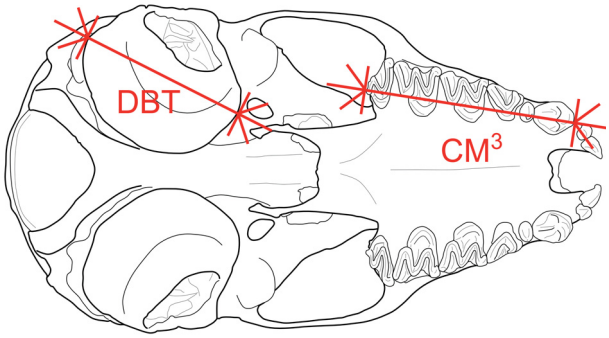

Fig. 1. Skull drawing of a *Plecotus austriacus* (specimen MHNG 1704.016) illustrating the two cranial measurements examined in this study (DBT and CM^3).

invasively under bat roosts, air-dried and then stored at room temperature in paper envelopes for periods ranging from several months to several years. Additional biopsy punches specifically sampled for monitoring programs were collected under appropriate licence and ethical approval (see Acknowledgments). Wing-membrane samples (3 mm in diameter) were collected with sterile biopsy punches according to standard procedures (Worthington-Wilmer & Barratt, 1996) and stored in ethanol. To further minimise the potentially stressful handling of live animals, minimal biological characteristics (gender, age, forearm and tibia length) and four standardised close-up pictures (profile, chin, thumb and foot) were taken. Animals were retained for a maximum of 20 min in cotton bags, before being released on the capture site.
As many samples contained only minute amounts of genetic material, DNA processing was done under a laminar flow hood sterilized with UV lights and with dedicated apparatus, to avoid cross-contamination. The tissue samples were extracted under sterile conditions using the DNeasy Blood & Tissue Kit (Qiagen, Switzerland) according to the manufacturer's protocol, whereas extracts from faecal samples were obtained with the DNA Stool Mini Kit (Qiagen, Switzerland) following the modifications suggested by Zeale *et al.* (2011). Purified DNA extracts were eluted in 200 µL of TE Buffer.
An initial group of 271 samples originating from Switzerland was sequenced for various fragments of the 16S gene (Appendix 2; DOI: 10.5281/zenodo.1041631). For the gel-based discrimination, we additionally attempted various amplifications with methods described below on 181 tissues and 97 guano extracts (Appendix 3; DOI: 10.5281/zenodo.1041631). We further assayed cross-amplifications on an array of ten other species of bats, including three other long-eared bats (*P. kolombatovici*, *P. teneriffae* and *P. gaisleri*) and several other genera of vespertilionids from the Western Palearctic region likely to share roosts with *Plecotus* (*Pipistrellus*, *Myotis*,

Eptesicus, *Vespertilio*, *Barbastella* and *Nyctalus*). As no tissue was available for *P. sardus*, we mined sequences from the GenBank (accession numbers AY175826, AY175822 and AY175819 from Mucedda *et al.*, 2002) in order to carry *in silico* alignments to estimate the cross-amplification specificity of our primers (see below).

Primer cocktails

In order to obtain size-specific amplicons that can be easily discriminated on agarose gels (see Kanuch *et al.*, 2007; Boston *et al.*, 2011), we first amplified reference samples of the three target species and sequenced them for a large fragment (540 bp) of the 16S gene in both directions, using the classical primers 16SAr and 16SBr (Palumbi *et al.*, 1991). These reference sequences were then aligned with the ClustalW algorithm (Thompson *et al.*, 1994) implemented in Sequencher 4.10.1 (Genecodes, USA). The same forward primer (16SAr) was used to amplify shorter, size-specific fragments, in combination with three newly designed reverse primers situated some 300-400 bp further downstream. The 16SauriR reverse primer matched exclusively sequences of *P. auritus*, the 16SaustR exclusively those of *P. austriacus* and the 16SmacrobR exclusively those of *P. macrobullaris*. Each of these new primers (Table 1) were designed to contain 5 to 8 specific mutations discriminating one species from the other two and were chosen to have a similar annealing temperature and no complementary 3' overlap with the other primers. These features allowed us to combine all three specific reverse and the forward primers in a single PCR cocktail in order to obtain size-specific amplicons.
Extracts containing only degraded DNA which either failed the PCR procedures described above or which were issued from guano samples of unknown age, were amplified for an even shorter fragment amplicon of the 16S gene (about 110 bp), using the primers MamP007F and MamP007R (Giguet-Covex *et al.*, 2014). This very short fragment was originally targeted to amplify ancient mammal DNA issued from soil samples, but its 79 hyper-variable nucleotide positions (Palumbi *et al.*, 1991) proved to have enough resolution for the discrimination of all species of *Plecotus*.

