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Description of a new species of the genus *Aselliscus* (Chiroptera, Hipposideridae) from Vietnam

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Trident bats found in mainland Southeast Asia are currently subsumed into a single species, *Aselliscus stoliczkanus*. In this study, we examined morphological and genetic data from different populations from Southeast Asia, with a special focus on specimens from Vietnam. Our analyses support the existence of a further species of *Aselliscus* in northeastern Vietnam that separated from *A. stoliczkanus* sensu lato (s.l.) during the late Miocene. Within the latter taxon, we identified five geographic lineages that diverged from each other during the Plio-Pleistocene epoch. Some of them may also correspond to further separate taxa, but additional molecular and morphological data are needed to test this hypothesis. Herewith, based on the combined evidences we describe the northeastern Vietnamese population as a separate species.

Key words: taxonomy, phylogeography, mtDNA, morphology, karst, bat, Southeast Asia

Introduction

Stoliczka's trident bat, *Aselliscus stoliczkanus* (original spelling is *Asellia stoliczkana*; type locality: Penang island, Peninsular Malaysia) (Dobson, 1871) is a small species of the family Hipposideridae that roosts in caves and forages in cluttered microhabitats in both intact and disturbed forests of northern Southeast Asia, from Myanmar and southern China in the North through Thailand, Laos and Vietnam to Pulau Tioman island, Peninsular Malaysia in the South (Fig. 1) (Lekagul and McNeely, 1977; Zubaid, 1988; Struebig *et al.*, 2005; Li *et al*., 2007; Bates *et al*., 2008; Francis, 2008). Its sister-species, *Aselliscus tricuspidatus*, is found on the Molucca Islands, in New Guinea, on the Bismarck Archipelago, on the Solomon Islands, on Vanuatu and adjacent small islands (Corbet and Hill, 1992; Simmons, 2005). The two species of *Aselliscus* overlap in body size, but *A. tricuspidatus* was known to have a slightly longer forearm and tail (Sanborn, 1952). They can be distinguished by several discrete morphological characters: i.e., the upper margin of the posterior noseleaf (Zubaid, 1988); the outline of the rostrum; the extent and position of the upper expansion of the zygoma; and the position and relative size of the second lower premolar (Sanborn, 1952).

Dobson's (1871) description was published just before Peters' (1871) paper, who described a new trident bat species from Myanmar (without precise locality) named *Phyllorhina trifida* (=*A. trifidus*), which was then treated as synonym of *A. stoliczkanus* by Dobson (1876). Later, Osgood (1932) described a new species, *Triaenops wheeleri* from northwestern Vietnam (locality: Muong Muon) also considered as a synonym of *A. stoliczkanus* by several authors (Tate, 1941; Sanborn, 1952; Corbet and Hill, 1992). Currently, trident bats found in Mainland Southeast Asia are regarded as representatives

of a single species, *A. stoliczkanus* (Lekagul and McNeely, 1977; Francis, 2008; Smith and Xie, 2008; Zhang L. *et al*., 2009; Kruskop, 2013; Thomas *et al*., 2013). This theory is also supported by their very similar echolocation calls (as expressed by the frequency of maximum energy, FmaxE) recorded in different regions of Southeast Asia, such as northeastern Vietnam (127 ± 2.6 kHz — Furey *et al.*, 2009), Thailand (126.43 kHz — Hughes *et al.*, 2010), Myanmar (126.68 ± 4.36 kHz — Khin, 2012), and southern China (120.3 ± 0.3 kHz in Sichuan and Guizhou, 118.4–119.3 in Yunan — Li *et al.*, 2007).

By contrast, Li *et al*. (2007) and Sun *et al*. (2009) found high levels of intraspecific variation in Cyt*b* sequences among specimens of *A. stoliczkanus* collected from southern China. With a broader taxonomic sampling, Francis *et al*. (2010) analysed DNA barcode sequences (COI) of *A. stoliczkanus* collected from Myanmar, Laos, Vietnam and southern China, and recovered three deeply divergent lineages that potentially represent distinct species. The results of previous molecular studies, therefore, have revealed that potential cryptic diversity might exist in *A. stoliczkanus*. However, this hypothesis needs to be confirmed by additional studies using other characteristics including further genetic markers, morphology or ecological data (Francis *et al*., 2010).

In this study, Cyt*b* and COI genes were sequenced for bats initially identified as *A. stoliczkanus* collected from different, so far mostly unstudied localities in Vietnam. Phylogeny and phylogeography of *A. stoliczkanus* in mainland Southeast Asia were reconstructed based on the newly generated sequences and those of previous studies. Morphological variation was assessed using the available specimens identified for the different genetic lineages of *A. stoliczkanus*. Based on the results, we address the taxonomic status of bats currently recognized as the Stoliczka's trident bat *A. stoliczkanus* in the region.

