MicroCT imaging reveals morphometric baculum differences for discriminating the cryptic species *Pipistrellus pipistrellus* and *P. pygmaeus*

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With the recent and continuing discovery of further cryptic bat species, it is essential to find morphological species discriminating characters. Pipistrellus pipistrellus (common pipistrelle) and Pipistrellus pygmaeus (soprano pipistrelle) have been recognized as separate species since 1997, but no reliable morphological species discriminating trait has yet been found. The most commonly used morphological species discrimination traits are 'wing vein' pattern and shape and color of the penis, but these have not been validated on sets of genetically identified specimens. The baculum (os penis) has long been used successfully in species discrimination in bats and other mammals. In this study, we tested the reliability of the established traits and demonstrated how to reliably separate the common pipistrelle and the soprano pipistrelle by simple baculum measurements. The bacula of museum specimens of these two species and of Pipistrellus hanaki were imaged with high-resolution microCT. Several measurements were taken on the size-calibrated volume images, and their value for species discrimination was tested by discriminant analysis with leave-one-out cross validation. We showed that P. pipistrellus and P. pygmaeus specimens can be discriminated by measuring the projected length, height, and width of the baculum (n = 48; all but one classified correctly). Geometric morphometrics was used to analyze and locate variations in baculum shape. Principal component analysis of baculum variation was not sufficient to separate these species. Most of the interspecific variation in baculum shape can be found in the proximal third (the base) of the baculum, and most individual variation can be observed in lateral view, especially in the dorsoventral curve. Quantitative details of morphology are becoming more important to distinguish cryptic species and understand their phylogeographic distributions. The simple baculum measurements can be used to classify single specimens and could be taken without microCT, on a resected baculum.

Key words: os penis, morphometrics, micro-CT, x-ray microtomography, Vespertilionidae

INTRODUCTION

Finding morphological characters to properly describe and discriminate newly discovered cryptic species is becoming an important challenge, with the growing number of species discovered due to the rise of molecular techniques in taxonomy. The problem posed by cryptic species to the traditional morphospecies concept has long been recognized (Mayr, 1942), and the widespread application of genetic tools has revealed that morphologically similar population groups can consist of several genetically distinct species (Baker *et al.*, 1995; Bruna *et al.*, 1996). In Palearctic bats, cryptic diversity has emerged as a major subject of research in the last decade. Cryptic species or species complexes in the Western Palearctic have been discovered in the genera *Eptesicus*, *Hypsugo*, *Myotis*, *Pipistrellus*, *Plecotus*, and *Rhinolophus* (Jones and Barlow, 2001; Mayer and von Helversen, 2001*a*, 2001*b*; von Helversen *et al.*, 2001; Hulva *et al.*, 2004*a*; Ibáñez *et al.*, 2006; Spitzenberger *et al.*, 2006; Mayer *et al.*, 2007; Benda and Vallo, 2012; Salicini *et al.*, 2013). Cryptic species frequently go undetected because their diagnostic features are in sensory modalities very different from our own, such as olfactory cues or the ultrasonic calls of microchiropteran bats (Davidson-Watts *et al.*, 2006).

The *Pipistrellus pipistrellus/Pipistrellus pygmaeus* species complex is an iconic example of this problem. The common pipistrelle, one of the most widespread European bat species and previously considered a monotypic taxon, actually comprises two cryptic species, Pipistrellus pipistrellus (Schreber, 1774) and Pipistrellus pygmaeus (Leach, 1825) (Jones and van Parijs, 1993; Barratt et al., 1995, 1997). Recently, further cryptic variation was discovered within the Mediterranean basin (Hulva et al., 2004a, 2007, 2010). The structure of the species complex comprises many lineages with small ranges in statu nascendi on Mediterranean islands (e.g., Pipistrellus hanaki on Crete) and peninsulas, and two sympatric continental sibling species. Thus the P. pipistrellus species complex is a remarkable model system for investigating links between morphology, echolocation calls, and niche partitioning (Davidson-Watts et al., 2006). In small Mediterranean demes (e.g., on Cyprus), hybridization has probably occurred in the past (Hulva et al., 2010). There is even some genetic evidence for hybridization still occurring in Central Europe (Sztencel-Jabłonka and Bogdanowicz, 2012).

Most of the recent studies on the genus Pipistrellus have focused on its genetic diversity, including the discovery of new cryptic species (e.g., Benda et al., 2004; Hulva et al., 2004a, 2007, 2010; Mayer et al., 2007; Racey et al., 2007; Bryja et al., 2009; Sztencel-Jabłonka and Bogdanowicz, 2012). Subsequently, the requirement has arisen to separate the new cryptic species morphologically as well as genetically. In the last decade a great number of studies have been published on one or both of the two cryptic species P. pipistrellus and P. pygmaeus, referring to differences in many respects and marking them as truly separate species. These have included studies on distribution (e.g., Mayer and von Helversen, 2001b; Benda et al., 2003; Řehák et al., 2004; Isaksen and Landsgard, 2007; Derouiche et al., 2009), habitat requirements (e.g., Davidson-Watts et al., 2006; Bartonička et al., 2008; Smith and Racey, 2009; Kaňuch et al., 2010; Kusch and Schmitz, 2013), behavior (e.g., Russ et al., 2005; Bartonička, 2008; Petrželková et al., 2009), physiology (e.g., Azzali et al., 2003; Abramochkin et al., 2006; Waters and Wong, 2007; Becker et al., 2013), and breeding biology (e.g., Aşan-Bayedmir and Albayrak, 2006; Bielik, 2007).

Even though much effort has been devoted to finding reliable morphologic species discriminating characters, so far none have been established. Still, the most useful methods to date for reliable determination of both species of the *P. pipistrellus*/

P. pygmaeus complex remain genetic sequence comparison and the analysis of echolocation calls (Mayer and von Helversen, 2001*b*; Benda *et al.*, 2003; Kaňuch *et al.*, 2007). Because the external characters of the two species are quite similar, many attempts to find distinguishing morphological parameters have failed (Pavlinić *et al.*, 2008). Measurable parameters like wing morphology (Barlow and Jones, 1999; Häussler *et al.*, 2000) and skull morphology (Barlow *et al.*, 1997; Sztencel-Jabłonka *et al.*, 2009) indicate that *P. pygmaeus* is slightly smaller than *P. pipistrellus*. However, these findings are limited by the fact that there is significant overlap in these characters between the two species (Barlow *et al.*, 1997).

Of the qualitative characters described, the 'wing vein' pattern, referring to the patterns of elastic bands and fibers in the bat wing (von Helversen and Holderied, 2003), and shape and color of the penis (Häussler et al., 2000) are used most frequently. Bartonička et al. (2010) also found that the distinctive musky smell of P. pygmaeus can be used to identify the species in live animals; however, this character can vary depending on age and physiological condition of the individual bat (Benda, personal observation). Qualitative characters can be useful if applied by someone with good knowledge of both species, for example the shorter muzzle of P. pygmaeus, an internarial ridge (hump between nostrils) in P. pygmaeus and different coloration of the face (Häussler et al., 2000; Ziegler et al., 2001; Sendor et al., 2002; Zöphel et al., 2002; Sattler, 2003; von Helversen and Holderied, 2003).