Amplifications

For the gel-based amplicon discrimination of the three target species, amplifications were achieved by multiplexing four primers (16SAr, 16SauriR, 16SaustR and 16SmacrobR) in a 25 µL reaction volume, including 2 µL 10× CoralLoad PCR Buffer (Qiagen, Switzerland), 1.6 µM $MgCl_2$, 0.16 mM dNTP, 0.1 µM of each primer, 4 µL Q-Solution, 0.5 U Taq Qiagen and 3 µL of extracted DNA. Owing to the different sources of DNA used, the DNA quantity and quality in the extract varied and different thermal cycling programs were necessary to optimize PCRs. For tissue extracts, containing typically 20-100 ng of bat DNA per µL, the cycling started with an initial denaturation at 94°C for 1.5 min, followed by 12 touchdown cycles consisting in 45 s denaturation at 94°C, 45 s annealing at a temperature set between 62 and 56°C (with a decreasing pitch of 0.5°C) and 1 min extension at 72°C, followed by 21 cycles with an annealing temperature set at 56°C and a final extension for 5 min at 72°C. For guano extracts, typically containing less than 2 ng of bat DNA per µL, the touchdown temperature was set between 58 to 52°C (with a decreasing pitch of 0.5°C) in order to provide less-stringent conditions for amplifications. These distinct annealing conditions were necessary to obtain only the targeted amplicons for older tissue samples and to be effective with guano extracts as well. To visualize the size-specific amplicons, 5 µL of PCR products were run for 30 minutes at 70 V on a 1.5% agarose gel containing 0.005% ethidium bromide. A 100 bp ladder (PeqLab, Germany) was run alongside the PCR products to estimate the size of the amplicons.
Amplification of the fragments intended to be sequenced was performed in a 25 µL reaction volume, including 2.5 µL CoralLoad PCR Buffer, 1.6 µM $MgCl_2$, 0.8 mM dNTP, 0.2 µM of each primer, 4 µL Q-Solution, 1 U

Table 1. Sequences and source of the different 16S primers used in this study. The amplicon size, expressed in number of base pairs (bp), corresponds to amplifications obtained with the 16Ar forward primer and one of the reverse primers. The MamP007 primer pairs produced an amplicon of about 110 bp.

Primer name	Sequence (5'-3')	Amplicon size	Reference
16SAr	CGCCTGTTTATCAAAAACAT		Palumbi *et al.* (1991)
16SBr	CCGGTCTGAACTCAGATCACGT	591 bp	Palumbi *et al.* (1991)
16auriR	GTTTAATTTGTCTCTTATAGATTAATGCTATAACTTG	291 bp	this study
16SaustR	GAGTTAGTCTTTATCTCGAGGTCG	352 bp	this study
16SmacrobR	TCATTGGCGGATCAATGTGTGG	416 bp	this study
MamP007F	CGAGAAGACCCTATGGAGCT		Giguet-Covex *et al.* (2014)
MamP007R	CCGAGGTCRCCCCAACC		Giguet-Covex *et al.* (2014)

Taq (Qiagen, Switzerland) and 3 µL of extracted DNA. Cycling conditions for MamP007F-MamP007R followed Giguet-Covex *et al.* (2014) and those for 16SAr-16SBr followed Mucedda *et al.* (2002). As they were short and easily read, the fragments were sequenced with the Sanger method in a single direction, using the forward primer used for amplification (either MamP007F or 16SAr).

Geographic range

Finally, all georeferenced specimens from Switzerland that could be identified molecularly (both here and in previous studies) were mapped on a 5 × 5 km grid in order to provide an updated view of the distribution of the three species living in this country. This map illustrates the six main biogeographical regions of Switzerland (i.e. Jura, Plateau, north side of the Alps, western inner Alps, eastern inner Alps and south side of the Alps, *sensu* Gonseth *et al.*, 2001).

RESULTS

Molecular identifications

A total of 278 DNA extracts of *Plecotus* spp. from various age and origins were tested for amplification with the species-specific primer cocktail. From these samples, 94/97 (97%) guano extracts, 74/74 (100%) wing biopsies and 61/107 (57%) ancient tissue extracts successfully amplified fragments of expected sizes. Of the failed samples, 45 were further tested for the very short amplicon with the MamP007 primers, which resulted in 35 (78%) positive PCR, all of which were successfully sequenced. The ten museum samples for which all our attempts to get amplification failed were most likely specimens that had been fixed in formalin before being stored in ethanol.