Materials and Methods

Taxonomic Sampling

Seventy-six trident bats (two *A. tricuspidatus* and 74 *A. stoliczkanus*) were included in the analyses (Appendix I). Most of the specimens were collected by the authors in the field with the use of mist nets (Ecotone, Gdańsk, Poland) and four-bank harp-traps. Captured bats were measured, photographed and initially identified using the field guide of Francis (2008). Tissue samples were collected from the muscle of the vouchers or from the patagium of the released bats, and preserved in 95% ethanol in two ml tubes. The voucher specimens are deposited in the following institutions: Institute of Ecology and Biological Resource, Hanoi, Vietnam (IEBR), Hungarian Natural History Museum, Budapest, Hungary (HNHM), and the Zoological Museum, Vietnam National University, University of Science, Hanoi (VNU) (see Appendix I).

DNA Extraction, Amplification and Sequencing

Total DNA was extracted using QIAGEN DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Two mitochondrial genes were sequenced in three laboratories for this study: the COI barcode fragment and the complete Cyt*b* gene. The primer sets used for PCR amplification of COI were UTyr/C1L705 (Hassanin *et al.*, 2012) or VF1d /VR1d (Ivanova *et al.*, 2007). The primer set used for PCR amplification of Cyt*b* was Mt-14724F/Cyb-15915R (Irwin *et al.*, 1991).

The PCR amplifications for the COI gene were performed as detailed in Tu *et al*. (2015). PCR products were purified using ExoSAP Kit (GE Healthcare, Buckinghamshire, UK) and sequenced in both directions using Sanger sequencing on an ABI 3730 automatic sequencer at the Centre National de Séquençage (Genoscope) in Evry (France); and on ABI 3500 at Biological Research Centre of the Hungarian Academy of Sciences (Hungary). The obtained COI sequences were then edited and assembled using Codoncode Alignment Version 3.7.1 (Codon Code Corporation). The PCR amplifications and DNA sequencing for the entire 1,140 nt Cyt*b* gene were done in the Infectious Disease Surveillance Center (NIID, Japan) as presented in Arai *et al.* (2012). The new Cyt*b* sequences were processed by using the Genetyx v11 software (Genetyx Corporation, Shibuya, Tokyo, Japan). All 38 sequences generated for this study were deposited in the EMBL/DDBJ/GenBank database (accession numbers KU161538–KU161575).

Phylogenetic Reconstruction

Specimens initially identified as *A. stoliczkanus* were sequenced for either COI (n = 20) or Cyt*b* genes (n = 18) (Appendix I). The new sequences were compared with 33 COI and 23 Cyt*b* sequences downloaded from GenBank (Appendix II). The phylogenetic trees were rooted using species belonging to the families Pteropodidae (*Pteropus scapulatus*, *Rousettus leschenaultii*), Megadermatidae (*Megaderma lyra*), Rhinolophidae (*Rhinolophus affinis*, *R. ferrumequinum*, *R. hipposideros*, *R. luctus*, *R. pearsonii*, *R. pusillus*) and Hipposideridae (*Hipposideros armiger*, *H. larvatus*, *H pomona*, *H. pratti*, *Coelops frithii*) (see Appendix II).

Sequences were aligned manually in PhyDe version 0.9971 (Müller *et al.*, 2010). No gaps and stop codons were found in the alignments of the mitochondrial COI and Cyt*b* protein-coding genes. The phylogenetic trees were reconstructed from two separate mitochondrial datasets, (1) COI (49 taxa and 657 nt), and (2) Cyt*b* (41 taxa and 1140 nt) using Bayesian inference (BI) with MrBayes v3.2 (Ronquist *et al.*, 2012). The best-fitting models of sequence evolution for both datasets (GTR+I+G) were selected with jModelTest v 2.1.4, using the Akaike Information Criterion (Posada, 2008).

Molecular Dating

Divergence times were estimated using the Bayesian approach implemented in BEAST v.2.1.3 (Bouckaert *et al.*, 2014)