The use of baculum shape (penis bone, os penis, os glandis, or os priapi) as a possible species specific trait has been proposed by Ziegler et al. (2001), who also cautioned that to use this trait will first require examining the bacular shape in statistically relevant numbers of individuals. Bats show a great variety in shape and size of the baculum. Nevertheless, its basic forms are clade-specific. Thus they provide key diagnostic criteria, which have been used successfully as taxonomic characters in bats and other mammals, particularly in the speciose groups rich in convergent phenotype evolution. The largest family of bats, Vespertilionidae, can serve as a typical example (cf., Horáček and Hanák, 1985; Hill and Harrison, 1987). Phylogenies were based on baculum shape even before molecular techniques were developed. The polyphyly of the genera Pipistrellus, Plecotus, Hypsugo, and Eptesicus was discovered because of their marked differences in baculum shape, e.g. the reason for the separation of *Pipistrellus subflavus* was its distinctive baculum (Hill and Harrison, 1987).

In this study we aimed to (1) critically evaluate the reliability of the taxonomic characters penis color and shape, and 'wing vein' pattern, which also are used in field identification, using genetically identified specimens; and (2) demonstrate that baculum shape and size can be used as discriminating characters to consistently separate *P. pipistrellus* and *P. pygmaeus*.

MATERIAL AND METHODS

Specimens of *P. pipistrellus* and *P. pygmaeus pygmaeus* were used to test the reliability of species discriminating characters, while the other specimens were described only for comparison. 'Wing vein' pattern was studied on all bats. Penis morphology was studied on all but five (four *P. pipistrellus*, one *P. hanaki*) of the male bats (these *P. pipistrellus* were added to sample later and only the 'wing vein' pattern was examined; the baculum of the *P. hanaki* holotype had been resected, thus there was no penis to study). Baculum characters were studied on all but six (*P. pipistrellus*) of the male specimens (which were added to the sample after the microCT scanning had been done).

Age Determination

Specimen age was estimated by the ossification of the metacarpophalangeal joint of the third finger (Baagøe, 1977).

Evaluation of External Characters

'Wing vein' pattern

To evaluate this character, patterns of the elastic bands between the forearm and the metacarpal of the fifth finger (Fig. 2) were drawn, photographed, and described. For evaluating the 'wing vein' pattern as discriminating character, we followed the description by von Helversen and Holderied (2003). The pattern typical for *P. pipistrellus* only contains one uninterrupted 'cell', while the typical pattern for *P. pygmaeus* contains a second uninterrupted cell right above the first one (Fig. 2).



FIG. 1. Map of collecting localities of specimens of *P. pipistrellus* (◊), *P. pygmaeus* (▲), *P. pygmaeus cyprius* (■), and *P. hanaki* (●). For location names and collection dates see Appendix (map taken from www.weltkarte.com/welt/weltatlas/weltkarte-blank-vektorgrafik.htm, image in the public domain)



FIG. 2. Typical patterns of elastic bands and fibers in the wing ('wing vein' pattern), in *P. pipistrellus* (left) and *P. pygmaeus* (right). Diagnostic features are marked with white lines. While both species have an uninterrupted 'wing cell' between forearm (at elbow-height) and metacarpal of the 5th finger, differences can be seen in the 'wing cell' directly above. In the pattern typical for *P. pipistrellus* (left), this 'cell' is intersected by smaller elastic bands. In the pattern typical for *P. pygmaeus* (right) this 'cell' is uninterrupted

Penis morphology

The penes of the male specimens were studied under a stereomicroscope. Shape, color, and presence or absence of a median stripe were described. Drawings and photographs were made. The penes were categorized as typical for *P. pipistrellus*, or typical for *P. pygmaeus* according to Dietz and von Helversen (2004) and Dietz *et al.* (2007), or as intermediate (e.g. with the shape typical for one species, but the color typical for the other species).

The typical *P. pipistrellus* penis is shaped like a dorsoventrally flattened club. Its color usually is (dark-) gray with a lighter, whitish median stripe on the dorsal side of the glans (Dietz and von Helversen, 2004). Compared to *P. pipistrellus*, the typical penis shape of *P. pygmaeus* is more uniform in diameter, but it also widens towards the glans. Depending on reproductive state, penis color of *P. pygmaeus* can be orange, skincolored, or yellowish. It usually does not have a lighter median stripe (Dietz *et al.*, 2007).

Evaluation of Baculum Characters

MicroCT imaging

High resolution X-ray microtomographic (microCT) imaging was used to visualize and measure the bacula. Unstained whole bats in 70% ethanol were imaged without further treatment or dissection. The bats where mounted in 70% ethanol in sealed plastic test tubes. The scans were made with a high-resolution lab-based Xradia MicroXCT system (Zeiss X-Ray Microscopy, Pleasanton, CA, USA; www.xradia.com), which is equipped with a microfocus tungsten source, secondary optical magnification of the scintillator images, and a $2k \times 2k$ cooled CCD camera. Projection images were taken every 0.25° over a rotation of 180° (plus the cone angle — Metscher, 2011) with 60 sec exposure, 4× optical magnification, and a source voltage of 40 kV at 4-8 W. Tomographic sections were reconstructed with the XMReconstructor software (version 8.1) supplied with the Xradia system, resulting in reconstructed isotropic voxel sizes of 2.0-2.5 µm.

Measurements

In the male specimens the following projected measurements were taken on thick sections of the microCT volume images of the baculum with the Xradia TXM3DViewer software (version 1.1.6) supplied with the Xradia system: length, width, height, width of the forked tip, width of each of the branches of the base, space between the branches of the base in the middle of their length, and length of the indentation in the base (Fig. 3). All measurements were taken by the same person (ANH).



FIG. 3. Schematic drawing of the baculum measurements; dorsal view (left) and lateral view (right). L — projected length, W — projected width, H — projected height, T — projected width of the forked tip, B — projected width of each of the branches of the base, S — space between the branches of the base in the middle of their length, and I — length of the indentation in the base (arrows)

Statistical analyses

A discriminant analysis with leave-one-out cross validation between *P. pipistrellus* and *P. pygmaeus* was used on all the baculum measurements and on just the projected length, width, and height using PAST (http://nhm2.uio.no/norlex/past/download. html; Hammer *et al.*, 2001). Summary statistics of penis and 'wing vein' pattern characters were used to determine a percentage of individuals correctly identified by these traits.