Overall, tissue samples tended to produce brighter bands than faecal material and were thus easier to discriminate on gels. The primer cocktail efficiently amplified a single product for *P. macrobullaris* and *P. austriacus* producing bright bands of about 400 and 350 bp, respectively (Fig. 2, lanes 1-2). For *P. auritus*, 126 of the 129 positive samples produced the expected single band at about 300 bp (Fig. 2, lane 3), but three samples produced a second band of about 400 bp (Fig. 2, lane 4). This unexpected double band pattern resulted from the primer 16SmacrobR (supposed to be specific to *P. macrobullaris*) annealing to sequences of *P. auritus* from Central and Eastern Europe. As shown by comparison with reference sequences, these *P. auritus* carry a different mitochondrial lineage from that of bats from Western Europe, that were called clades 'east' and 'west', respectively, by Juste *et al.* (2004). As these eastern *P. auritus* were the only bats resulting in a double-banded pattern on the agarose gels, this method thus allowed a simple discrimination of both lineages of *P. auritus*, and did not compromise other species identification.

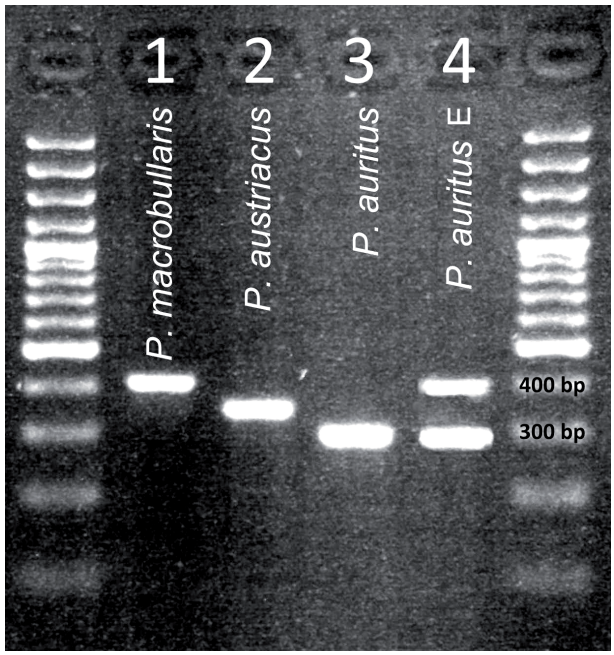

Fig. 2. Species-specific patterns of amplification of 16S fragments obtained in a single PCR cocktail. These fragments were resolved on a 1.6% agarose gel run for about 30 min at 60 V/m. A 100 bp molecular ladder was run on each side of the pictured agarose gel. Amplification products of diagnostic sizes appear on lane 1 for *P. macrobullaris* (at about 400 bp), on lane 2 for *P. austriacus* (350 bp), on lane 3 for the 'west' clade of *P. auritus* (300 bp) and on lane 4 for the 'east' clade of *P. auritus* (two bands at about 300 and 400 bp, respectively).

Cross-amplification of other bat species did not produce any visible band on the gel, even for extracts issued from good-quality tissue samples. The only exception was a very faint band of about 350 bp, i.e. in principle diagnostic for *P. austriacus*, obtained for one of the three *P. kolombatovici* tested (NMP 48725). Finally, in silico comparisons with *P. sardus* showed that none of the three specific reverse primers should amplify the 16S gene fragment for this species, as they all exhibit 3 to 5 mutations within the targeted annealing sequence.

Additional identifications and range reassessment

Additional samples from Switzerland (n = 271) were genetically identified using direct Sanger sequencing for full or partial 16S (see Appendix 2). Depending on the primers used, sequences ranged from 50 to 540 bp, and after alignment, were collapsed into seven unique haplotypes. The longest reads were deposited in the GenBank (accession numbers MF423092 to MF423098) and correspond to five distinct haplotypes of *P. auritus* (PaurHap1, 2, 3, 4 and 5), to a single haplotype of *P. austriacus* (PausHap1) and one of *P. macrobullaris*

(PmacHap1). Shorter reads were attributed to one of these seven haplotypes using a 100% matching score threshold (for a detailed list of specimens and haplotypes see Appendix 2). Besides these newly genotyped samples, 263 molecularly identified individuals (sequenced for either the COI or cyt-*b* mitochondrial genes) issued from previous publications (Ashrafi *et al.*, 2010; Mattei-Roesli, 2010; Rutishauser *et al.*, 2012) were also recovered to produce a total dataset of 700 *Plecotus* samples from Switzerland (Fig. 3). This comprehensive dataset includes 177 grid-cells occupied by *P. auritus*, 45 by *P. austriacus* and 82 by *P. macrobullaris*. These georeferenced and molecularly identified samples thus cover a large part of the country, except the northern slopes of the Alps, which are only marginally represented (Fig. 3). Thirteen grid-cells, mostly located in the Jura Mountain and Geneva province, have occurrences of both *P. auritus* and *P. austriacus*, and 17 from the inner and southern slopes of the Alps show sympatry between *P. macrobullaris* and *P. auritus*. A single grid-cell located in the Geneva province supports the molecular evidence for the sympatric occurrence of all three species in Switzerland.

