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Multiple morphological characters needed for field identification of cryptic long-eared bat species around the Swiss Alps

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Abstract

The identification of cryptic species may significantly change our view about their distribution, abundance, ecology and therefore conservation status. In the European Alps, molecular studies have revealed the existence of three sibling species of plecotine bats *Plecotus auritus*, *Plecotus austriacus* and, very recently, *Plecotus macrobullaris*. Knowledge of the ecological niche partitioning of cryptic species is a requisite to develop sound conservation policies. Yet, this requests the development of unambiguous identification methods easily applicable in the field. This study investigates the reliability of several morphological methods used for species recognition and proposes a new identification key for field workers. We captured 214 *Plecotus* bats from 29 sites in four bioregions within Switzerland, collected biopsy punches for genetic analysis, described and measured external morphological characters. All three species occurred as mono-specific colonies, except at one site where *P. auritus* and *P. macrobullaris* shared the same church attic. Qualitative traits alone did not allow a reliable separation of the three species. A series of multivariate analyses conducted on external linear measurements resulted in a discriminant function enabling correct species classification with a 97.5% probability. Compared with genetic analysis, our multivariate morphological method represents a valuable, rapid and cost-effective alternative.

Introduction

Cryptic species are a group of species that are morphologically alike and hence difficult to distinguish based on external features, despite being genetically distinct (Jones, 1997). Cryptic species have been discovered in many taxa (e.g. in ants: Schlick-Steiner *et al.*, 2006; in fish: Kon *et al.*, 2006; in bats: Ruedi, Arlettaz & Maddalena, 1990; Arlettaz, Ruedi & Hausser, 1993; Kiefer & Veith, 2001; Mayer, Dietz & Kiefer, 2007). Before their distinction, cryptic species were biogeographically and ecologically confounded. This calls for a careful re-evaluation of their distribution and ecological requirements, especially when it comes to conservational issues (Arlettaz *et al.*, 1997*a*; Arlettaz, 1999; Sattler *et al.*, 2007).

Chiroptera are one of the most speciose groups of mammals with >1100 species described so far and recent discoveries of many cryptic species (Harris *et al.*, 2006; Ceballos & Ehrlich, 2009), which calls for investigations of the species' ecological needs. With 22% of species classified as threatened worldwide, bats are among the most threatened vertebrates. In temperate biomes, bats are often closely associated with humans. This is due to their dependence on man-made structures for roosting and reproducing, a fact that increases their vulnerability. Currently, 42 species of bats are recognized in Europe, with 21% classified as threatened or near threatened (IUCN Red list, 2009). Bats not only require a complex network of seasonal roosting sites but they are also very selective regarding foraging habitats (Entwistle, Racey & Speakman, 1996; Arlettaz, 1999; Bontadina *et al.*, 1997 Bontadina, Schofield & Naef-Daenzer, 2002; Popa-Lisseanu, Bontadina & Ibanez, 2009). A proper understanding of all these requisites is essential for implementing an efficient conservation action.

The genus *Plecotus* (long-eared bats) is widespread in the Palaearctic (Spitzenberger *et al.*, 2006). It has been subjected to several studies in Europe (e.g. Entwistle *et al.*, 1996; Kiefer *et al.*, 2001, 2002; Juste *et al.*, 2004; Dietrich *et al.*, 2006; Spitzenberger *et al.*, 2006). From the 1960s until recently, two sympatric *Plecotus* species were recognized in Europe: *Plecotus auritus* (Linnaeus, 1758) and *P. austriacus* (Fischer, 1829). With the continuing development of molecular techniques, five European species of plecotine bats are now recognized: *P. auritus*, *P. austriacus*, *Plecotus*

Table 1 Study sites with coordinates, altitude, sample size (*n* = number of individuals), genetically identified species and haplotype (the new haplotype for *Plecotus auritus* is in italics)