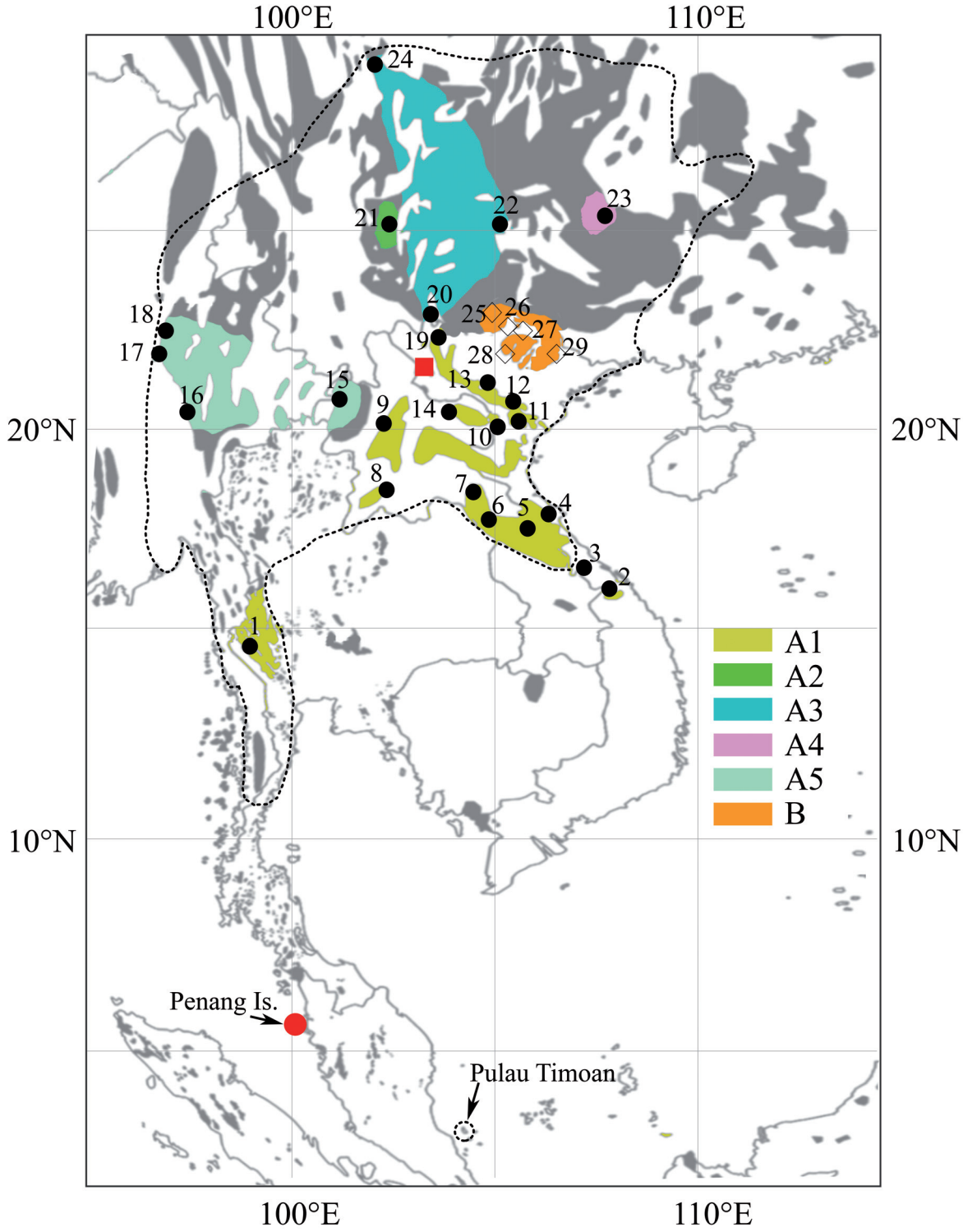

FIG. 1. Distribution area (dot line) of *Aselliscus stoliczkanus* s.l. (Li *et al*., 2007; Bates *et al*., 2008) and taxonomic sampling used for this study. Map of karst (shaded) in the mainland of Southeast Asia (modified from Ford and Williams, 2007). Type locality: *A. stoliczkanus* (circle, in red); *A. wheeleri* (full square, in red). Symbols represent the geographical origins of bats of clade A (full circles) and clade B (empty diamonds) of *A. stoliczkanus* identified by genetic and morphological analyses (Figs. 2 and 4). Clade A: Subclade A1 (1 — Sai Yok; 2 — Dakrong; 3 — Bac Huong Hoa; 4 — Phong Nha - Ke Bang; 5, 6, 7 — Hin Nam No region; 8 — Phou Khao Khouay; 9 — Luoang Phrabang; 10 — Xuan Lien; 11 — Ngoc Lac; 12 — Cuc Phuong; 13 — Xuan Son; 14 — Nam Et NBCA; 19 — Ta Phin, Sa Pa); Subclade A2 (21 — Yunnan (Li *et al*., 2007)); Subclade A3 (20 — Yunnan (Sun *et al*., 2009); 22 — Guizhou; and 24 — Shichuan); Subclade A4 (23 — Guizhou, Libo) and Subclade A5 (15 — Louang Namtha; 16, 17, 18 — Myanmar); Clade B: 25 — Khau Ca; 26 — Phia Oac-Phia Den; 27 — Ba Be; 28 — Na Hang; and 29 — Huu Lien

using a Cyt*b* alignment of 29 taxa. As no calibration point (fossil record or biogeographic event) is sufficiently accurate for the family Hipposideridae, divergence times were estimated using mutation rates drawn from a normal distribution centred at 0.0175 nucleotide substitutions per site per lineage per Mya with a standard deviation of 0.0075, root age fixed at 59 ± 6 Mya, and a common ancestor of *Aselliscus* and *C. frithii* fixed at 16 ± 1.5 Mya. These priors were chosen in agreement with divergence rates previously estimated for different groups of mammals, including bats (Arbogast and Slowinski, 1998; Hulva *et al.*, 2004) and based on recent molecular dating estimates on the family Hipposideridae (Foley *et al.*, 2015). We applied a GTR+I+G model of evolution (as selected by jModelTest) and a relaxed-clock model with uncorrelated lognormal distribution for substitution rates. Node ages were estimated using a Yule speciation prior and 10^8 generations, with tree sampling every 1000 generations, and a burn-in of 10%. Adequacy of chain mixing and MCMC chain convergence were assessed using the ESS values in Tracer v.1.6. The chronogram was reconstructed with TreeAnnotator v.1.7.5 and visualized with FigTree v.1.4.1 (Rambaut, 2009).