Geometric morphometrics enables the statistical analysis of the shape of morphological structures based on Cartesian landmark coordinates, rather than linear, areal, or volumetric variables (Weber et al., 2001; Bogdanowicz, 2009; Mitteröcker and Gunz, 2009). Images of virtual thick sections of the bacula from microCT volume images were taken in lateral and in dorsal view, with the Xradia software package. All landmarks were set by the same person (ANH) on all of the images using tpsDig (http://life.bio.sunysb.edu/morph/; Adams et al., 2004; Mitteröcker and Gunz, 2009). On each dorsal image, 3 landmarks and 60 semi-landmarks were set. On each lateral image, one landmark and 50 semi-landmarks were set. The landmark sets were analyzed with tpsRelw (http://life.bio.sunysb.edu/morph/; http://www.wolfram.com/mathematica/new-in-9/?fp=middle; Adams et al., 2004; Mitteröcker and Gunz, 2009) and Mathematica (http://www.morpho-tools.net/index.html; Mitteröcker and Gunz, 2009; Madsen, 2013).

The used landmark configurations were superimposed by generalized Procrustes analysis (Rohlf and Slice, 1990) to standardize orientation, location and size. Semi-landmarks were allowed to slide along their corresponding curve to minimize bending energy. Bending energy is a measure of local shape differences between individuals and the average shape (Bookstein, 1997). We visualized average shape for all three species and average differences in shape between the species. We performed between-group principal component analyses (Mitteröcker and Bookstein, 2011) for dorsal and lateral shape.

RESULTS

'Wing Vein' Pattern

We were able to correctly classify 78.9% of the bats (n = 95; *P. pipistrellus* and *P. pygmaeus*) by the 'wing vein' pattern trait (Fig. 2 and Table 1) as proposed by von Helversen and Holderied (2003). Of the 66 *P. pipistrellus* specimens 58 were correctly identified, and two were classified as *P. pygmaeus*. Of the 29 *P. pygmaeus pygmaeus* 17 were correctly identified, and four were classified as *P. pipistrellus*.

Of the six *P. pygmaeus cyprius* only one showed a modified *P. pygmaeus* 'wing vein' pattern. This bat had three uninterrupted 'wing cells'. Two bats of this population showed a *P. pipistrellus*-like 'wing vein' pattern. *Pipistrellus hanaki* specimens exhibited a *P. pipistrellus*-like pattern most often. A *P. pygmaeus* like pattern was observed on two specimens. Different patterns on the left and right wing of the same bat where observed in six *P. pipistrellus*, eight *P. pygmaeus*, one *P. pygmaeus cyprius*, and five *P. hanaki*.

Penis Shape and Color

We were able to classify correctly 72.0% of the bats (n = 50, *P. pipistrellus* and *P. pygmaeus*) by penis shape and color (Table 2). Of the six *P. pygmaeus cyprius*, three had a *P. pygmaeus*-like penis, while the other three showed an intermediate penis color and shape. In *P. hanaki*, penis color and shape were most often intermediate between *P. pipistrellus* and *P. pygmaeus*.

Baculum Biometrics

A discriminant analysis was performed to determine whether baculum measurements can be used to discriminate *P. pipistrellus* and *P. pygmaeus*. The discriminant analysis between *P. pipistrellus* (n =30) and *P. pygmaeus pygmaeus* (n = 18; excluding the presumably hybrid population from Cyprus; Hulva *et al.*, 2010) using projected length (L), width (W), and height (H) of the baculum (Fig. 3) yielded the following discriminant function:

x = (L*0.030194 + W*0.06156 + H*0.024837) - 78.2545

New specimens can be classified by multiplying the measurements in μ m with the discriminant function factors for length (0.030194) width (0.06156) and height (0.024827) and subtracting the offset value (78.2545) from the resulting sum (Hammer *et al.*, 2001; Hammer, 2012). Specimens with a resulting value of x greater than 0 should be classified as *P. pipistrellus* and specimens with x less than 0 as *P. pygmaeus*. This function correctly assigned all but one of the bats in our sample (97.92%) to its genetically determined species (Fig. 4). This was verified by leave-one-out cross validation with each of the specimens.

TABLE 1. Percentage of specimens exhibiting typical 'wing vein' pattern for *P. pipistrellus* and *P. pygmaeus* (classified after von Helversen and Holderied, 2003)

Character	<i>P. pipistrellus</i> $(n = 66)$	P. pygmaeus $(n = 29)$	<i>P. hanaki</i> (<i>n</i> = 18)
P. pipistrellus pattern (%)	87.9	13.8	61.1
P. pygmaeus pattern (%)	3.0	58.6	11.1
Left and right wing different (%)	9.1	27.6	27.8

Character	<i>P. pipistrellus</i> $(n = 32)$	P. pygmaeus $(n = 18)$	<i>P. hanaki</i> (<i>n</i> = 8)
P. pipistrellus shape & color (%)	68.8	22.2	0
P. pygmaeus shape & color (%)	31.2	77.8	25.0
Intermediate (%)	0	0	75.0

TABLE 2. Percentage of specimens exhibiting typical penis shape and color for *P. pipistrellus* and *P. pygmaeus* (classified after Dietz and von Helversen, 2007)

Geometric Morphometrics of the Baculum

The variation of baculum shape within and between the three Pipistrellus species was visualized (Figs. 5 and 6) and quantified with geometric morphometrics, but the species could not be classified reliably by between-group principal component analyses of 2D shape variation, applied laterally or dorsally (Fig. 7). However, this analysis showed that most of the interspecific variation in baculum shape can be found in the proximal third (the base) of the baculum (Figs. 5 and 6). Generally, in the baculum of P. pipistrellus the two branches of the proximal bifurcated base are broader, shorter, and more clubshaped than in P. pygmaeus and in most P. hanaki. The branches of the base in the P. pipistrellus baculum are closer together, with a smaller angle than in P. pygmaeus and in most P. hanaki. The distal tip of the baculum also shows differences between the species: the baculum of P. pipistrellus tapers uniformly towards the forked tip gradually, while the baculum of P. pygmaeus typically narrows into



FIG. 4. Discriminant analysis, classifying *P. pipistrellus* (white, n = 30) and *P. pygmaeus* (black, n = 18) by projected measurements of baculum length, width, and height. One specimen of *P. pipistrellus* (NMP 94542) was misclassified as *P. pygmaeus*

a slender neck just proximal to the forked tip, where it widens again to a width similar to that of the *P. pipistrellus* baculum tip. *P. hanaki* displays an intermediate shape. Most of the individual variation can be observed in lateral view, in the shape of the curve (Fig. 5). In some of the bats, the baculum is curved evenly, while in others the base is rather straight and stands at an angle to the rest of the bone, which can have different degrees of curvature as well.

DISCUSSION

Pipistrellus pipistrellus and *P. pygmaeus* show a broad overlap in variation ranges of almost all phenotypic characters, including those for which certain discrimination value was suggested (Häussler *et al.*, 2000; von Helversen and Holderied, 2003). We reexamined this matter using a set of genetically identified specimens and found that only baculum measurements can be used to reliably distinguish males of these cryptic bat species.