Sites (canton)	Latitude	Longitude	Elevation (m)	Date	Species	*n*	Haplotype
Arbaz (VS)	46°12′	7°21′	1100	16.08.2006	*P. macrobullaris*	1	macHT1
Ayent (VS)	46°16′	7°24′	1000	18.08.2006	*P. macrobullaris*	8	macHT1
Basse Nendaz (VS)	46°11′	7°18′	1000	9.08.2006	*P. macrobullaris*	5	macHT1
Blitzingen (VS)	46°26′	8°11′	1300	3.08.2006	*P. auritus*	5	aurHT3
Col de Bretolet (VS)	46°08′	6°47′	1960	7.09.2006	*P. auritus*	3	aurHT3
Collex (GE)	46°16′	6°07′	450	4.09.2006	*P. austriacus*	7	ausHT1
Gampel (VS)	46°18′	7°44′	640	4.08.2006	*P. macrobullaris*	10	macHT1
Gouffre Cathy Arzier (VD)	46°29′	6°08′	1300	4.09.2006	*P. auritus*	14	aurHT3, *aurHT7*
Grengiols (VS)	46°22′	8°05′	900	3.08.2006	*P. macrobullaris*	16	macHT1
Grimsuat (VS)	46°15′	7°23′	850	17.08.2006	*P. macrobullaris*	3	macHT1
Isérables (VS)	46°10′	7°15′	1000	19.08.2006	*P. auritus*	1	aurHT4
Kirchrued (AG)	47°17′	8°5′	500	26.08.2006	*P. auritus*	7	*aurHT7*
Lax (VS)	46°23′	8°7′	1050	28.07.2006	*P. macrobullaris*	18	macHT1
Lens (VS)	46°15′	7°23′	1100	18.08.2006	*P. macrobullaris*	3	macHT1
Leytron, mine (VS)	46°11′	7°12′	617	6.08.2006	*P. macrobullaris*	2	macHT1
Ittenthal (AG)	47°31′	8°03′	410	26.08.2006	*P. austriacus*	1	ausHT1
Mandach (AG)	47°32′	8°11′	490	22.08.2006	*P. austriacus*	27	ausHT1
Mönthal (AG)	47°31′	8°08′	500	21.08.2006	*P. austriacus*	4	ausHT1
Obergesteln (VS)	46°30′	8°19′	1360	2.08.2006	*P. auritus*	5	aurHT3
Obergesteln (VS)	46°30′	8°19′	1360	2.08.2006	*P. macrobullaris*	6	macHT1
Pfyn (VS)	46°18′	7°36′	420	7.08.2006	*P. auritus*	8	aurHT3, aurHT4
Poteux Cave (VS)	46°10′	7°10′	1000	29.07.2006	*P. macrobullaris*	12	macHT1
Salins (VS)	46°12′	7°21′	600	15.08.2006	*P. auritus*	17	*aurHT7*
Sembrancher (VS)	46°04′	7°09′	740	31.07.2006	*P. macrobullaris*	8	macHT1
Thalheim (AG)	47°26′	8°06′	460	2.08.2006	*P. austriacus*	1	ausHT1
Ulrichen (VS)	46°30′	8°18′	1340	30.07.2006	*P. macrobullaris*	2	macHT1
Vens, pond (VS)	46°05′	7°06′	1250	30.07.2006	*P. auritus*	1	*aurHT7*
Vens, cave (VS)	46°05′	7°06′	1200	08.08.2006	*P. auritus*	3	aurHT4, *aurHT7*
Wiler-Guttet (VS)	46°19′	7°40′	1260	8.08.2006	*P. macrobullaris*	14	macHT1
Zeihen (AG)	47°28′	8°05′	450	25.08.2006	*P. austriacus*	2	ausHT1

VS, Valais; GE, Geneva; VD, Vaud; AG, Aargau.