Morphological Analyses

Forty-eight specimens initially identified as *A. stoliczkanus* and two *A. tricuspidatus* were analysed for craniodental characters. Some of those were also examined for external (n = 22), and bacular (n = 8) features (Appendix I). All examined specimens were adults, as confirmed by the presence of fully ossified metacarpal-phalangeal joints.

External measurements were taken to the nearest 0.1 mm from alcohol-preserved museum specimens. These included: forearm length (FA) from the extremity of the elbow to the extremity of the carpus with the wings folded; the third finger metacarpal ($3rd^{mt}$) and the first phalanx ($3rd^{1}$); the fourth finger metacarpal ($4th^{mt}$) and the first phalanx ($4th^{1}$); the fifth finger metacarpal ($5th^{mt}$) and the first phalanx ($5th^{1}$); tibia length (Tib) from the knee joint to the ankle.

Craniodental measurements were taken to the nearest 0.01 mm using digital calipers under stereomicroscope. These include the greatest length of skull (GLS) from the most anterior part of the upper canine to the most posteriorly projecting point of the occipital region; the condylo-canine length (CCL) from the exoccipital condyle to the most anterior part of the canine; the greatest width across the upper canines (C^1C^1) between their buccal borders; the greatest width across the crowns of the last upper molars (M^3M^3) between their buccal borders; the greatest width of the skull across the zygomatic arches (ZB); the greatest distance across the mastoid region (MB); the greatest width of the braincase (BW); maxillary toothrow length (CM^3) from the anterior of the upper canine to the posterior of the crown of the 3^{rd} upper molar; mandible length (ML) from the anterior rim of the alveolus of the 1st lower incisor to the most posterior part of the condyle; mandibular toothrow length (CM_3) from the anterior of the lower canine to the posterior of the crown of the 3rd lower molar; upper canine length (UCL) from the cingular ridge to the tip of the upper canine; and lower canine length (LCL) from the cingular ridge to the tip of the lower canine (Fig. 5).

In order to test the morphometric affinities of the studied specimens, principal component analyses (PCA) were done in PAST (Hammer *et al.*, 2001) on log-transformed morphometric measurements for both sexes combined. The PCAs also included mensural data published for the holotypes (or type series) of *A. stoliczkanus*, and its synonyms, *A. trifidus* and *A. wheeleri* to check their relationships with the newly acquired material. The equalities of means of all morphological measurements and PC scores obtained from PCAs between different taxa were tested by one-way analysis of variance (ANOVA) followed by Tukey HSD multiple comparison test for unequal sample sizes (or Tukey-Kramer) or *T*-test (Zar, 1999). Only statistically significant PCs ($P \leq 0.05$) were selected for interpretation.

Results

Phylogeography of Aselliscus *Based on mtDNA Sequences*

The Bayesian trees reconstructed from the analyses of COI and Cyt*b* gene sequences show similar patterns (Fig. 2). Accordingly, the genus *Aselliscus* was found to be a monophyletic (PP = 1) sister-group of *Coelops* and *Hipposideros* (Fig. 2). Within *Aselliscus*, *A. tricuspidatus* and *A. stoliczkanus* were found to be reciprocally monophyletic (Fig. 2).

Within *A. stoliczkanus*, two highly divergent clades, named A and B, can be distinguished on both Cyt*b* and COI trees (PP = 1; Fig. 2). The pairwise nucleotide distances between the two clades estimated from Cyt*b* and COI sequences are 10.0–10.9% and 10.7–13.5%, respectively (Fig. 2 and Appendix III). The clade A comprises bats from the Southeast Asian mainland (including southern China), with the exception of the limestone areas of Ha Giang, Bac Kan, Tuyen Quang and Lang Son provinces in northeastern Vietnam, where only individuals belonging to clade B were collected (Fig. 1).

Based on levels of genetic divergence in mtDNA sequences, clade A can be further divided into different subclades, namely A1, A2, and A3 on the Cyt*b* tree and A1, A4, and A5 on the COI tree. The pairwise nucleotide differences between these subclades based on Cyt*b* and COI sequences are 4.1–6.3% and 4.9–6.8%, respectively. Bats of these subclades might also be separated geographically from each other: A1 — central to northwestern Indochina; A2 — Yunnan, China; A3 — Yunnan, Guizhou, and Sichuan, China; A4 — Guizhou, China; and A5 — northwestern Laos to Upper Myanmar (Fig. 1). The pairwise nucleotide distances calculated from Cyt*b* and COI sequences within the subclades of clade A and B are < 3% and < 3.8%, respectively (Fig. 2 and Appendix III).

