Vespertilio 15: 55–62, 2011
ISSN 1213-6123

Reliability of field determination in three cryptic whiskered bats (*Myotis alcathoe*, *M. mystacinus*, *M. brandtii*) and basic biometric characters: evidence from the Czech Republic

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Abstract. The Alcathoe bat (*Myotis alcathoe*), the whiskered bat (*Myotis mystacinus*) and the Brandt's bat (*Myotis brandtii*) represent three cryptic species living in sympatry across much of Europe. Although their determination based solely on external characters has been widely used in field research, there is no study addressing the reliability of such determination. Based on material of bats identified with the help of genetic methods, we aimed (1) to analyse the reliability of field determination, (2) to compare basic quantitative measurements (forearm length, length of tibia, body mass) and (3) to provide information on the reliability of using these measurements for field identification of these three species based on material from the Czech Republic. Fourteen of 359 individuals (3.9%) were originally erroneously determined based on external characters. Eight per cent of bats originally determined as *M. alcathoe* were in fact different species. In all *M. mystacinus* bats, the original species determination was confirmed using the molecular analysis (i.e. 0% determination error). Four per cent of bats (5 inds.) originally determined as *M. brandtii* were assigned to *M. mystacinus* using molecular methods. The three species significantly differed in forearm length, the length of tibia and body mass. Although a considerable overlap of marginal values always existed, the lowest one was recorded between *M. alcathoe* and *M. brandtii*. The best model for discrimination among the three species included sex, forearm length and body mass. However, ca. 69–94% correctness of assignment based solely on these three variables stresses the importance of using a combination of both metric and qualitative characters (i.e. colouration, ear and dental morphology) to further improve the reliability of determination.

***Myotis alcathoe*, *Myotis mystacinus*, *Myotis brandtii*, determination, external morphology**

Introduction

The Alcathoe bat (*Myotis alcathoe*), the whiskered bat (*Myotis mystacinus*) and the Brandt's bat (*Myotis brandtii*) represent three distinct species from the *Myotis mystacinus* morpho-group living in sympatry across most of Europe (Dietz et al. 2007). Although they are not closely related species (see Ruedi & Mayer 2001), their morphology is so similar that until the 1960s they were treated as a single species. Gauckler & Kraus (1970) and Hanák (1970) showed *M. brandtii* (Eversmann, 1845) to represent a species separate from *M. mystacinus* (Kuhl, 1817), based on

body size and dental and penial morphology. Since that time, *M. mystacinus* and *M. brandtii* were easily distinguished in the field based on the presence of prominent cingular cusp on the upper large premolar (P^4) and the widened distal part of penis in the latter species (Gauckler & Kraus 1970, Hanák 1970, 1971). However, the influx of molecular methods into bat taxonomy at the beginning of the 21st century revealed another species within the morpho-group, whose existence had been predicted based on the results of karyologic studies by Volleth (1987) and field observations by von Helversen (1989). This new species was described as *Myotis alcathoe* von Helversen et Heller, 2001 (von Helversen et al. 2001) and the growing literature has confirmed its sympatric (and even syntopic) occurrence over much of the European continent (Niermann et al. 2007, Spitzenberger et al. 2008, Lučan et al. 2009, Jan et al. 2010, Bashta et al. 2011).

External characters used for field identification of these three bats (summarised by Dietz et al. 2007) are as follows:

Myotis alcathoe – smallest European *Myotis*; at first sight it resembles *Myotis daubentonii* but is clearly smaller; dorsal pelage is uniformly brown or reddish brown; face and ears are pale-coloured; feet are smaller than in *M. mystacinus* and *M. brandtii*; tragus is short, it does not reach the notch on the posterior edge of the ear or only scarcely; the large upper premolar (P^4) bears a distinct cingular cusp that is, however, not so prominent as in *M. brandtii*; penis is evenly narrow or slightly thickened at the end; forearm length <32.8 mm, fifth finger length <44 mm, third finger length <56 mm, thumb length <4.7 mm, tibia length <14.8 mm, foot length <5.8 mm.