Skull measurements

As expected, the measured skulls labelled as *P. auritus* in museum collections exhibited the smallest cranial dimensions, *P. austriacus* the greatest and *P. macrobullaris* intermediate values. Among the 194 studied skulls, 75 (39%) were associated with tissue samples that could be genetically identified (plain symbols in Fig. 4). The bivariate plot of the two cranial measurements overlaid with these molecular identifications shows that these dimensions overlap considerably among species, especially between *P. macrobullaris* and *P. austriacus* (Fig. 4). If one considers the limits proposed by Blant *et al.* (2008) to identify those skulls based on DBT and CM^3 (grey bars on Fig. 4), several molecularly identified specimens fall outside these limits and indeed were mislabelled in the museum collections. In particular, three molecularly identified *P. austriacus* with particularly small tooth rows (CM^3<5.7 mm in Appendix 1) would fall below the lowest values reported in this species (Spitzenberger *et al.*, 2002; Benda & Ivanova, 2003; Blant *et al.*, 2008). Conversely, due to its particularly large upper tooth row (CM^3=5.78 mm),

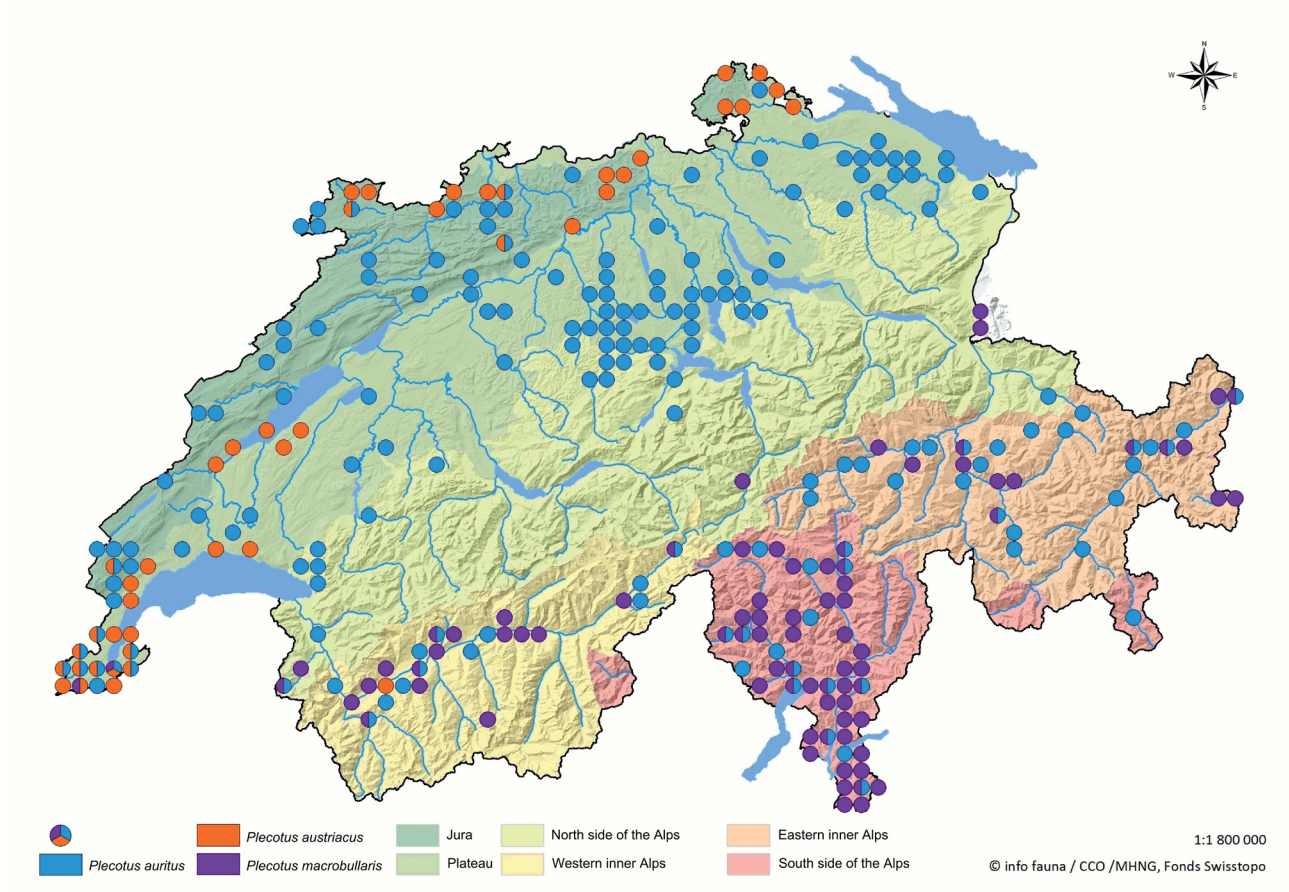

Fig. 3. Map of Switzerland depicting the six biogeographical regions occurring in this country (Gonseth *et al*., 2001) and the occurrences of 700 genetically identified *Plecotus* samples. Plain symbols represent locations of *P. auritus* (in blue), *P. austriacus* (in orange) and *P. macrobullaris* (in violet). Symbols with more than one colour represent areas of sympatry. Map produced by the Centre Suisse de Cartographie de la Faune.