Molecular Dating

Within the genus *Aselliscus*, the split between *A. tricuspidatus* and *A. stoliczkanus* took place around 14.3 Mya, whereas clades A and B of

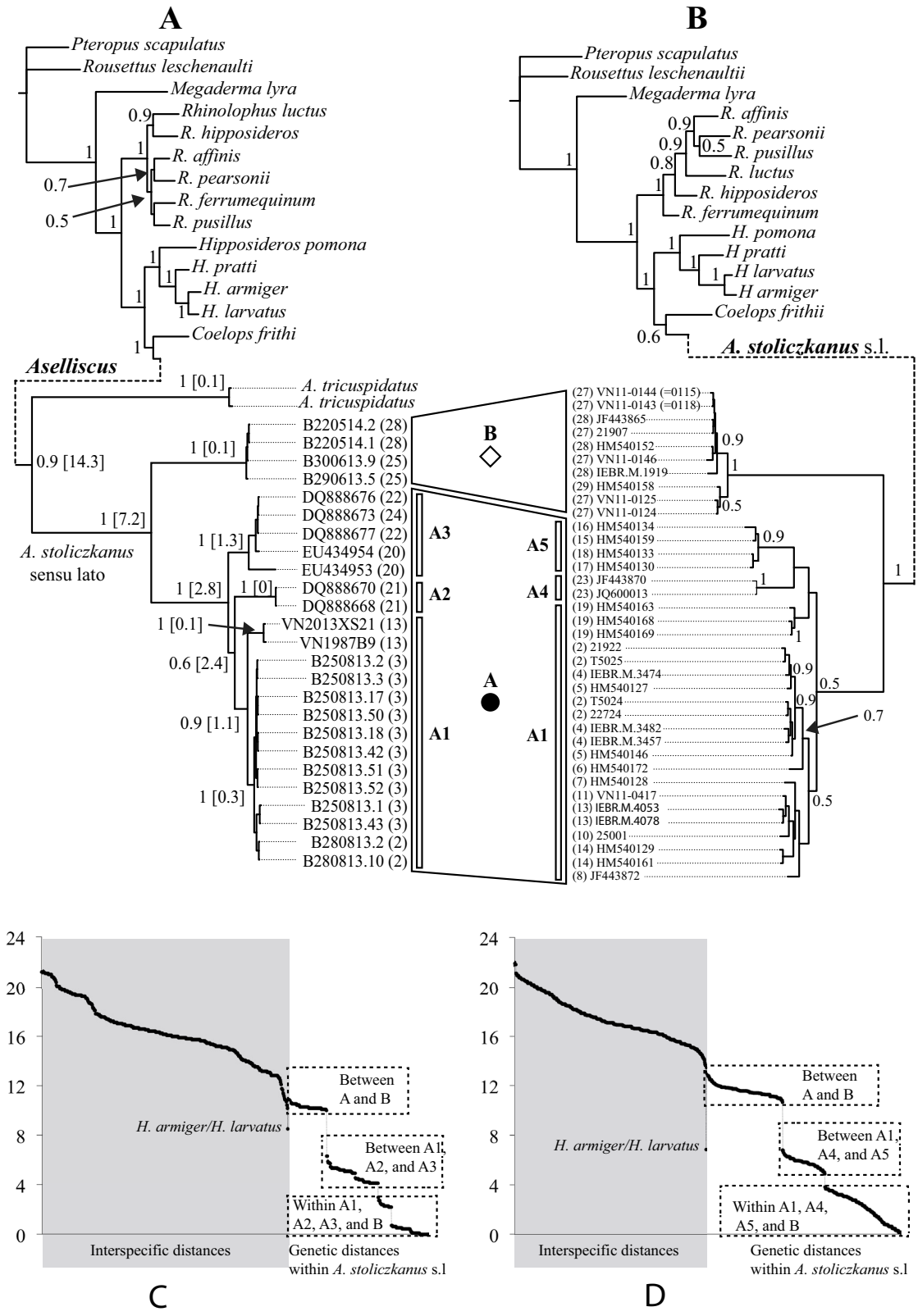

FIG. 2. Phylogenetic and pairwise distance analyses of mtDNA sequences. Bayesian trees reconstructed from Cyt*b* (A) or COI sequences (B). The numbers on nodes represents posterior probabilities. The numbers in brackets are divergence times estimated from Cyt*b* sequences (see Appendix IV for details). The number in parentheses after the name of the sequences indicates the geographical origin of specimen examined (see Fig. 1 and Appendices I and II for details). The two figures below show pairwise nucleotide distances (K2P) calculated from Cyt*b* (C). and COI sequences (D). The distances were ranged in two categories corresponding to interspecific comparisons and intraspecific comparisons within *A. stoliczkanus* s.l., and they were ranked in descending order

A. stoliczkanus diverged from each other around 7.2 Mya (Fig. 2 and Appendix IV). Within clade A of *A. stoliczkanus*, the three subclades (A1, A2, and A3) diversified during the late Pliocene and early Pleistocene (2.8–2.4 Mya) (Fig. 2 and Appendix IV).