Myotis mystacinus – slightly larger than *M. alcathoe*; dorsal pelage is very dark, frequently with yellowish tips giving bi-coloured ("frosty") appearance; face and ears are dark brown to black; tragus extends beyond the notch on the posterior edge of the ear; penis is evenly narrow for its whole length; tragus, tibia, foot and thumb lengths larger than in *M. alcathoe* (see above); cingular cusp on the large upper premolar as well as prominent protoconuli on upper molars are mostly missing or are very minute; the second small premolars in both jaws (P^3 and P_3) are markedly smaller than the first ones (P^2 and P_2).

Myotis brandtii – similar or same in size to *M. mystacinus*; dorsal pelage has light-golden hair tips; skinny parts on the face and the base and inner part of ears are pale (pinkish); tragus extends behind notch on the posterior edge of the ear; high cingular cusp on the large upper premolar (P^4) which is equal in height or even higher than the second small premolar (P^3); the two small upper premolars (P^2 and P^3) are almost equal in size; penis is club-shaped at its end.

Although basic external qualitative and quantitative characters discriminating *M. alcathoe, M. mystacinus* and *M. brandtii* were well defined (see above) and have been frequently used (see e.g. Niermann et al. 2007, Lučan et al. 2009, Danko et al. 2010), there is little information on the reliability of discrimination based on these characters particularly in *M. alcathoe*. This bat is a rare species and field workers do not often have an opportunity to train their identification skills on a large number of individuals. Moreover, the size characters given in the literature were taken from material originating from different parts of Europe, thereby they can include possible geographic variation, while there may be lower variation within a smaller geographic region (e.g. within Central Europe) which could be useful-to-know for local researchers to improve the reliability of their identification.

The aim of this study was (1) to analyse the reliability of field identification of *M. alcathoe, M. mystacinus* and *M. brandtii*, (2) to compare basic field measurements (forearm length, tibia length, body mass) in genetically identified individuals, and (3) to provide information on the reliability of discrimination based on these measurements for field identification of the respective three species based on relatively extensive data from the Czech Republic.

Material and methods

During various fieldworks focused on bats, *Myotis alcathoe, M. mystacinus* and *M. brandtii* were captured using mist-netting in suitable habitats (foraging sites, swarming sites) or by hand nets at roosting sites. Upon their identification based on external qualitative and quantitative characters summarized above, wing membrane samples were taken from each individual using the sterile biopsy punch (Worthington Wilmer & Barratt 1996) and stored in 96% ethanol. Sex, age, forearm length and body mass were recorded in most of the sampled bats (n=310), while the length of tibia was only measured in a subsample of each species (n=77) only. Forearm length was taken including wrist. Only measurements of full-grown bats were used for analyses while juveniles measured before 15 July were excluded because of the possibility they did not reach adult size at that time.

Genomic DNA was extracted from the wing punch, with the DNeasy Blood & Tissue kit (QIAGEN). All individuals were genotyped for 12 microsatellite loci, which were selected for their ability to distinguish among the three species (Zima et al. 2011). Methodology of the PCR amplification, fragment analysis, genotyping and details of the microsatellites will be described elsewhere (Zima et al., in prep.). All individual genotypes were analysed as a single dataset, with three distinct "populations", which were represented by individuals of one of the three species. The most probable "population" of origin for each individual was determined using the Bayesian assignment test implemented in the GeneClass software (Cornuet et al. 1999). Original field determination was then compared with true species identity based on the molecular genetic analysis.

Given the normal distribution of the data we used factorial analysis of variance (ANOVA) to test the effect of species and sex on the forearm and tibia lengths and the body mass. We used Tukey test for post-hoc comparisons. The discriminant function analysis (DFA) was used to find best discriminating variables. In the first run we used the dataset including species, sex, forearm length, tibia length and body mass. Given the missing data on tibia length, this dataset was ca. four-times smaller (n=77) than the dataset used for final model that included sex, forearm length and body mass only (n=310). All analyses were performed using the Statistica 8.0 (Statsoft Inc.) software. If not specified, the values are presented as mean±S.D.