Our study indicates that the 'wing vein' pattern species discriminating character should not be relied upon. Even if most of the P. pipistrellus specimens exhibit the pattern von Helversen and Holderied (2003) proposed, too many P. pygmaeus exhibit this pattern also. External shape and color of the penis might be slightly more reliable than the 'wing vein' pattern as a field character, but still should not be relied upon alone. They can be very useful in combination with other characters, such as face length and color, internarial ridge, and the smell of the bat, if used by someone with good knowledge of both species. In ethanol preserved or dried material, the colors fade and the shape is often hardly recognizable, so in this sample we were only able to classify 72% of the specimens correctly (fewer than with the 'wing vein' pattern trait).

A correct species identification can be achieved with the aid of a discriminant function calculated as a weighted sum of measurements of the projected length, width, and height of the baculum. The discriminant function was determined only for the two continental sibling species, which inhabit most of



FIG. 5. Average shape of the bacula of *P. pipistrellus* (left), *P. pygmaeus* (middle), and *P. hanaki* (right), in dorsal view (upper row) and in lateral view (lower row), calculated using geometric morphometrics

the Western Palearctic (the common pipistrelle *P. pipistrellus* sensu stricto and the soprano pipistrelle *P. pygmaeus* sensu stricto). The *P. pipistrellus* species complex includes several (mostly allopatric) populations inhabiting peninsulas and islands of the Mediterranean basin. These small Mediterranean populations show higher diversification in morphology and the situation is more complicated (Evin *et al.*, 2011). In the Mediterranean basin, reticulate evolution presumably occurred, and some populations are of hybrid origin (Cyprus, Maghreb).

Applying discrimination criteria to hybrid population is problematic, owing to many phenomena affecting their phenotypes, including higher variability of hybrids and occurrence of transgressive phenotypes. Therefore, for testing species discriminating characters, we did not include the six P. pygmaeus *cyprius*. These bats are from the morphologically and genetically distinct population on Cyprus and show different baculum characteristics (Fig. 7). They were originally classified as a subspecies of P. pygmaeus with mitochondrial DNA analysis (Benda et al., 2007). Later analysis showed their nuclear DNA to be closer to P. pipistrellus and there is evidence for hybridization between the two species in their distant past on Cyprus (Hulva et al., 2010).

Similarly the misclassification of one specimen in the discriminant function might also be due to hybridization. The one P. pipistrellus specimen (NMP 94542) that was misclassified as P. pygmaeus with the discriminant analysis of baculum measurements stems from the Maghrebian population in Morocco. The misclassified specimen shows intermediate character states, possibly owing to a hybrid origin. Phylogenetic distance of the Maghrebian P. pipistrellus from the continental form may be related to divergent evolutionary pathways of both lineages (Hulva et al., 2004b). A distant position of this lineage was observed also in the morphospace derived from geometric morphometric analysis of skulls (Evin et al., 2011). Moreover, cryptic diversity in mtDNA combined with homogenous nuclear pool indicates past reticulate evolution (Hulva et al., 2010). Hybridization may lead to peculiar phenotypic evolution, including transgressive phenotypes (Renaud et al., 2009). While the Maghrebian population is presumably sedentary and perhaps it was never in contact with the *P. pygmaeus* lineage, the opposite is true for P. pipistrellus sensu stricto, in which character displacement could not be excluded (Hulva et al., 2010). Considering the hypothesized



FIG. 6. Deformation grids showing exaggerated shape differences (1.5 times standard deviation) between the average shapes of *P. pipistrellus* (left) and *P. pygmaeus* (right), visualized using geometric morphometrics



FIG. 7. Between-group principal component analysis of dorsal baculum shape of *P. pipistrellus* (\diamond , *n* = 32), *P. pygmaeus* (\blacktriangle , *n* = 18), *P. hanaki* (\blacklozenge , *n* = 9), and *P. pygmaeus cyprius* (\blacksquare , *n* = 6)

role of the baculum in sexual selection (Eberhard, 1985; Arnqvist, 1998; Danielsson and Askenmo, 1999; House and Simmons, 2002; Hosken and Stockley, 2004; Lüpold *et al.*, 2004) and the possibility of interspecific hybridization of both forms (Hulva *et al.*, 2010; Sztencel-Jabłonka and Bogdanowicz, 2012), divergent selection may also act on penile and bacular morphology.

Owing to the lack of anatomical landmarks on the baculum, we applied 2D geometric morphometrics to virtual thick-sections of 3D microCT imaging data. In geometric morphometric analyses it is possible to use a large number of semi-landmarks, which are allowed to slide along a curve between neighboring landmarks, with only one fixed anatomical landmark. We were not able to classify the three species P. pipistrellus, P. pygmaeus, and P. hanaki with between-group principal component analysis (Fig. 7). However, it was very useful for visualizing and finding areas on the penis bone with maximal variation. In lateral view, the dorsoventral curve of the baculum outline shows large and overlapping individual variation, so it is not useful as a species discrimination character (Fig. 5). In dorsal view, most of the between-species variation could be observed in the base (the proximal third) of the baculum (Figs. 5 and 6).

The baculum measurements we introduced as a new reliable species discriminating character can be taken most precisely and non-destructively on museum material with microCT imaging. However, researchers who do not have access to a microCT system can still use this discriminating character to identify specimens by resecting the baculum and measuring it under a standard light microscope. High definition 2D X-ray imaging could also be used, if special care is taken to take radiographs at the correct angles. This, and the fact that a single specimen can now be identified by using the discriminant function we introduced here, makes the baculum a very good trait for discriminating dead P. pipistrellus and P. pygmaeus, especially for museum specimens. Unfortunately the equivalent of the baculum in females, the os clitoridis or baubellum, has seldom been studied in bats (Brown, 1967; Long and Frank, 1968) and has not been found in the pipistrelle species under study. Thus there is no comparable trait to use for species identification in females. The search for a reliable species identification character to be used on live bats in the field will also have to continue.