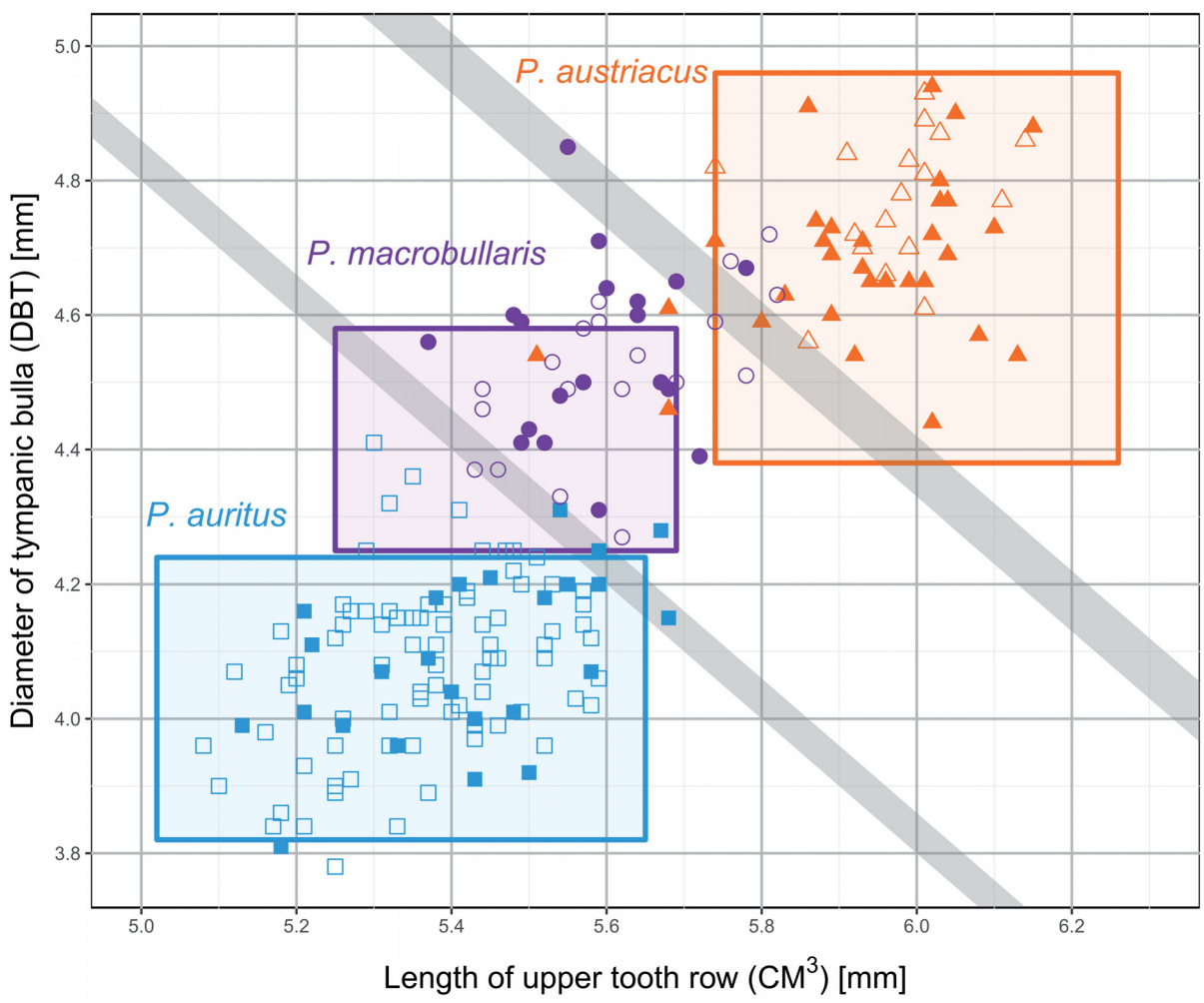

Fig. 4. Bivariate plot of the length of upper tooth row (CM^3) versus diameter of tympanic bulla (DBT) of 194 skulls of *Plecotus*. Blue squares represent skulls of *P. auritus*, violet circles skulls of *P. macrobullaris* and orange triangles skulls of *P. austriacus*. Plain symbols indicate genetically identified individuals, while hollow ones are from animals examined for skull morphology only. Coloured boxes indicate the species-specific measurement ranges given by Benda & Ivanova (2003) for Central European *Plecotus* and the grey bars represent the limit values of the two cranial measurements proposed by Blant *et al.* (2008) to identify the three species.

one genetically confirmed *P. macrobullaris* clearly falls within the morpho-space occupied by *P. austriacus*. A further *P. macrobullaris*, identified as such by external characters, had even greater dimensions (Fig. 4), but as it was fixed in formalin prior to storage in ethanol, its identification could not be confirmed genetically. We therefore suspect that more marginal records of genetically unverified animals on this scatter plot have doubtful identifications.

DISCUSSION

Molecular techniques

Reducing sequencing costs is desirable when financial resources are limiting, in particular for long-term monitoring programs of cryptic species, or when dealing with multiple non-invasive samples (such as faeces, hairs, etc.) that do not allow species recognition by other means than genetics. In the context of cryptic species recognition, cheaper methods based on gel discrimination of size-specific amplicons have been developed to circumvent these limitations (Kanuch *et al.*, 2007; Boston *et al.*, 2011). Similar protocols to save the purification and sequencing steps are proposed here, and provide a reliable, time- and cost-effective alternative that allows the discrimination of the three long-eared bat species in Switzerland. The new gel-based method also discriminates two major European lineages of *P. auritus* (the 'east' and 'west' lineages proposed by Juste *et al.*, 2004) by producing a unique double-banded pattern in the eastern lineage. Although we recommend to test the validity of this method with appropriate reference