Results

Reliability of Field Determination

Altogether 359 individuals of *M. alcathoe, M. mystacinus* and *M. brandtii* were sampled at 18 localities (6 of them hosting *M. alcathoe*, 13 *M. mystacinus*, 9 *M. brandtii*) and identified to species using molecular genetic methods. Of these, 14 individuals (3.9%) were erroneously identified in the field based on external characters. Of 113 bats originally identified as *M. alcathoe* six were genetically identified as *M. mystacinus* (4 males, 2 females) and three as *M. brandtii* (2 males, 1 female), which means that 8% of the bats originally determined as *M. alcathoe* were in fact different species. In all 121 individuals of *M. mystacinus*, the original species determination was confirmed using the molecular genetic analysis (i.e. 0% determination error). Of 125 bats originally determined as *M. brandtii*, five (5 females) were assigned to *M. mystacinus* using molecular methods (4% determination error). While 9 of 14 erroneously determined bats were captured at a swarming site, where high numbers of bats (usually >100 in a netting event) of up to 16 species are usually captured, the remaining five misidentified bats were sampled at much less "busy" sites. Thirteen of these bats were adults and one was a juvenile.

External Characters

The average forearm length [in millimetres] was 31.9±0.82 (n=90) in *M. alcathoe*, 34.7±1.2 (n=118) in *M. mystacinus*, and 35.8±1.1 (n=102) in *M. brandtii*. It significantly differed between the three species ($F_{2,304}$=297.5; p<0.0001) but also varied with sex within each species sample ($F_{1,304}$=30.3; p<0.0001). While males and females did not differ in forearm length in *M. alcathoe* (p=0.56), males were significantly smaller than females in the two remaining species, *M. mystacinus* (p<0.001) and *M. brandtii* (p<0.05). Both sexes of *M. alcathoe* had a smaller forearm length than all *M. mystacinus* and *M. brandtii* (p<0.001 in all cases). While males of *M. mystacinus* had a smaller forearm length than both sexes of *M. brandtii* (p<0.001 in both cases), females of

Table 1. Biometric data given separately for each species and sex of *Myotis alcathoe*, *M. mystacinus* and *M. brandtii*. CI – confidence intervals, min – minimum, max – maximum
Tab. 1. Biometrické údaje v závislosti na druhu a pohlaví u *Myotis alcathoe*, *M. mystacinus a M. brandtii*. CI – konfidenční interval, min – minimum, max – maximum

	sex / pohlaví	n	mean / průměr	S.D.	–95 CI	+95 CI	min	max
forearm length / délka předloktí [mm]								
Myotis alcathoe	♂♂	47	31.8	0.7	31.6	32.0	30.3	33.5
	♀♀	43	32.1	0.9	31.8	32.4	30.0	33.6
Myotis mystacinus	♂♂	26	33.9	0.9	33.5	34.2	31.7	35.6
	♀♀	92	35.0	1.2	34.7	35.2	31.8	38.2
Myotis brandtii	♂♂	31	35.3	1.1	34.9	35.7	32.6	37.4
	♀♀	71	36.0	1.1	35.7	36.2	32.9	38.2
tibia length / délka tibie [mm]								
Myotis alcathoe	♂♂+♀♀	46	14.7	0.7	14.5	14.9	12.8	16.0
Myotis mystacinus	♂♂+♀♀	23	15.9	0.6	15.6	16.1	14.7	16.9
Myotis brandtii	♂♂+♀♀	8	16.2	1.1	15.3	17.1	14.5	17.4
body mass / hmotnost [g]								
Myotis alcathoe	♂♂	46	4.4	0.5	4.3	4.6	3.6	5.5
	♀♀	42	4.8	0.7	4.6	5.1	3.5	6.8
Myotis mystacinus	♂♂	26	4.9	0.5	4.7	5.1	4.0	5.8
	♀♀	91	5.7	0.6	5.6	5.9	4.5	7.8
Myotis brandtii	♂♂	31	5.9	0.9	5.6	6.2	4.8	9.0
	♀♀	68	6.4	0.7	6.2	6.5	4.7	8.5

M. mystacinus had a smaller forearm length than females of *M. brandtii* ($p<0.001$) but did not differ from males of *M. brandtii* ($p=0.59$). Detailed data on forearm length for each species and sex are given in Table 1. Despite statistically significant differences in forearm length among the