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LITERATURE CITED

- ABRAMOCHKIN, D. V., G. S. SUKHOVA, and L. V. ROZEN-SHTRAUKH. 2006. Effect of acetylcholine on the action potential in bat atrium and ventricle. Doklady Biological Sciences, 407: 121–122.
- ADAMS, D. C., F. J. ROHLF, and D. E. SLICE. 2004. Geometric morphometrics: ten years of progress following the 'revolution'. Italian Journal of Zoology, 71: 5–16.
- ARNQVIST, G. 1998. Comparative evidence for the evolution of genitalia by sexual selection. Nature, 393: 784–786.
- AŞAN-BAYEDMIR, N., and I. ALBAYRAK. 2006. A study on the breeding biology of some bat species in Turkey (Mammalia: Chiroptera). Turkish Journal of Zoology, 30: 103–110.
- AZZALI, G., M. L. ARCARI, B. SPAGGIARI, and G. ROMITA. 2003. Ultrastructural aspects of the follicular cells of the pars tuberalis in bats related to the seasonal cycle. The Anatomical Record, 273A: 763–771.
- BAAGØE, H. 1977. Age determination in bats. Videnskabelige Meddelelser Dansk Naturhistorisk Forening, 140: 53–92.
- BAKER, A. J., C. H. DAUGHERTY, R. COLBOURNE, and J. L. MC-LENNAN. 1995. Flightless brown kiwis of New Zealand possess extremely subdivided population structure and cryptic species like small mammals. Proceedings of the National Academy of Sciences of the United States of America, 92: 8254–8258.
- BARLOW, K. E., and G. JONES. 1999. Roosts, echolocation calls and wing morphology of two phonic types of *Pipistrellus pipistrellus*. Zeitschrift für Säugetierkunde, 64: 257–268.
- BARLOW, K. E., G. JONES, and E. M. BARRATT. 1997. Can skull morphology be used to predict ecological relationships between bat species? A test using two cryptic species of pipistrelle. Proceedings of the Royal Society, 264B: 1695–1700.
- BARRATT, E. M., M. W. BRUFORD, T. M. BURLAND, P. A. RACEY, and R. K. WAYNE. 1995. Characterization of mitochondrial DNA variability within the microchiropterean genus *Pipistrellus*: approaches and applications. Symposia of the Zoological Society of London, 67: 377–386.
- BARRATT, E. M., R. DEAVILLE, T. M. BURLAND, M. W. BRUFORD, G. JONES, P. A. RACEY, and R. K. WAYNE. 1997. DNA answers the call of pipistrelle bat species. Nature, 387: 138–139.
- BARTONIČKA, T. 2008. Cimex pipistrelli (Heteroptera, Cimicidae) and the dispersal propensity of bats: an experimental study. Parasitology Research, 104: 163–168.
- BARTONIČKA, T., A. BIELIK, and Z. ŘEHÁK. 2008. Roost switching and activity patterns in the soprano pipistrelle, *Pipistrellus pygmaeus*, during lactation. Annales Zoologici Fennici, 45: 503–512.
- BARTONIČKA, T., P. KAŇUCH, B. BÍMOVÁ, and J. BRYJA. 2010. Olfactory discrimination between two cryptic species of bats *Pipistrellus pipistrellus* and *P. pygmaeus*. Folia Zoologica, 59: 175–182.
- BECKER, N. I., J. A. ENCARNAÇÃO, M. TSCHAPKA, and E. K. V. KALKO. 2013. Energetics and life-history of bats in comparison to small mammals. Ecological Research, 28: 249–258.
- BENDA, P., and P. VALLO. 2012. New look on the geographical variation in *Rhinolophus clivosus* with description of a new horseshoe bat species from Cyrenaica, Libya. Vespertilio, 16: 69–96.

- BENDA, P., P. HULVA, M. ANDREAS, and M. UHRIN. 2003. Notes on the distribution of *Pipistrellus pipistrellus* complex in the Eastern Mediterranean: first records of *P. pipistrellus* for Syria and of *P. pygmaeus* for Turkey. Vespertilio, 7: 87–95.
- BENDA, P., P. HULVA, and J. GAISLER. 2004. Systematic status of African populations of *Pipistrellus pipistrellus* complex (Chiroptera: Vespertilionidae), with a description of a new species from Cyrenaica, Libya. Acta Chiropterologica, 6: 193–217.
- BENDA, P., V. HANÁK, I. HORÁČEK, P. HULVA, R. LUČAN, and M. RUEDI. 2007. Bats (Mammalia: Chiroptera) of the eastern Mediterranean. Part 5. Bat fauna of Cyprus: review of records with confirmation of six species new for the island and description of a new subspecies. Acta Societatis Zoologicae Bohemicae, 71: 71–130.
- BIELIK, A. 2007. Letová aktivita netopierov z reprodukčnej kolónie *Pipistrellus pygmaeus* v laktačnom a postlaktačnom období s využitím telemetrie a detektoringu. M.Sci. Thesis, Masarykova Univerzita, Brno, 61 pp.
- BOGDANOWICZ, W. 2009. Analysis of bat morphology. Pp. 409– 435, *in* Ecological and behavioral methods for the study of bats, 2nd edition (T. H. KUNZ and S. PARSONS, eds.). Johns Hopkins University Press, Baltimore, 901 pp.
- BOOKSTEIN, F. 1997. Landmark methods for forms without landmarks: morphometrics of group differences in outline shape. Medical Image Analysis, 1: 225–243.
- BROWN, R. E. 1967. Bacula of some New World molossid bats. Mammalia, 31: 645–667.
- BRUNA, E. M., R. N. FISHER, and T. J. CASE. 1996. Morphological and genetic evolution appear decoupled in Pacific skinks (Squamata: Scincidae: *Emoia*). Proceedings of the Royal Society, 263B: 681–688.
- BRYJA, J., P. KAŇUCH, A. FORNŮSKOVÁ, T. BARTONIČKA, and Z. ŘEHÁK. 2009. Low population genetic structuring of two cryptic bat species suggests their migratory behaviour in continental Europe. Biological Journal of the Linnean Society, 96: 103–114.
- DANIELSSON, I., and C. ASKENMO. 1999. Male genital traits and mating interval affect male fertilisation success in the water strider *Gerris lacustris*. Behavioral Ecology and Sociobiology, 46: 149–156.
- DAVIDSON-WATTS, I., S. WALLS, and G. JONES. 2006. Differential habitat selection by *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus* identifies distinct conservation needs for cryptic species of echolocating bats. Biological Conservation, 133: 118–127.
- DEROUICHE, S., M. DEVILLE, M. L. TAYLOR, H. AKBAR, J. GUILLOT, L. E. CARRETO-BINAGHI, M. POTTIER, E. M. ALIOUAT, C. M. ALIOUAT-DENIS, E. DEI-CAS, *et al.* 2009. Pneumocystis diversity as a phylogeographic tool. Memórias do Instituto Oswaldo Cruz, 104: 112–117.
- DIETZ, C., and O. VON HELVERSEN. 2004. Illustrated identification key to the bats of Europe. Version 1.0 — electronic publication, 72 pp. Available at http://www.fledermausdietz.de/publications/publications.html.
- DIETZ, C., O. VON HELVERSEN, and D. NILL. 2007. Handbuch der Fledermäuse Europas und Nordwestafrikas. Franckh-Kosmos Verlags GmbH, Stuttgart, 400 pp.
- EBERHARD, W. G. 1985. Sexual selection and animal genitalia. Harvard University Press Cambridge, MA, x + 244 pp.
- EVIN, A., I. HORAČEK, and P. HULVA. 2011. Phenotypic diversification and island evolution of pipistrelle bats (*Pipistrellus pipistrellus* group) in the Mediterranean region inferred

from geometric morphometrics and molecular phylogenetics. Journal of Biogeography, 38: 2091–2105.