Morphological and Morphometric Comparisons

Clade B differs from clade A by its distinctively robust and longer upper and lower canines (Fig. 5, Table 1). Bacula extracted from specimens of clade A and B of our *A. stoliczkanus* and *A. tricuspidatus* (after Topál, 1975) are presented in Fig. 3. Accordingly, the two nominal species show strong differences in the size and the shape of the baculum that are listed below for *A. tricuspidatus* followed by the comparable features of *A. stoliczkanus* presented in parentheses. The length is approximately 1mm (significantly longer than 1 mm); S-shaped in the right lateral view and the ventrally projecting apical lappet turns sharply to the left (bow-shaped or relatively straight). The basal portion is dorsoventrally flattened and with a dorsal knob (the basal portion is widened and with two or three relatively visual lobes). The shaft is distally tapering to the widening base of the strongly flattened, truncate apical lappet (the shaft tapers slightly from the basal portion to the blunt tip and is ventrally flattened but slightly concave near the basal portion, and dorsally convex). In contrast, the bacular morphology observed in clades A and B of *A. stoliczkanus* s.l. is overlapping, although the ventral margin of the basal portion of the examined specimens of the first clade is triangular while in the latter clade two of three presented specimens is rectangular. However, as presented in Topál (1975), the bacular morphology of various sibling species of the families Hipposideridae and Rhinolophidae tends to overlap in size and shape. This biological phenomenon might have also been encountered in different clades of the *A. stoliczkanus* complex.

Specimens with no corresponding genetic data were assigned into the molecular groups of clade A and B based on the above morphological features and their geographic origin. This initial identification was then checked by PCA on morphometric

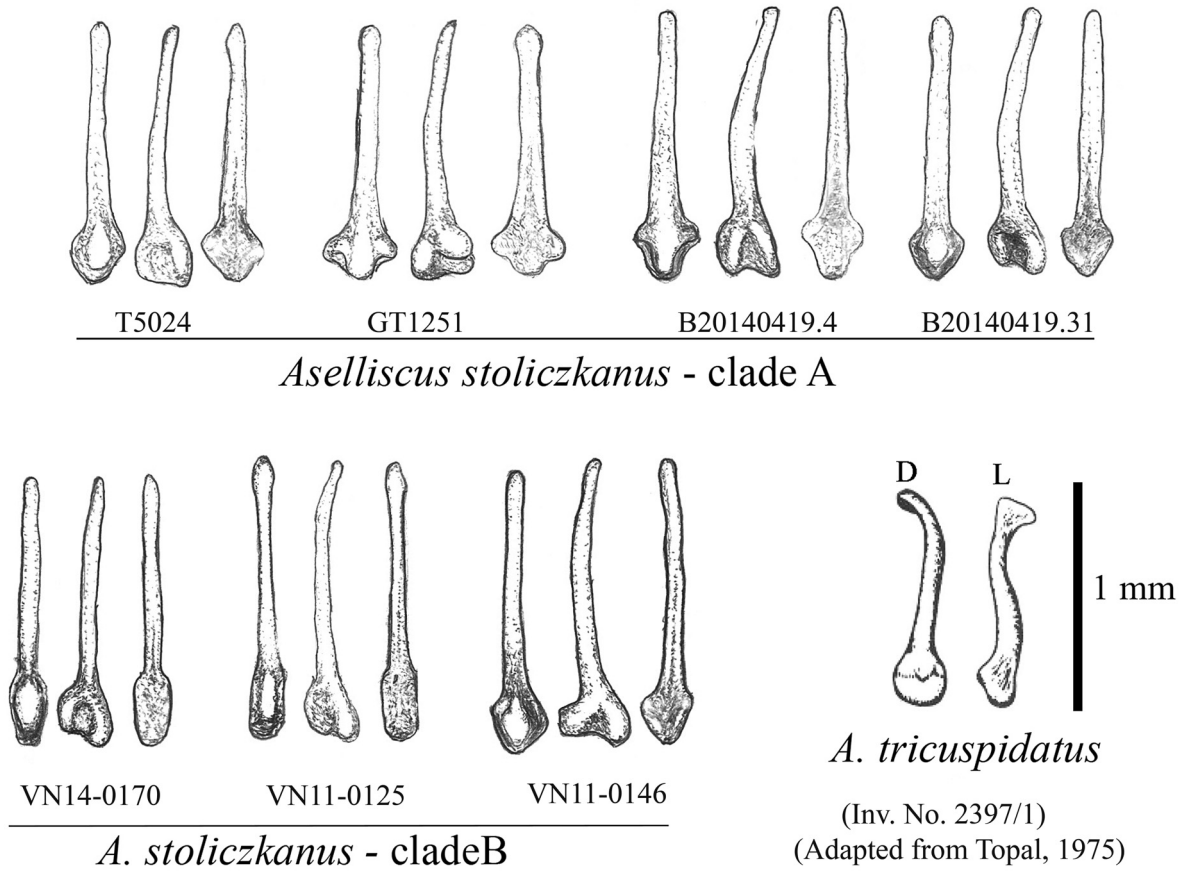

FIG. 3. Bacula of specimens of clade A and B of *A. stoliczkanus* and *A. tricuspidatus*. From left to right: *A. stoliczkanus* s.l. (dorsal, lateral, and ventral view); *A. tricuspidatus* (dorsal and later view)