kolombatovici Dulic, 1980, *Plecotus macrobullaris* Kuzjakin, 1965 and the Sardinian insular endemic *P. sardus* Mucedda, Kiefer, Pidinchedda & Veith, 2002. Based on phylogenetic analysis, Juste *et al.* (2004) distinguished two major clades of Plecotine bats in Europe, the *auritus* group including *P. auritus*, *P. macrobullaris* and *P. sardus*; and the *austriacus* group, which contains *P. austriacus* and *P. kolombatovici*. In Central Europe, the presence of the two sibling species *P. auritus* and *P. austriacus* has been known for some time. Recently, however, molecular evidence has proven the existence of a third sympatric cryptic species, *P. macrobullaris*, which occurs mostly in the Alpine region (Kiefer & Veith, 2001; Kiefer *et al.*, 2002; Spitzenberger *et al.*, 2006). In the European Alps, *P. macrobullaris* appears to occur sympatrically with *P. auritus* and in the vicinity of *P. austriacus* (Juste *et al.*, 2004).

Reliable identification of these three species is difficult, and so far only possible with certainty through molecular methods (Kiefer *et al.*, 2002; Spitzenberger, Haring & Tvrtkovic, 2002). However, these methods are time consuming and relatively expensive, with the results not instantaneously available to field workers. Therefore, in order to complement the current distribution and conservation status of Central European *Plecotus*, an easy identification method is needed. The present study aims, firstly, to evaluate the reliability of morphological characters already proposed for species identification, and secondly to build an updated, refined identification kit for field workers in Central Europe, which is a pre-requisite for any ecological and conservation studies in this area.

Materials and methods

Field sampling and data collection

In summer 2006, we sampled 214 *Plecotus*, which were assumed to belong to the three target species, at 29 sites in southern, northern and western Switzerland (Table 1). We captured *Plecotus* bats in four out of the six bioregions in Switzerland (Jura Mountains, Plateau, Western Central Alps and Northern Alps; Gonseth *et al.*, 2001), where we expected the species to occur sympatrically. Most of the capture sites were already known as nocturnal or colonial roosts of long-eared bats (data bank of the Swiss Bat

Conservation centres). Sites were not randomly chosen, but were selected in order to obtain a representative, as far as possible balanced sample including all three species. Bats were captured using mist and hand nets, typically near entrances to roosts. In a few cases, we captured individuals on the wing above ponds or in the roost vicinity.

The following eight external measurements were taken from all captured adult individuals according to Dietz & Von Helversen (2004) and Tvrtkovic, Pavlinic & Haring (2005). We used either a dial caliper (accuracy 0.01 mm) to measure: length of forearm (FA), thumb length without claw (TH), tibia length (TIB) and hind foot without claw (HF); or a steel ruler (accuracy 0.5 mm) to measure: ear length (EARL), ear width (EARW), tragus length (TRAGL) and tragus width (TRAGW) (Appendix S1). Additionally, a number of qualitative characters were recorded: general fur colour, especially on the back (three levels: white brown, brown and grey), colour of fur on the throat (three levels: brown, brownish grey and whitish grey), penis shape (three levels: narrowing toward the end, club shape and cylindrical pointed at the tip), and the density and the position of hairs on the hind foot (three levels: long and upright hairs on the whole hind foot, long sticking hairs at toes and short hairs on toes). The presence of a triangular pad on the lower lip (TP) as well as the sex were also recorded. A biopsy punch (4 mm diameter) of the wing membrane was collected from all individuals and stored in ethanol for subsequent genetic analyses.

Molecular species identification

Skin samples were dissolved in lysing buffer and proteinase K at 55 °C for 24 h. Total DNA was extracted from fresh skin tissue using a high pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany). DNA was extracted according to the protocol suggested by the manufacturer. PCR amplification to RNA was performed with primers, using standard procedures. The 550 bp fragment of the rRNA 16s gene was obtained using the primers L 15975 and 16425 (Wilkinson & Chapman, 1991). The PCR cycling procedure was as follows: denaturation step for 60 s at 95 °C, 39 cycles, primer annealing for 90 s at 55 °C and extension for 120 s at 72 °C. PCR products were purified using the 'High pure PCR product purification kit' (Boehringer, Mannheim, Germany). The amplified gene fragments were sequenced using a capillary ABI prism 377 sequencer (Applied Biosystems, Darmstadt, Germany). Then, sequence alignments were carried out using DNA SEQUENCER and MEGA 3.1 software.