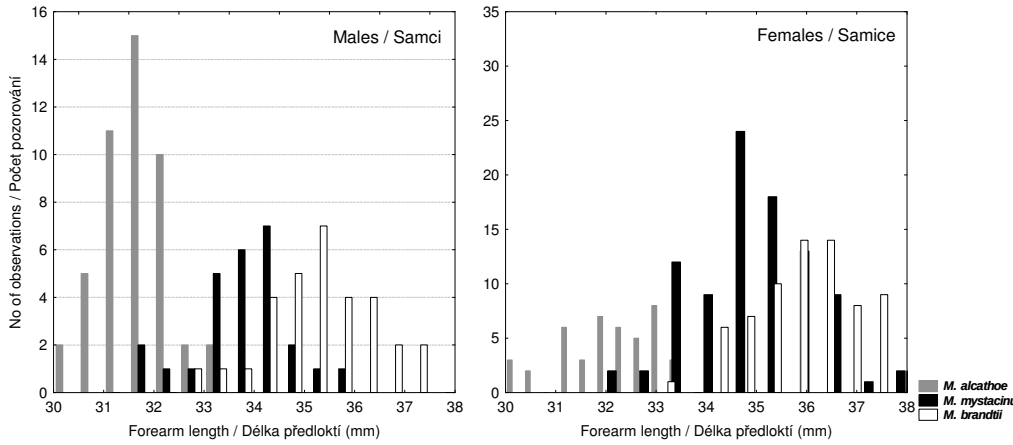

Fig. 1. Distribution of forearm length in males (left) and females (right) of *Myotis alcathoe*, *M. mystacinus* and *M. brandtii*.
Obr. 1. Rozložení délek předloktí u samců (vlevo) a samic (vpravo) *Myotis alcathoe*, *M. mystacinus* a *M. brandtii*.

species, there was a considerable overlap (Fig. 1). In males, the overlap of forearm lengths was 43.8% between *M. alcathoe* and *M. mystacinus*, 73.7% between *M. mystacinus* and *M. brandtii* and 7.7% between *M. alcathoe* and *M. brandtii*. In females, the overlap of forearm lengths was 29.6% between *M. alcathoe* and *M. mystacinus*, 96.9% between *M. mystacinus* and *M. brandtii* and 9.6% between *M. alcathoe* and *M. brandtii*.

The average length of tibia [in millimetres] was 14.7±0.7 (n=46) in *M. alcathoe*, 15.9±0.6 (n=23) in *M. mystacinus*, and 16.2±1.1 (n=8) in *M. brandtii*. While species identity had a significant effect on the length of tibia ($F_{2,71}$=12.6; p<0.0001), sex did not ($F_{1,71}$=0.98; p=0.16). *M. alcathoe* had a smaller tibia length than either of the remaining two species (p<0.001 in both cases) but *M. mystacinus* did not significantly differ from *M. brandtii* (p=0.44). Data on tibia length are given in Table 1. The overlap in tibia lengths was 55.1% between *M. alcathoe* and *M. mystacinus*, 80.6% between *M. mystacinus* and *M. brandtii* and 51.9% between *M. alcathoe* and *M. brandtii*.

The average body mass [in grams] was 4.6±0.6 in *M. alcathoe* (n=88), 5.6±0.7 in *M. mystacinus* (n=117) and 6.2±0.8 in *M. brandtii* (n=99). Species identity ($F_{2, 298}$=108.4; p<0.0001) and sex ($F_{1, 298}$=44.8; p<0.0001) had a significant effect on body mass. Males of *M. alcathoe* had a slightly (p=0.06) lower body mass than females and both sexes had a lower body mass than all *M. mystacinus* and *M. brandtii*, except for males of *M. mystacinus* which did not differ from females of *M. alcathoe* (p=0.9). Males of *M. mystacinus* had a lower (p<0.001) body mass than females and also had a lower body mass than both sexes of *M. brandtii* (p<0.001 in both cases). Females of *M. mystacinus* (p<0.001) had a lower body mass than females of *M. brandtii*, but did not differ (p=0.9) from males of *M. brandtii*. Detailed data on body mass are given in Table 1. In males, the overlap in body mass was 72.2% between *M. alcathoe* and *M. mystacinus*, 57.9% between *M. mystacinus* and *M. brandtii*, and 24.7% between *M. alcathoe* and *M. brandtii*. In females, it was 84.2% between *M. alcathoe* and *M. mystacinus*, 98.1% between *M. mystacinus* and *M. brandtii*, and 63.6% between *M. alcathoe* and *M. brandtii*.