TABLE 1. Selected external and craniodental measurements (in mm) of *Aselliscus* spp. Values are given as $\bar{x} \pm$ SD, *n*, min–max. Level of statistical significance (*P*) of intraspecific variation within *A. stoliczkanus* s.l. based on *T*-test. Acronyms and definitions for measurements are given in the Materials and Methods section

Character	*A. tricuspidatus*		*A. stoliczkanus** (holotype)	*A. trifidus*** (holotype)	*A. wheeleri**** (type series)	Variation within *A. stoliczkanus* s.l.				*P*-level
						Clade A		Clade B		
FA		39.4–43.6+	39.5	40.0	42.0, *6*	42.4 ± 0.8, *12*	41.0–43.4	42.8 ± 0.8, *10*	41.1–43.7	ns
$3nd^{mt}$	–		29.0	27.5	31.5, *6*	30.4 ± 0.9, *12*	29.1–32.5	31.3 ± 0.9, *10*	29.7–32.5	<0.05
$3rd^{1}$	–		13.6	14.2	15.0, *6*	14.9 ± 0.5, *12*	14.1–15.8	15.2 ± 0.4, *10*	14.7–15.9	ns
$4th^{mt}$	–		30.5	29.5	31.0, *6*	30.5 ± 0.8, *12*	29.7–32.5	31.6 ± 1.0, *10*	30.1–33.3	<0.01
$4th^{1}$	–		10.5	11.4	12.0, *6*	12.2 ± 0.4, *12*	11.6–12.9	12.4 ± 0.3, *10*	12.0–13.2	ns
$5th^{mt}$	–		25.5	23.5	28.0, *6*	26.1 ± 0.5, *12*	25.2–27.3	27.2 ± 0.6, *10*	26.0–28.0	<0.001
$5th^{1}$	–		12.0	12.2	12.5, *6*	12.6 ± 0.4, *12*	11.8–13.3	12.6 ± 0.4, *10*	12.1–13.2	ns
Tib	–		16.8	16.5	18.0, *6*	18.6 ± 0.5, *12*	17.8–19.4	18.7 ± 0.5, *10*	17.8–19.7	ns
GLS	15.29 ± 0.08, *2*	15.23–15.34	14.4	–	15 (holotype)	14.84 ± 0.16, *29*	14.54–15.17	15.20 ± 0.16, *17*	14.94–15.52	<0.001
CCL	13.15 ± 0.11, *2*	13.07–13.22	–	–	13 (holotype)	12.91 ± 0.15, *29*	12.69–13.26	13.18 ± 0.16, *17*	12.97–13.55	<0.001
$C^{1}C^{1}$	3.58 ± 0.13, *2*	3.49–3.67	–	–	–	3.27 ± 0.11, *29*	2.94–3.44	3.45 ± 0.11, *17*	3.19–3.61	<0.001
$M^{3}M^{3}$	5.34 ± 0.01, *2*	5.33–5.35	–	–	–	5.21 ± 0.12, *29*	4.88–5.43	5.42 ± 0.12, *17*	5.18–5.63	<0.001
ZB	7.47 ± 0.04, *2*	7.44–7.49	7.4	–	7.4 (holotype)	7.41 ± 0.11, *28*	7.21–7.64	7.66 ± 0.09, *17*	7.49–7.84	<0.001
MB	6.84 ± 0.08, *2*	6.78–6.9	7.0	–	7.1 (holotype)	7.08 ± 0.09, *29*	6.91–7.25	7.29 ± 0.08, *17*	7.10–7.45	<0.001
BW	5.99 ± 0.01, *2*	5.98–5.99	6.1	–	–	6.06 ± 0.10, *29*	5.88–6.28	6.18 ± 0.08, *17*	6.04–6.31	<0.001
CM^{3}	5.59 ± 0.04, *2*	5.56–5.61	4.9	–	5.2 (holotype)	5.15 ± 0.08, *29*	4.96–5.32	5.37 ± 0.06, *17*	5.28–5.49	<0.001
ML	9.94 ± 0.11, *2*	9.86–10.02	8.8	–	–	9.05 ± 0.10, *28*	8.78–9.29	9.41 ± 0.10, *17*	9.15–9.58	<0.001
CM_{3}	5.95 ± 0.05, *2*	5.91–5.98	5.2	–	–	5.43 ± 0.10, *28*	5.23–5.63	5.68 ± 0.06, *17*	5.57–5.77	<0.001
UCL	–	–		–	–	1.71 ± 0.06, *21*	1.59–1.81	1.95 ± 0.06, *14*	1.87–2.04	<0.001
LCL	–	–		–	–	1.30 ± 0.05, *21*	1.21–1.37	1.51 ± 0.05, *14*	1.42–1.64	<0.001

+ — Robson *et al.*, 2012 (and reference therein); * — Sanborn, 1952; ** — Peters, 1871; *** — Osgood, 1932; ns — not significant

measurements. *T*-tests indicate that most examined external and craniodental characters of bats in clade A are generally smaller than those in clade B (Table 1).