Morphometric species identification model

In order to identify the species based on morphological characteristics, we built a discriminant model on the prior genetically identified individuals (see Arlettaz, Ruedi & Hausser, 1991; Arlettaz *et al.*, 1997*a*). We had to exclude four individuals from the dataset due to poor quality sequences from the molecular analysis. Therefore, we used the quantitative and qualitative morphometric measurements from 210 genetically identified individuals of all three species to create an identification model. Initially, a principal component analysis (PCA) was carried out on the eight quantitative variables to describe the overall multivariate structure of the dataset. Then, we used discriminant analysis (DA) and a multinomial logit (MNL) model to group individuals based on morphological characters. The analysis was performed using the program R 2.4.1 (R Development Core Team, 2006).

DA

We tested the assumptions of both multivariate normality and common variance–covariance matrix. Firstly, we performed a Mardia test, according to Timm (2002), for estimating multivariate normality, which revealed significant deviations from normality. We transformed the data using a Box–Cox transformation (Sokal & Rohlf, 1995), which still resulted in a deviation from normality. Therefore, eight outliers detected by the Mardia test were excluded from the dataset to obtain a normal distribution (Tabachnik & Fidell, 2001).

The assumption of a common variance–covariance matrix was tested with Box's *M*-test, using SYSTAT 10 software. In spite of slight covariance differences, most likely due to uneven sample sizes, we used both linear (LDA) and quadratic (QDA) discriminant analysis (see Wahl & Kronmal, 1977; Tabachnik & Fidell, 2001). The model was built using 80% of the data, with a subsequent cross validation conducted with the remaining 20% data to derive a misclassification rate. We performed both LDA and QDA, including the quantitative variables, to compare error rates of the two methods for the same dataset. Finally, the outliers excluded during the first step of the modelling procedure were tested in the model (see Tabachnik & Fidell, 2001).

MNL model

The model was built using all eight continuous and two categorical variables (sex and TP) using 80% of the dataset as training data and the remaining 20% for model validation. The modelling procedure excluded missing values, which caused four of the qualitative variables not being further considered. We used a stepwise search method based on the AIC criterion for selecting the best model (Faraway, 2006), with the better performing models being those with low AIC values.

Results

Molecular species identification

All three *Plecotus* species were present in our sample: 104 *Plecotus macrobullaris* (84♀, 20♂), 64 *P. auritus* (40♀, 24♂) and 42 *P. austriacus* (25♀, 17♂) (Table 1). At 24 sites, where several individuals were caught, we have the genetic

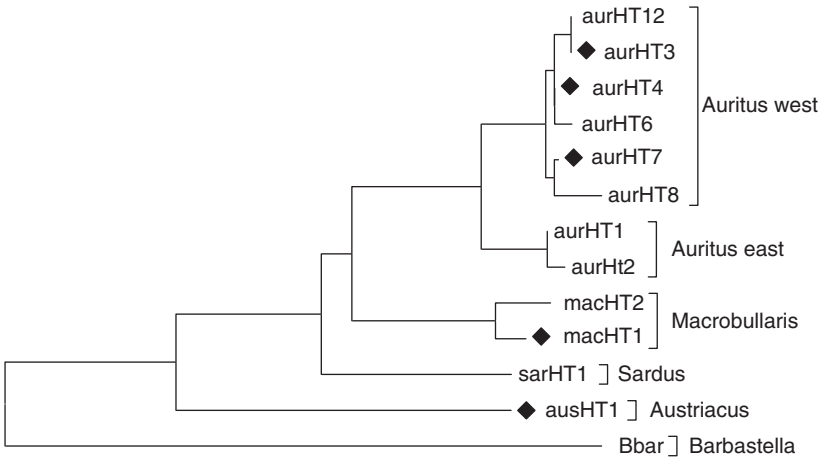

Figure 1 Phylogenetic tree drawn from published haplotypes of the genus *Plecotus* in Europe, with *Barbastella barbastellus* acting here as an outgroup. Species found in this study are depicted with a diamond. Reference numbers in gene bank: macHT1 (AY 531628), aurHT1 (AF 629230), aurHT3 (AF 326106), aurHT4 (AF 529229) and ausHT1 (AY 134022).