samples when applied to other geographic areas (e.g. in the Iberian or Italian peninsulas, Ibáñez *et al.*, 2006; Galimberti *et al.*, 2012), it proved to be efficient in the Alpine region. It enabled routine identification of most samples and can be used to facilitate further studies where these long-eared bats occur in sympatry. The relatively small size of the amplicons makes this approach robust even for identifying mummies or relatively recent guano, which can be collected easily when monitoring roost occupancy without disturbing the colonies.

When the primer cocktail failed to amplify visible products on agarose gels, the amplification of an even shorter fragment (MamP007) was a good alternative to obtain usable PCR products. Although it implies the sequencing of the amplicons, this mini-16S fragment was successfully amplified in museum specimens stored in denatured ethanol for nearly half a century and in all guano samples of unknown age. This highly variable fragment was well-suited to discriminate all three species of *Plecotus* considered here, including the 'west' (corresponding to haplotypes PaurHap1 to 4 in Appendix 2) and 'east' (corresponding to PaurHap5) clades reported in *P. auritus* that only differ by few transitions in this fragment (Fig. 5). We thus strongly recommend the use of this mini-16S fragment when dealing with predictably low-quality DNA samples, e.g. those issued from necropsies. As this fragment amplifies a large array of other mammalian species as well (Giguet-Covex *et al.*, 2014), we anticipate that this versatile mini-16S fragment will be of much broader interest in forensic mammal identification. However, proper comprehensive reference sequences of all mammal species deposited in public repositories are currently lacking and hinder a quick identification based on this small 16S gene fragment. Thus, a more extensive use of this potentially well-discriminating marker is still awaiting the construction of more complete reference databases.