External Quantitative Characters and Their Discrimination Power

The best model in DFA in the restricted dataset included sex ($F_{2, 69}$=4.93; p<0.01), forearm length ($F_{2, 69}$=14.55; p<0.0001), and body mass ($F_{2, 69}$=9.10; p<0.001), while the effect of tibia length was not significant ($F_{2, 69}$=2.20; p=0.12). Therefore, we built a new model including sex, forearm length and body mass only, which enabled us to considerably enlarge the analysed dataset (see Methods). All variables in a new model were also highly significant (sex: $F_{2, 299}$=11.10; p<0.0001; forearm length: $F_{2, 299}$=130.58; p<0.0001; body mass: $F_{2, 299}$=14.30; p<0.0001). Based on these three variables, the model correctly classified 94.3% of *M. alcathoe*, 70.9% of *M. mystacinus* and 68.7% of *M. brandtii*.

Forearm Length in Erroneously Determined Individuals

Forearm length of all *M. mystacinus* (n=6) and *M. brandtii* (n=3) misidentified as *M. alcathoe* was well bellow the mean values and confidence limits for the respective species. It ranged between 31.7–33.1 mm and 32.6–33.2 mm in misidentified *M. mystacinus* and *M. brandtii*, respectively. Forearm length of all *M. mystacinus* (n=5) misidentified as *M. brandtii* ranged between 34.8–36.1 mm, which is well within or slightly above the mean values and confidence limits of *M. mystacinus*.

Discussion

Our results showed that despite high precision in field identification, there were some misidentifications in our material and these were unevenly distributed among the three species of bats under

study. While no *M. alcathoe* was confused with the other two species, 11/132 individuals (8.3%) of *M. mystacinus* were confused with either *M. alcathoe* (4.5%) or *M. brandtii* (3.8%), and 3/128 individuals (2.3%) of *M. brandtii* were confused with *M. alcathoe*. This fact generally reflects the enormous phenotypic variation in the morpho-group, as well as the higher degree of overlap in external morphological characters between *M. mystacinus* and *M. brandtii* than between one of the latter species and *M. alcathoe*.

It is worth mentioning, however, that most of the erroneously identified bats (9/14) were captured and determined under busy circumstances, i.e. bat researchers were forced to quickly process a high number of individuals and, consequently, the error in determination could be higher than under usual conditions when a researcher can carefully inspect a captured bat. Furthermore, all individuals of *M. mystacinus* or *M. brandtii* that were confused with *M. alcathoe* were unusually small individuals and their size fell within the range of the latter species, which most probably affected the evaluation of the individual more than the other discrimination characters (e.g. ear colouration or dentition traits).

Spitzenberger et al. (2008) pointed out that as the fur and membrane colour of subadult individuals of *M. alcathoe* and *M. mystacinus* are similar, reliable records based on field identification should be restricted to adult individuals. However, despite our material consisted of a mixture of both adults and juveniles (the latter made up ca. 25% of all inds.), most of the misidentified bats in our analysis were adults. Hence we assume that the determination bias was mostly due to abnormal size (quantitative character) rather than to colouration (qualitative character).

Our analysis revealed that the three species significantly differ in forearm length, tibia length as well as body mass and that there is no overlap of forearm length values lying within 95% confidence limits when sex is taken into account. Application of these values as determination criteria may further improve the reliability of field determination for populations from Central Europe. Although the combination of sex, forearm length and body mass alone is not sufficient for a reliable discrimination among the three species (cf. ca. 69–94% correctness in determination by the results of DFA), inclusion of further discrimination characters (particularly the qualitative ones, see Introduction) may obviously largely improve precision as demonstrated by ca. 92–100% correct field determination in our study.