evidence for the presence of mono-specific populations, except in Obergesteln (canton of Valais), where two nursery colonies of *P. macrobullaris* and *P. auritus* shared the same church attic. *P. macrobullaris* was found between 600 and 1360 m altitude in this study (Table 1). A phylogenetic analysis confirmed the identification of all three species in our sample, and showed the existence of three haplotypes of *P. auritus*, one haplotype of *P. macrobullaris* and one haplotype of *P. austriacus* (Fig. 1). All mitochondrial haplotypes except one were found in previous studies (Kiefer, 2007; Benda *et al.*, 2004). Haplotype aurHT7 was recorded for the first time; it was found at four locations in three cantons (Table 1).

Biometric species identification

Qualitative traits did not reliably separate the three species. For example, we found an overlap in fur coloration from brown to grey in all three species, possibly reflecting different age classes. Also, the majority of individuals had some long and visible hairs on the feet and around the toes, rendering this identification criterion difficult to apply. Yet, *P. macrobullaris* was the only species to bear a TP, with almost 95% of individuals (98 out of 104) possessing this pad. Altogether, the qualitative characters did not allow a reliable species separation by applying the currently existing identification keys (Spitzenberger *et al.*, 2006; Dietz, Von Helversen, & Nill, 2009) in the field.

Results of the PCA of the eight quantitative external characters showed that the first component explained 35.2% of the overall variance. This component was mostly correlated with variables expressing the body size like:FA, TIB, TRAGL and TRAGW. The second component explained 23.6% of the total variance and correlated mainly with size of extremities, including ears like:TH, HF, EARW and again TRAGW. The third component explained only 7% of variation and was thus disregarded. There was a large overlap between the three species in the multivariate space (Fig. 2). The low level of variance explained by the PCA further informs about weak correlations between single characters. Therefore, all eight variables were used for the DA.

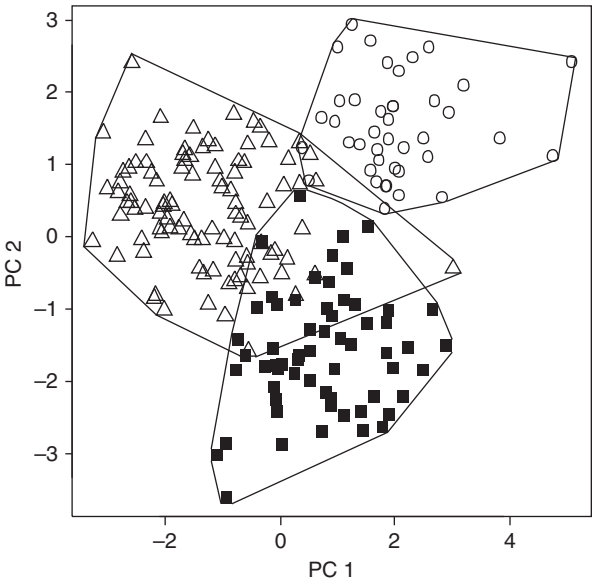

Figure 2 Relationship between first and second factors (PC) of a principal component analysis on eight external morphological characters. PC1 and PC2 explained 35.2 and 23.6% of the total variance, respectively. Untransformed data were used for the three species *Plecotus austriacus* (○), *Plecotus macrobullaris* (Δ) and *Plecotus auritus* (■). The species groups are encompassed by polygons.