Critical evaluation of discriminant cranial characters

Several discriminant cranial measurements have been proposed to identify specimens of *Plecotus* in Western Europe (Häussler & Braun, 1991; Benda & Ivanova, 2003; Benda *et al.*, 2004; Spitzenberger *et al.*, 2006; Pavlinić & Đaković, 2015) including Switzerland (Blant *et al.*, 2008), but no independent identification method was used to validate their efficiency. By measuring DBT and CM^3 for a large number of skulls from Central Europe (n = 194), we show here that the three sympatric *Plecotus* do not cluster into distinct morpho-groups, but rather fall within a continuum of skull dimensions (Fig. 4). Furthermore, we could obtain genetic identification for 75 of the measured individuals with the genetic methods described here (plain symbols in Fig. 4), which suggest much more morphological overlap than anticipated. The skulls of *P. auritus* are indeed smaller and with a relatively small tympanic bulla, and barely overlap with the larger-sized *P. macrobullaris*. However, genetically identified *P. austriacus*, which have supposedly the largest dimensions in this morpho-space, fall clearly below the usual limits given for the species. Three small individuals of *P. austriacus* have the same dimensions as typical *P. macrobullaris* (Fig. 4) and thus would be misidentified if only those two skull measurements were used. These results stress the difficulty to identify the morphologically intermediate-sized *P. macrobullaris* based on skull characters only. Due to this large size overlap, there is probably no simple linear combination of skull measurements that would identify *P. macrobullaris* with certainty, and their discrimination is thus limited to the smallest and largest sections of this morpho-space (Fig. 4). Fortunately, if external characters can be examined, *P. macrobullaris* can be reliably recognised by the shape of the chin pad, as suggested by Spitzenberger *et al.* (2002) or Kiefer & Veith (2002). Indeed, all living individuals that were sampled here and identified a posteriori as *P. macrobullaris* by genetic methods had a typical triangular, laterally highly concave chin pad that

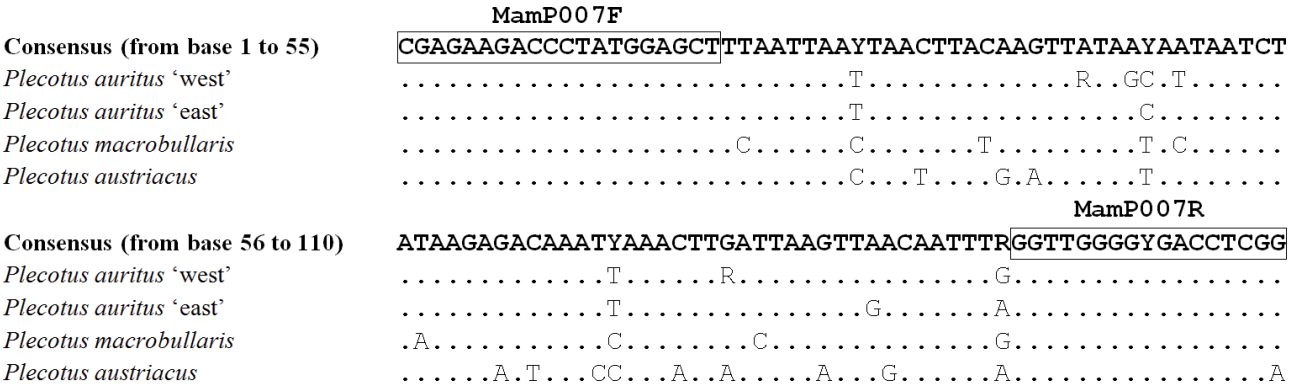

Fig. 5. Alignment of typical 16S sequences of different *Plecotus* lineages obtained with the MamP007 primer pair (framed); the expected amplicon size is 110 bp (including primers). Alignment dots represent identical nucleotides.

was absent (or of a different shape) in the other species. To better illustrate this character, we compiled a range of close-ups from the three genetically identified species (Fig. 6).

The presence of few atypical specimens that conflict between a genetic and a morphologic diagnose (as shown in Fig. 4) raises a further question: are these animals just extremes in their cranial dimensions or do they represent interspecific hybrids? As all previous taxonomic studies (including this one) were based on mitochondrial markers (e.g. Mayer & Helversen, 2001; Kiefer *et al.*, 2002; Juste *et al.*, 2004; Spitzenberger *et al.*, 2006), which are inherited from the mother without contribution from the father (Ballard & Whitlock, 2004), they are not appropriate to detect the occurrence of putative interspecific hybrids (Andriollo *et al.*, 2015). Furthermore, cases of mitochondrial gene introgression are rare in bats, but at least known in few other vespertilionids (Berthier *et al.*, 2006; Artyushin *et al.*, 2009), and thus this phenomenon could also occur, albeit very rarely, in long-eared bats. The use of biparentally inherited markers such as nuclear microsatellites will be necessary to address this question in further studies.

Fig. 6. Close-up views of the lower mandible of *Plecotus* bats illustrating the shape of the chin pad in the three species. Pictures were taken from genetically identified adult long-eared bats from Switzerland or France. In the first column is *P. auritus*, in the middle column *P. macrobullaris* and in the third *P. austriacus*. Notice the particular shape of the chin pad of *P. macrobullaris*, with elongated tip and distinctly concave sides.