- HAMMER, Ø. 2012. PAST PAleontological STatistics Version 2.17. Reference manual. Natural History Museum, University of Oslo, Oslo, 229 pp.
- HAMMER, Ø., D. A. T. HARPER, and P. D. RYAN. 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica, 4: 1–9. http:// palaeo-electronica.org/2001_1/past/issue1_01.htm.
- HÄUSSLER, U., A. NAGEL, M. BRAUN, and A. ARNOLD. 2000. External characters discriminating sibling species of european pipistrells, *Pipistrellus pipistrellus* (Schreber, 1774) and *P. pygmaeus* (Leach, 1825). Myotis, 37: 27–40.
- HILL, J. L., and D. L. HARRISON. 1987. The baculum in the Vespertilioninae (Chiroptera: Vespertilionidae) with a systematic review, a synopsis of *Pipistrellus* and *Eptesicus*, and the descriptions of a new genus and subgenus. Bulletin of the British Museum (Natural History) Zoology, 52: 225–305.
- HORÁČEK, I., and V. HANÁK. 1985. Generic status of *Pipistrellus savii* and comments on classification of the genus *Pipistrellus* (Chiroptera, Vespertilionidae). Myotis, 23–24: 9–16.
- HOSKEN, D. J., and P. STOCKLEY. 2004. Sexual selection and genital evolution. Trends in Ecology and Evolution, 19: 87–93.
- HOUSE, C. M., and L. W. SIMMONS. 2002. Genital morphology and fertilization success in the dung beetle *Onthophagus taurus*: an example of sexually selected male genitalia. Proceedings of the Royal Society, 270B: 447–455.
- HULVA, P., I. HORÁČEK, P. P. STRELKOV, and P. BENDA. 2004a. Molecular architecture of *Pipistrellus pipistrellus/Pipistrellus pygmaeus* complex (Chiroptera: Vespertilionidae): further cryptic species and Mediterranean origin of the divergence. Molecular Phylogenetics and Evolution, 32: 1023–1035.
- HULVA, P., I. HORÁČEK, P. P. STRELKOV, and P. BENDA. 2004b. Phylogeography of *Pipistrellus pipistrellus/P. pygmaeus* complex (Chiroptera: Vespertilionidae). Bat Research News, 45: 120.
- HULVA, P., P. BENDA, V. HANÁK, A. EVIN, and I. HORÁČEK. 2007. New mitochondrial lineages within the *Pipistrellus pipi-strellus* complex from Mediterranean Europe. Folia Zoologica, 56: 378–388.
- HULVA, P., A. FORNŮSKOVÁ, A. CHUDÁRKOVÁ, A. EVIN, B. ALLE-GRINI, P. BENDA, and J. BRYJA. 2010. Mechanisms of radiation in a bat group from the genus *Pipistrellus* inferred by phylogeography, demography and population genetics. Molecular Ecology, 19: 5417–5431.
- IBÁÑEZ, C., J. L. GARCÍA-MUDARRA, M. RUEDI, B. STADELMANN, and J. JUSTE. 2006. The Iberian contribution to cryptic diversity in European bats. Acta Chiropterologica, 8: 277–297.
- ISAKSEN, K., and M. LANDSGARD. 2007. The soprano pipistrelle *Pipistrellus pygmaeus* confirmed as a wintering species in Norway. Fauna (Oslo), 60: 212–225.
- JONES, G., and K. E. BARLOW. 2001. Cryptic species of echolocating bats. Pp. 345–349, *in* Echolocation in bats and dolphins (J. A. THOMAS, C. F. MOSS, and M. VATER, eds.). University of Chicago Press, Chicago, xxvii + 604 pp.
- JONES, G., and S. M. VAN PARIJS. 1993. Bimodal echolocation in pipistrelle bats: are cryptic species present? Proceedings of the Royal Society, 251B: 119–125.
- KAŇUCH, P., P. HÁJKOVÁ, Z. ŘEHÁK, and J. BRYJA. 2007. A rapid PCR-based test for species identification of two cryptic

bats *Pipistrellus pipistrellus* and *P. pygmaeus* and its application on museum and dropping samples. Acta Chiropterologica, 9: 277–282.

- KAŇUCH, P., A. FORNŮSKOVÁ, T. BARTONIČKA, J. BRYJA, and Z. ŘEHÁK. 2010. Do two cryptic pipistrelle bat species differ in their autumn and winter roosting strategies within the range of sympatry? Folia Zoologica, 59: 102–107.
- KUSCH, J., and A. SCHMITZ. 2013. Environmental factors affecting the differential use of foraging habitat by three sympatric species of *Pipistrellus*. Acta Chiropterologica, 15: 57–67.
- LONG, C. A., and T. FRANK. 1968. Morphometric variaton and function in the baculum, with comments on correlation of parts. Journal of Mammalogy, 49: 32–43.
- LÜPOLD, S., A. G. MCELLIGOTT, and D. J. HOSKEN. 2004. Bat genitalia: allometry, variation and good genes. Biological Journal of the Linnean Society, 83: 497–507.
- MADSEN, M. Ø. 2013. Phenotypic divergence of the three-spined stickleback (*Gasterosteus aculeatus*) — relating body shape and habitat. M.Sci. Thesis, Universität Wien, Vienna, 71 pp.
- MAYER, F., and O. VON HELVERSEN. 2001*a*. Cryptic diversity in European bats. Proceedings of the Royal Society, 268B: 1825–1832.
- MAYER, F., and O. VON HELVERSEN. 2001b. Sympatric distribution of two cryptic bat species across Europe. Biological Journal of the Linnean Society, 74: 365–374.
- MAYER, F., C. DIETZ, and A. KIEFER. 2007. Molecular species identification boosts bat diversity. Frontiers in Zoology, 4: 5.
- MAYR, E. 1942. Systematics and the origin of species. Columbia University Press, New York, xxxv + 334 pp.
- METSCHER, B. D. 2011. X-ray microtomographic imaging of intact vertebrate embryos. Cold Spring Harbor Protocols, 2011: 1462–1471.
- MITTERÖCKER, P., and F. BOOKSTEIN. 2011. Linear discrimination, ordination, and the visualization of selection gradients in modern morphometrics. Evolutionary Biology, 38: 100–114.
- MITTERÖCKER, P., and P. GUNZ. 2009. Advances in geometric morphometrics. Evolutionary Biology, 36: 235–247.
- PAVLINIĆ, I., N. TVRTKOVIĆ, and D. HOLCER. 2008. Morphological identification of the soprano pipistrelle (*Pipistrellus pygmaeus* Leach, 1825) in Croatia. Hystrix — Italian Journal of Mammalogy (N.S.), 19: 47–53.
- PETRŽELKOVÁ, K. J., N. C. DOWNS, J. ZUKAL, and P. A. RACEY. 2009. A comparison between emergence and return activity in pipistrelle bats *Pipistrellus pipistrellus* and *P. pygmaeus*. Acta Chiropterologica, 8: 381–390.
- RACEY, P. A., E. M. BARRATT, T. M. BURLAND, R. DEAVILLE, D. GOTELLI, G. JONES, and S. B. PIERTNEY. 2007. Microsatellite DNA polymorphism confirms reproductive isolation and reveals differences in population genetic structure of cryptic pipistrelle bat species. Biological Journal of the Linnean Society, 90: 539–550.
- ŘEHÁK, Z., T. BARTONIČKA, and A. BIELIK. 2004. Distributional status of *Pipistrellus pipistrellus* (Schreber, 1774) and *P. pygmaeus* (Leach, 1825) in the Czech Republic: results of mapping. Bat Research News, 45: 145.
- RENAUD, S., P. ALIBERT, and J.-C. AUFFRAY. 2009. Mandible shape in hybrid mice. Naturwissenschaften, 96: 1043–1050.
- ROHLF, F., and D. SLICE. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. Systematic Biology, 39: 40–59.