DA

The comparison between cross validation results of both linear and quadratic DA showed similar error rates (Table 2). Therefore, the linear discriminant function was preferred because of its simpler form. Most of the specimens (97.5%, i.e. 39 out of 40) were correctly classified, with only one individual misclassified, which gives an error rate of 2.5%

Table 2 Classification tables obtained from linear discriminant analysis (LDA), quadratic discriminant analysis (QDA) and multinomial logit model (MNL) for *Plecotus macrobullaris* (Pmac), *Plecotus auritus* (Paur) and *Plecotus austriacus* (Paus)

	Predicted species											
	LDA				QDA				MNL			
Actual species	Pmac	Paur	Paus	Error	Pmac	Paur	Paus	Error	Pmac	Paur	Paus	Error
Pmac	20	1	0	0.1	20	1	0	0.1	18	2	0	0.1
Paur	0	11	0	0	0	11	0	0	2	10	0	0.2
Paus	0	0	8	0	0	0	8	0	0	0	8	0
Apparent error rate				0.017				0.017				0.09
Overall error rate				0.025				0.025				0.1

Apparent error rates (misclassifications for each group, divided by the group sample size) and overall error rates of cross validation tests.

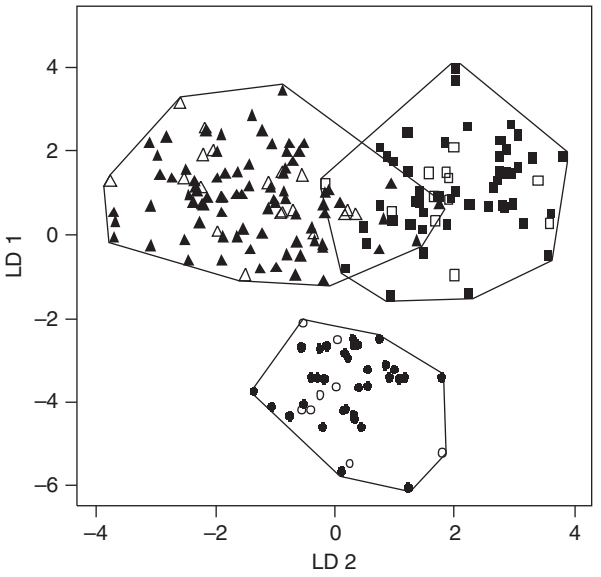

Figure 3 Scatter plot of the scores of the linear discriminant factor 1 and 2 obtained from both training data (full symbols) and validating data (empty symbols) for the three species *Plecotus austriacus* (○), *Plecotus macrobullaris* (Δ) and *Plecotus auritus* (□). Species groups are enclosed by a polygon.

(Table 2). A scatter plot of the scores of the linear discriminant functions 1 (LD1) and 2 (LD2) shows a clear separation of *P. austriacus* from the other two species by the first discriminant function. Yet, *P. macrobullaris* and *P. auritus* overlap, with LD2 alone not allowing a correct separation (Fig. 3). The discriminant functions in Table 3 were used for calculating classification equations for the three species (an Excel file for species determination is provided as Appendix S2 in the supporting information). Species identity can be determined based on the species-specific function (out of the three functions) yielding the largest discriminant score. The outliers were also tested with the discrimination functions, resulting in only one of the eight outliers being misclassified. There was only one actual *P. macrobullaris* identified as *P. austriacus*, most likely due to mistakes in measuring the animal or bad data recording. This outcome anyway indicates a high model performance.

MNL model

Based on the AIC value, the stepwise search method elected a model including five variables: FA, TRAGL, EARW, HF and presence or absence of TP (Table 4). Cross validation results of the MNL model showed a larger error rate (10%, i.e. four individuals out of 40) for this model compared with the DA. Apparent and overall error rates of the three models are shown in Table 2.

Discussion

This study shows that field identification of all three species of *Plecotus* bats within and around the Swiss Alps is possible with a high accuracy by applying a simple but powerful set of linear functions. In contrast, due to intermediate or often indistinct traits, no reliable identification could be achieved using the classical external characters which had been proposed in previous studies (Tvrtkovic *et al.*, 2005; Spitzenberger *et al.*, 2006; Dietz *et al.*, 2009).