It is virtually useless to compare our measurements with those published for *M. mystacinus* prior to the end of 20th century (e.g. Hanák 1965, 1970, 1971, Benda & Tsytsulina 2000) as they most probably contain mixed data for *M. mystacinus* and *M. alcathoe* (see also Benda et al. 2003). However, it is possible to carry out such comparison for the other two species. The forearm and tibia lengths of *M. alcathoe* in our material are in accordance with the values reported from other European countries, e.g. Slovakia (Benda et al. 2003, Danko et al. 2010), Spain (Agirre-Mendi et al. 2004), Poland (Niermann et al. 2007, Bashta et al. 2011), Czech Republic (Řehák et al. 2008), Austria (Spitzenberger et al. 2008), Germany (Schorcht et al. 2009) and Ukraine (Bashta et al. 2011). The overall variation in forearm length was somewhat larger than reported in the original species diagnosis by von Helversen et al. (2001) but smaller than reported by Dietz et al. (2007). The upper limits of body mass in our material (6.8 g for females) exceeded the values given by von Helversen et al. (2001) as well as those by Dietz et al. (2007). However, the values at the upper limit of the range were obtained from pregnant females.

Also in *M. brandtii* our data on forearm and tibia lengths and body mass well correspond with the published information (e.g. Hanák 1965, 1970, 1971, Benda & Tsytsulina 2000, Dietz et al. 2007). Only the lower limit in forearm length in our material was somewhat smaller than in majority of the above cited studies.

Souhrn

Spolehlivost určení tří kryptických druhů netopýrů (*Myotis alcathoe, M. mystacinus, M. brandtii*) podle vnějších znaků a jejich základní biometrické údaje: zkušenosti z České republiky. Netopýr alkathoe, netopýr vousatý a netopýr Brandtův jsou nepříbuzné, ale morfologicky velmi podobné druhy žijící sympatricky na většině evropského území. Přestože v rámci terénní praxe jsou tyto druhy dnes víceméně rutinně odlišovány na základě vnější morfologie, doposud nebyla provedena žádná analýza, která by spolehlivost takovéhoto určování ověřila. Cílem naší studie bylo (1) pomocí molekulárně genetických metod ověřit správnost terénního určení druhu, (2) porovnat proměnlivost základních a v terénu standardně zaznamenávaných vnějších rozměrů (délka předloktí a holeně, tělesná hmotnost) a (3) ověřit spolehlivost jejich použití pro správné druhové určení na základě materiálu z území České republiky.

Čtrnáct z celkem 359 jedinců (3,9 %) těchto tří druhů bylo v terénu určeno nesprávně. Osm procent z počtu 113 jedinců původně určených jako *M. alcathoe* náleželo ve skutečnosti k jednomu ze dvou ostatních druhů (6 *M. mystacinus,* 3 *M. brandtii*). U všech jedinců (celkem 121) určených jako *M. mystacinus* byla správnost tohoto určení potvrzena. U pěti ze 125 jedinců (4 %) původně určených jako *M. brandtii* byla pomocí molekulárně genetických metod přiřazena druhová příslušnost k *M. mystacinus*. Většina chybně určených zvířat byla tvořena dospělci, avšak v případě záměn s *M. alcathoe* velikostně výrazně podprůměrnými jedinci. Dalším možným faktorem nesprávného určení (kromě samotné matoucí velikosti) mohla být skutečnost, že většina nesprávně identifikovaných jedinců byla určována během odchytů, při nichž bylo zpracováváno velké množství netopýrů, čímž mohla být míra pozornosti výzkumníků ovlivněna.

Všechny tři studované druhy se vzájemně významně lišily v délkách předloktí a holeně i v tělesné hmotnosti, přičemž vzájemný překryv byl vždy nejmenší mezi *M. alcathoe* a *M. brandtii*. Nejlepší model pro mezidruhovou diskriminaci zahrnoval pohlaví, délku předloktí a váhu jedince. Na základě diskriminační analysy byla s využitím těchto tří proměnných správnost druhové determinace 69–94 %. Tato skutečnost zdůrazňuje nutnost zohlednění nemetrických znaků (zbarvení srsti, čenichu, ucha, tvar a velikost tragu (kozlíku), penisu, a jednohrotých zubů), pro dosažení co možná nejvyšší míry spolehlivosti druhového určení těchto tří druhů.

Acknowledgements

We thank M. Andreas, T. Březinová, V. Hanák, H. Jahelková, M. Kipson, M. Lučanová, J. Neckářová, T. Svačina, M. Šálek, T. Vlasatá, A. Zieglerová for their help in the field. The research was supported by the Grant Agency of the Academy of Sciences of the Czech Republic (CR) (# KJB601410903) and the Ministry of Culture of the CR (# MK00002327201).

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received on 20 November 2011