- RUSS, J. M., G. JONES, and P. A. RACEY. 2005. Responses of soprano pipistrelles, *Pipistrellus pygmaeus*, to their experimentally modified distress calls. Animal Behaviour, 70: 397–404.
- SALICINI, I., C. IBÁÑEZ, and J. JUSTE. 2013. Deep differentiation between and within Mediterranean glacial refugia in a flying mammal, the *Myotis nattereri* bat complex. Journal of Biogeography, 40: 1182–1193.
- SATTLER, T. 2003. Ecological factors affecting the distribution of the sibling species *Pipistrellus pygmaeus* and *Pipistrellus pipistrellus* in Switzerland. M.Sci. Thesis, Universität Bern, Bern, 78 pp.
- SENDOR, T., I. ROEDENBECK, S. HAMPL, M. FERRERI, and M. SI-MON. 2002. Revision of morphological identification of pipistrelle bat phonic types (*Pipistrellus pipistrellus* Schreber, 1774). Myotis, 40: 11–17.
- SMITH, P. G., and P. A. RACEY. 2009. Selection of timber mortises in a church roof by *Pipistrellus* sp. at 52 degrees N. Acta Chiropterologica, 11: 205–207.
- SPITZENBERGER, F., P. P. STRELKOV, H. WINKLER, and E. HARING. 2006. A preliminary revision of the genus *Plecotus* (Chiroptera, Vespertilionidae) based on genetic and morphological results. Zoologica Scripta, 35: 187–230.
- SZTENCEL-JABLONKA, A., and W. BOGDANOWICZ. 2012. Population genetics study of common (*Pipistrellus pipistrellus*) and soprano (*Pipistrellus pygmaeus*) pipistrelle bats from central Europe suggests interspecific hybridization. Canadian Journal of Zoology, 90: 1251–1260.
- SZTENCEL-JABŁONKA, A., G. JONES, and W. BOGDANOWICZ. 2009. Skull morphology of two cryptic bat species: *Pipi-strellus pipistrellus* and *P. pygmaeus* — a 3D geometric

morphometrics approach with landmark reconstruction. Acta Chiropterologica, 11: 113–126.

- VON HELVERSEN, O., and M. HOLDERIED. 2003. Zur Unterscheidung von Zwergfledermaus (*Pipistrellus pipistrellus*) und Mückenfledermaus (*Pipistrellus mediterraneus/pygmaeus*) im Feld. Nyctalus (N.F.), 8: 420–426.
- VON HELVERSEN, O., K. G. HELLER, F. MAYER, A. NEMETH, M. VOLLETH, and P. GOMBKÖTÖ. 2001. Cryptic mammalian species: a new species of whiskered bat (*Myotis alcathoe* n. sp.) in Europe. Naturwissenschaften, 88: 217–223.
- WATERS, D. A., and J. G. WONG. 2007. The allocation of energy to echolocation pulses produced by soprano pipistrelles (*Pipistrellus pygmaeus*) during the wingbeat cycle. The Journal of the Acoustical Society of America, 121: 2990–3000.
- WEBER, G. W., K. SCHÄFER, H. PROSSINGER, P. GUNZ, P. MIT-TERÖCKER, and H. SEIDLER. 2001. Virtual anthropology: the digital evolution in anthropological sciences. Journal of Physiological Anthropology and Applied Human Science, 20: 69–80.
- ZIEGLER, T., A. FEILER, and U. ZÖPHEL. 2001. New data on the genital morphology of the midge bat *Pipistrellus pygmaeus* (Leach, 1825) from Germany (Mammalia: Chiroptera: Vespertilionidae). Zoologische Abhandlungen (Dresden), 51: 435–444.
- ZÖPHEL, U., T. ZIEGLER, A. FEILER, and S. POCHA. 2002. First records of the midge bat, *Pipistrellus pygmaeus* (Leach, 1825), for Saxony (Mammalia: Chiroptera: Vespertilionidae). Faunistische Abhandlungen Staatliches Museum für Tierkunde Dresden, 22: 411–422.

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Appendix

List of the material examined

Pipistrellus hanaki Hulva et Benda, 2004 — Libya: 1 3, 2 ♀♀ (NMP 49890–49892 [S+A]), 'Arqub Ash Shafshaf, 18 May 2002, leg. M. Andreas, P. Benda, V. Hanák, A. Reiter and M. Uhrin; – 4 ♀♀ (NMP 49894–49897 [S+A]), Wadi Al Kuf, Al Bayda, 19 May 2002, leg. M. Andreas, P. Benda, V. Hanák, A. Reiter and M. Uhrin; $-1 \stackrel{?}{\circ}, 2 \stackrel{\bigcirc}{\circ} (\text{NMP 49903},$ 49904 [S+A], 49902 [A]), Wadi Al Kuf, Massah, 20 May 2002, leg. M. Andreas, P. Benda, V. Hanák, A. Reiter and M. Uhrin; -1 Q (NMP 49884 [S+A]), Wadi Al Minshiyah, 17 May 2002, leg. M. Andreas, P. Benda, V. Hanák, A. Reiter and M. Uhrin. Greece, Crete: 1 d (NMP 92344 [S+A]), Monastiraki, 2 June 2008, leg. P. Benda, P. Georgiakakis and V. Hanák; - 5 33 (NMP 92349-92351, 92353 [S+A], 92352 [A]), Rouva forest, 4 June 2008, leg. P. Benda, P. Georgiakakis and V. Hanák; – 1 ♂ (NMP 92323 [S+A]), Zaros, 25 May 2008, leg. P. Benda and V. Hanák.