Our results confirm the presence of the cryptic species *P. macrobullaris* in the Swiss Alps (Kiefer & Veith, 2001; Juste *et al.*, 2004) and evidence the syntopic (roost sharing) occurrence of *P. macrobullaris* and *P. auritus*. While mixed colonies of *P. auritus* and *P. austriacus* are well known (e.g. Beck & Schelbert, 1994), there is to our knowledge only one previous report of a mixed colony involving *P. macrobullaris* with another *Plecotus* species (*P. kolombatovici;* Croatia; Pavlinić, 2008). According to the principle of competitive exclusion (Hutchinson, 1957, 1978), sympatric distribution as well as shared nursery roosts of sibling species signify species-specific differentiation in resource utilization, insofar as this co-existence is stable, which is very likely here (Arlettaz, Perrin & Hausser, 1997*b*; Arlettaz, Godat & Meyer, 2000; Arlettaz, 1999).

The availability of accurate, rapid and cost-effective identification methods based on morphological characters easily assessed in the field – contrary to non-instantaneous and expensive genetic tools – is advantageous for any ecological, behavioural and conservational studies. The novel identification key proposed here for identifying *Plecotus* will facilitate the task of bat ecologists and conservationists within Switzerland and might be valid also for the

Table 3 Constants and classification coefficient functions for the three *Plecotus* species

	Constant	FA	TH	TIB	HF	EARL	EARW	TRAGL	TRAGW
Pmac	−1373.50	19.820	30.389	7.405	−0.737	17.685	23.308	24.605	18.204
Paur	−1266.68	18.691	32.405	7.230	2.124	17.226	22.122	22.453	14.415
Paus	−1281.60	21.937	26.501	0.949	−5.166	17.761	25.386	21.919	19.251

FA, length of forearm; TH, thumb length without claw; TIB, tibia length; HF, hind foot without claw; EARL, ear length; EARW, ear width; TRAG, tragus length and TRAGW, tragus width (see Appendix S2 in supporting information).

Table 4 Multinomial logit model selection results with AIC, corrected AIC values and relative weights

Model	AIC value	AICc	Delta AICc	AIC weight
Species∼FA + TRAGL + EARW + HF + factor(TP)	27.8250	29.4087	0.0000	1.0000
Species∼FA + EARW + HF + factor(TP)	52.3060	53.4115	24.0027	0.0000
Species∼TRAGL + EARW + HF + factor(TP)	63.0838	64.1893	34.7805	0.0000
Species∼FA + TRAGL + EARW + HF	69.2416	70.3471	40.9384	0.0000
Species∼FA + TRAGL + HF + factor(TP)	82.4746	83.5801	54.1714	0.0000
Species∼FA + TRAGL + EARW + factor(TP)	128.6731	129.7786	100.3699	0.0000

EARW, ear width; FA, length of forearm; TP, triangular pad on the lower lip; TRAGL, tragus length.

rest of the European Alps. The qualitative characters proposed earlier did not work well in our study. For instance, the fur criteria proposed by Kiefer & Veith (2001: denser, longer and whiter throat fur in *P. macrobullaris* compared with *P. austriacus* and *P. auritus*) did not perform well in our study area. The same holds true for a whitish grey fur on the throat and belly reported for *P. macrobullaris* by Spitzenberger *et al.* (2002), Tvrtkovic *et al.* (2005) and Dietz *et al.* (2009). In our sample, only six out of 104 individuals of *P. macrobullaris* had this character. In contrast, a TP, another distinctive feature proposed to separate *P. macrobullaris* (e.g. Tvrtkovic *et al.*, 2005; Dietz *et al.*, 2009) was present in 95% of our *P. macrobullaris*. This character is thus partly discriminant: individuals harbouring a lip pad almost certainly belong to *P. macrobullaris*. However, it is not always easy to appraise the presence of a lip pad in all individuals, due to the variation in colour and shape of the triangle. Some field experience with *Plecotus* bats is necessary in this respect. Presence of long hair on the hind feet was also claimed to be a good criterion for species separation (e.g. Dietz *et al.*, 2009) but our evaluation suggests that this character cannot confidently differentiate any of the three species, at least in our sample. In most cases, longer hairs were visible in *P. auritus* and shorter hairs in *P. austriacus*, while *P. macrobullaris* had somewhat intermediate hairs, but the judgment was never definitive. Tvrtkovic *et al.* (2005) suggested the use of a bivariate scatter plot of TH regressed against HF to separate Croatian *P. auritus* and *P. austriacus*. This did not work on our sample, which shows a considerable overlap (34%) between the two species (Fig. 4). Finally, Tvrtkovic *et al.* (2005) and Dietz *et al.* (2009) have suggested penis shape as a good character for separating the males of the three species (*P. macrobullaris*: cylindrical penis); *P. auritus* (penis narrowing toward the end); and *P. austriacus* (club-shaped penis). The observation of the few male specimens in our sample ($n = 25$) confirmed