Revised species ranges

Given that most previous distribution ranges of the three species of *Plecotus* in Switzerland were only based on morphology (Hausser, 1995) or in combination with genetic characters but on scattered localities (Mattei-Roesli, 2010; Rutishauser *et al.*, 2012), we compiled the occurrence of all genetically identified specimens (n = 429) with those already available from literature to provide a revised distribution map for the whole country (Fig. 3). Global geographic coverage is important as locally clumped occurrences might bias downstream model-based analyses such as ecological niche modelling (Rebelo *et al.*, 2012; Razgour *et al.*, 2013). One example is provided here by the geographically widespread *P. auritus*. Indeed, the modelled distribution for *P. auritus* issued from a more scattered sampling (Rutishauser *et al.*, 2012) essentially predicted that alpine valleys provided optimal habitats, with the Jura or the Swiss Plateau only marginally suitable for that species. Our revised distribution map, derived from genetically identified samples, shows instead that *P. auritus* is well represented in all biogeographic regions and the most common and widespread long-eared bat outside the Alpine range.

Despite its broad European distribution (Razgour *et al.*, 2013), *P. austriacus* is localised in Switzerland and considered a critically endangered species (Bohnenstengel *et al.*, 2014). Modelled distributions (Rutishauser *et al.*, 2012) predicted that the whole Plateau region should be suitable for that species, however we failed to confirm its presence over most of this region, despite an extensive sampling of guano pellets collected in more than 70 grid cells; all these samples proved to belong to *P. auritus* (Fig. 3). Given this sampling effort and the uncertainty of previous morphology-based identifications, *P. austriacus* seems to be truly absent from most of the central and oriental parts of the Swiss Plateau (Fig. 3). We also concur with Mattei-Roesli (2010) that this species is absent from the southern slopes of the Swiss Alps, where only *P. auritus* and *P. macrobullaris* can be found. Its absence from that region, as well as from the eastern inner Alps, is puzzling since appropriate habitats are available (Rutishauser *et al.*, 2012) and the species inhabits similar areas further away in neighbouring regions of Austria or France (Spitzenberger, 2001; LPO, 2014a). Although *P. austriacus* has been reported as nearly ubiquitous in Italy (Lanza, 2012), all records from the northern regions (e.g. Preatoni *et al.*, 2000) predate the recognition of *P. macrobullaris* (see Kiefer & Veith, 2002) and thus likely concern misidentifications, while a more recent survey in Piedmont did not recover this species (Debernardi & Patriarca, 2007). To the best of our knowledge, all genetically identified *P. austriacus* from Italy (Galimberti *et al.*, 2012; Razgour *et al.*, 2013) come from areas south of the Po river basin, i.e. well away from the Alps. Hence the northern limits of distribution of *P. austriacus* in Italy still need to be molecularly ascertained. For the Alpine region, Rutishauser *et al.* (2012) hypothesised this species might be outcompeted by *P. macrobullaris*, as an explanation for their parapatric distributions. The occurrence of stable colonies of all three species breeding sometimes within a radius of two kilometres in the Geneva province (Gilliéron *et al.*, 2015, Fig. 3) suggests that this hypothesis does not apply elsewhere. Furthermore, *P. austriacus* and *P. macrobullaris* also breed in sympatry in Corsica (Courtois *et al.*, 2011) without apparent competitive exclusion.

Our extended geographic sampling further confirms that most records of *P. macrobullaris* are from mountainous areas characterized by abrupt topology and fit the expectations of broad and fine-scale modelling conducted for the species across its entire range by Alberdi *et al.* (2014). In particular, it is well represented in inner valleys and southern slopes of the Alps (Mattei-Roesli, 2010; Ashrafi *et al.*, 2013) and is also confirmed from several valleys of the Graubünden province (Fig. 3). The altitudinal range occupied by molecularly identified *P. macrobullaris* might however be biased towards the most easily accessible roosts in the bottom of the valleys, as suggested by Alberdi *et al.* (2014). Hence, although extensive, the area covered by our sampling is still not ideal to be representative of the full regions occupied by the three species. Likewise, more samples from the northern slopes of the Swiss Alps should also be analysed for a complete understanding of the distribution of these species of *Plecotus* in Switzerland, and in particular to define the northern limits of occurrence of *P. macrobullaris*.

Despite these limitations, the increasingly efficient and relatively simple genetic methods proposed to identify cryptic species, including *Plecotus* bats as exemplified here, are providing more accurate distribution maps that will help develop better conservation strategies. These new methods also point to the need for better morphological diagnose for long eared-bats. One of the caveats mentioned earlier is the inability of such mitochondrially-based approaches to detect hybrid or introgressed individuals. This problem needs to be addressed with technologically more sophisticated approaches and is particularly needed for the areas where species ranges overlap (Fig. 3), and where hybridization might occur.

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