Pipistrellus pipistrellus (Schreber, 1774) — Czech Republic: $1 \Leftrightarrow (NMP 90013 [S+A])$, Brno, 19 August 2000, leg. Z. Řehák. Iran: $1 \swarrow (NMP 90832 [S+A])$, Emam Qoli, 24 May 2006, leg. P. Benda and A. Reiter; $-1 \Leftrightarrow (NMP 90776 [S+A])$, Karizbalagh, 17 May 2006, leg. P. Benda and A. Reiter; $-1 \diamondsuit$

(NMP 90791 [S+A]), Shurlaq, 18 May 2006, leg. P. Benda and A. Reiter; - 2 ♂♂, 2 ♀♀ (NMP 90838, 90840, 90841 [S+A], 90839 [A]), Tunel-e Golestan, 26 May 2006, leg. P. Benda and A. Reiter; – 1 ♀ (NMP 48113 [S+A]), Yazd, 4 May 1997, leg. P. Benda. Lebanon: 3 $\bigcirc \bigcirc$ (NMP 93534–93536 [A]), Adonis, Nahr Ibrahim, 1 June 2010, leg. P. Benda and M. Uhrin; $-4 \stackrel{?}{\circ} \stackrel{?}{\circ}$, 1 $\hfill \ensuremath{\mathbb{Q}}$ (NMP 91895, 91896. le102, le103, le105 [S+A]), Afqa Cave, 26 June 2006, 17 January 2008, leg. P. Benda, I. Horáček, P. Hulva, R. Lučan and M. Uhrin; – 2 ♀♀ (NMP 93531, 93532 [A]), Balaa, 31 May 2010, leg. P. Benda and M. Uhrin; -1 ♂ (NMP le101 [S+A]), Er Rouais Cave, 26 June 2006, leg. I. Horáček, P. Hulva and R. Lučan; $-2 \stackrel{\bigcirc}{\downarrow} \stackrel{\bigcirc}{\downarrow}$ (NMP 93547, 93548 [A]), Faraya El Mzar, 2 July 2010, leg. P. Benda and M. Uhrin; -1 $^{\circ}$, 1 $^{\circ}$ (NMP 91902, 91903 [S+A]), Hagel El Aazime, 18 January 2008, leg. P. Benda, I. Horáček, R. Lučan and M. Uhrin; -1 ♀ (NMP 93572 [A]), Jenta, 8 June 2010, leg. P. Benda and M. Uhrin; -6 ♂♂ (NMP le19–le22, le24 [S+A], le26 [A]), Jezzine, 23 June 2006, leg. I. Horáček, P. Hulva and R. Lučan; $-2 \Im \Im$ (NMP le12, le13 [S+A]), Nahr Es Safa, 22 June 2006, leg. I. Horáček, P. Hulva and R. Lučan; – 1 ♂ (NMP le118 [S+A]), Qadicha Cave, 27 June 2006, leg. I. Horáček, P. Hulva

APPENDIX. Continued

and R. Lučan; $-2 \stackrel{?}{\supset} \stackrel{?}{\supset}, 1 \stackrel{?}{\subsetneq}$ (NMP le169, le193, le212 [S+A]), Ras El Assi, 29 June 2006, leg. I. Horáček, P. Hulva and R. Lučan. Morocco: 4 ♂♂, 3 ♀♀ (NMP 94540–94545, 94546 [S+A]), Bekrite, 28 April 2008, leg. P. Benda, J. Červený, A. Konečný and P. Vallo. Slovakia: 2 33, 5 99 (NMP 90016– 90022 [S+A]), Ernya Cave, 20 April 2003, leg. P. Benda, V. Hanák and M. Uhrin. – 1 ♂, 1 ♀ (NMP 90014, 90015 [S+A]), Tisovec, 24 July 2002, leg. P. Benda, E. Hapl and M. Uhrin. Syria: 2 ♀♀ (NMP 48084, 48085 [S+A]), Ar Rawda, 1 July 1998, leg. M. Andreas, P. Benda & M. Uhrin; $-1 \stackrel{\bigcirc}{\downarrow}$ (NMP 48902 [S+A]), Baniyas, 31 May 2001, leg. M. Andreas, P. Benda, A. Reiter and D. Weinfurtová; - 4 33 (NMP 48981-48984 [S+A]), Maalula, 30 April 2001, leg. R. Lučan; -1 Q (NMP 49986 [S+A]), Ras Al Bassit, 29 April 2001, leg. R. Lučan; – 1 ♂, 1 ♀ (NMP 48871, 48872 [S+A]), Sarghaya, 28 May 2001, leg. M. Andreas, P. Benda, A. Reiter and D. Weinfurtová; - 3 강강 (NMP 48060-48062 [S+A]), Slinfeh, 29 June 1998, leg. M. Andreas, P. Benda and M. Uhrin.

 Pipistrellus pygmaeus (Leach, 1825)
 Cyprus: 1

 \circlearrowleft (NMP 90420 [S+A]), Agios Nikolaos, Kelefou bridge, 16 April

 2005, leg. P. Benda and V. Hanák; $-4 & \checkmark$ (NMP 90413, 90414, 90416, 90417 [S+A]), Kalidonia Trail, Troodos, 12 April 2005, leg. P. Benda, V. Hanák and I. Horáček; $-1 & \checkmark$

[S+A]), Troodos Forest, 11 April 2005, leg. P. Benda and V. Hanák. Czech Republic: 1 ♀ (NMP 90135 [S+A]), Kolence u Třeboně, 1 August 2002, leg. V. Hanák; – 1 ♀ (NMP 90139 [S+A]), Lednice, 22 June 2001, leg. Z. Řehák; – 2 $\bigcirc \bigcirc$ (NMP 90023, 90136 [S+A]), Malý Ratmírov, 2 July 2002, leg. V. Hanák; - 1 ♂ (NMP 90138 [S+A]), Mláka u Třeboně, 5 August 2002, leg. V. Hanák; - 1 4 (NMP 90137 [S+A]), Třeboň, 29 July 2002, leg. V. Hanák. Greece: 1 3 (NMP 49021 [S+A]), Artiki, 25 August 2001, leg. P. Benda; - 1 d (NMP 49030 [S+A]), Anthiro, 31 August 2001, leg. P. Benda; -1 ♂, 1 ♀ (NMP 49040, 49041 [S+A]), Dimitra, 1 September 2001, leg. P. Benda; – 1 👌 (NMP 49016 [S+A]), Simopoulo, 23 August 2001, leg. P. Benda; - 1 \circlearrowleft (NMP 48738 [S+A]), Sparti, 16 September 1996, leg. P. Benda and M. Uhrin. Iran: 5 ♂♂, 4 ♀♀ (NMP 90873–90877, 90879, 90880 [S+A], 90878, 90881 [A]), Ali Abad, 28 May 2006, leg. P. Benda and A. Reiter; - 5 33 (NMP 90885-90887, 90889 [S+A], 90888 [A]), Tutaki, 3 June 2006, leg. P. Benda and A. Reiter. **Turkey**: $2 \ 3 \ 3$, $1 \ 9$ (NMP 47946, 90011 [S+A], 47947 [A]), Velika Köprüsü, 30 August 1996, 7;; May 2001, leg. M. Andreas, P. Benda, A. Reiter, M. Uhrin and D. Weinfurtová.