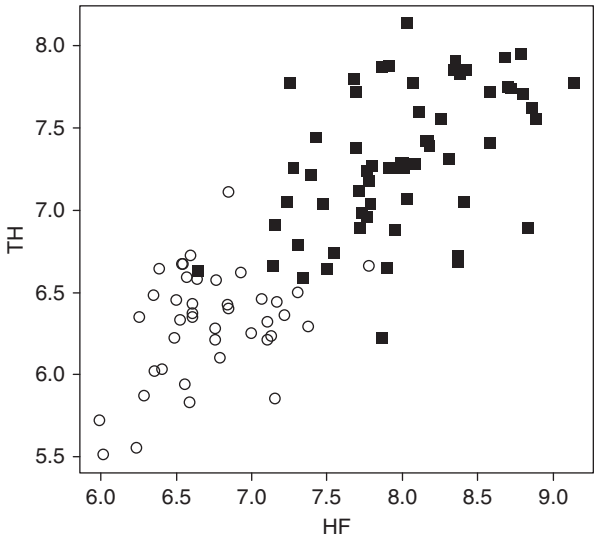

Figure 4 Scatter plot of hind foot (HF) against thumb length (TH) from *Plecotus auritus* (■) and *Plecotus austriacus* (○).

this, with 96% of individuals correctly identified. However, penis shape is not very helpful for assessing the identity of nursery populations from which males are most of the time absent.

We conclude that there is no simple solution to achieve a reliable identification of long-eared bats within the alpine region of Switzerland; neither qualitative characters nor bivariate graphs offer a reliable alternative to multivariate approaches. While none of the already proposed identification characters are reliable, using them singly or in combination does not render a trustworthy recognition. Based on the multilinear combination of eight characters, we achieved a high probability (97.5%) of correct species classification.

By checking several individuals per colony, our method represents a major improvement compared with other methods proposed earlier on. As most colonies are monospecific, three individuals belonging to the same species increase the probability of a correct identification to more than 99%. Our discriminant functions will greatly facilitate the field identification of *Plecotus* bats within and around the Swiss Alpine region. The validation of this method in the rest of the Alps and Central Europe has to be verified. These are crucial steps paving the way for future investigations of long-eared bats' distribution, ecology and conservation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. External measurements of long-eared bats (*Plecotus spp.*) in Switzerland. Number of individuals (n), mean, standard deviation (SD), min and max for eight variables (FA: length of forearm, TH: thumb length without claw, TIB: tibia length, HF: hind foot without claw, EARL: ear length, EARW: ear width, TRAG: tragus length and TRAGW: tragus width). All measurements are in mm.

Appendix S2. Description of measurements of ear length (EARL) and width (EARW) in living *Plecotus* bats: (a), as performed in this study: the ear must be stretched both in length and width along the steel ruler to reach the correct position, which provides maximum values. Ear width was not measured according to the combination of two measures as suggested in Dietz & Von Helversen (2004), but as a single measure, in its broadest section (middle part). Ear length was taken with a ruler first positioned at the base of the ear, inserted in its "V" shape opening, while stretching the ear upwards to its tip. It must be noted here that the maximum ear length and width in living *Plecotus* are definitely shorter than in dead specimens (b) due to the absence of folds on the distal edge of the ear in the latter individuals.

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