Although type specimens of *A. stoliczkanus*, *A. trifidus*, and *A. wheeleri* (housed in different museums) were not available for direct assessment by the authors, selected craniodental measurements had been published in previous studies (Peters, 1871; Osgood, 1932; Sanborn, 1952). PCAs were conducted on external and craniodental datasets including our own measurements and published data available for type materials. PCA based on eight external morphometric measurements of 22 bats representing clades A (n = 12) and B (n = 10) and the type specimens of *A. stoliczkanus*, *A. trifidus*, and *A. wheeleri* (after Peters, 1871; Osgood, 1932; Sanborn, 1952) reveal that only PC1 (explaining 62.9% of total variance) shows a significant difference (ANOVA; $P < 0.05$) between taxa (Fig. 4A and Table 2). Based on PC1, there are two distinct clusters: (1) the holotype of *A. stoliczkanus* and *A. trifidus* and (2) bats of clade A and B, and the type series (represented as mean of type series) of *A. wheeleri*. Within the first cluster, two type specimens of *A. stoliczkanus* and *A. trifidus* can be separated by PC2, but this separation is not statistically significant.

PCA was performed on 10 craniodental measurements for 46 specimens investigated (*A. tricuspidatus* (n = 2), clade A (n = 27) and clade B (n = 17) of *A. stoliczkanus*). In addition, we also performed PCAs on two datasets that included our new data and the available morphometric data for the holotypes of *A. stoliczkanus* and *A. wheeleri* from the literature (Osgood, 1932; Sanborn, 1952). In the latter analyses, our new data were re-scaled to the same level of precision of measurements acquired from the literature. All these analyses reveal that the two first PCs (PC1 and PC2) show significant differences between the taxa (ANOVA; $P < 0.05$) (Fig. 4B–E). Factor loadings for these PCs are presented in Table 3. Accordingly, figure 4B–E shows a clear separation of *A. tricuspidatus* from *A. stoliczkanus* s.l. Within *A. stoliczkanus* s.l., the PC plots from different datasets indicate significant separation between bats of clade A and B (Fig. 4B–4E). In relation to the holotypes of *A. stoliczkanus* and *A. wheeleri*, the analyses of different datasets show nearly similar results that include the strong affinity among the holotype of *A. wheeleri* and the bats of clade A (Fig. 4B–E), and the separation of different couples of the following taxa: the holotypes of *A. stoliczkanus* and *A. wheeleri* / the bats of clade B

TABLE 2. Factor loadings of characters for the two first PCs obtained from the principal component analysis of eight external measurements of *Aselliscus* spp. Acronyms and definitions for measurements are given in the Materials and Methods section

Character	PC 1	PC 2
FA	0.26	-0.13
$3rd^{mt}$	0.44	0.31
$3rd^{1}$	0.35	-0.31
$4th^{mt}$	0.31	0.45
$4th^{1}$	0.40	-0.53
$5th^{mt}$	0.39	0.43
$5th^{1}$	0.24	0.15
Tib	0.39	-0.32
Eigenvalue	0.0012	0.0003
% variance	62.9	16.5

Table 3. Factor loadings of characters for the two first PCs obtained from PCAs based on different datasets of craniodental measurements of *Aselliscus* spp. Acronyms and definitions for measurements are given in the Materials and Methods section

Character	Dataset							
	10 characters (B)		7 characters (C)		4 characters (D)		3 characters (E)	
	PC 1	PC 2	PC 1	PC 2	PC 1	PC 2	PC 1	PC 2
GLS	0.20	0.03	0.27	0.05	0.40	-0.10	0.42	0.81
CCL	0.18	0.08						
$C^{1}C^{1}$	0.58	-0.61						
$M^{3}M^{3}$	0.32	0.47						
ZB	0.22	0.37	0.27	0.45	0.44	0.43	0.64	0.06
MB	0.16	0.39	0.19	0.65	0.36	0.70	0.64	-0.59
BW	0.16	0.30	0.19	0.45				
CM^{3}	0.36	-0.04	0.51	-0.22	0.72	-0.56		
ML	0.34	-0.09	0.45	-0.32				
CM_{3}	0.38	0.11	0.57	-0.15				
Eigenvalue	0.0009	0.0002	0.0006	0.0001	0.0003	0.0001	0.0002	0.00003
% variance	67.6	12.4	70.9	15.7	69.7	22.7	76.3	14.9

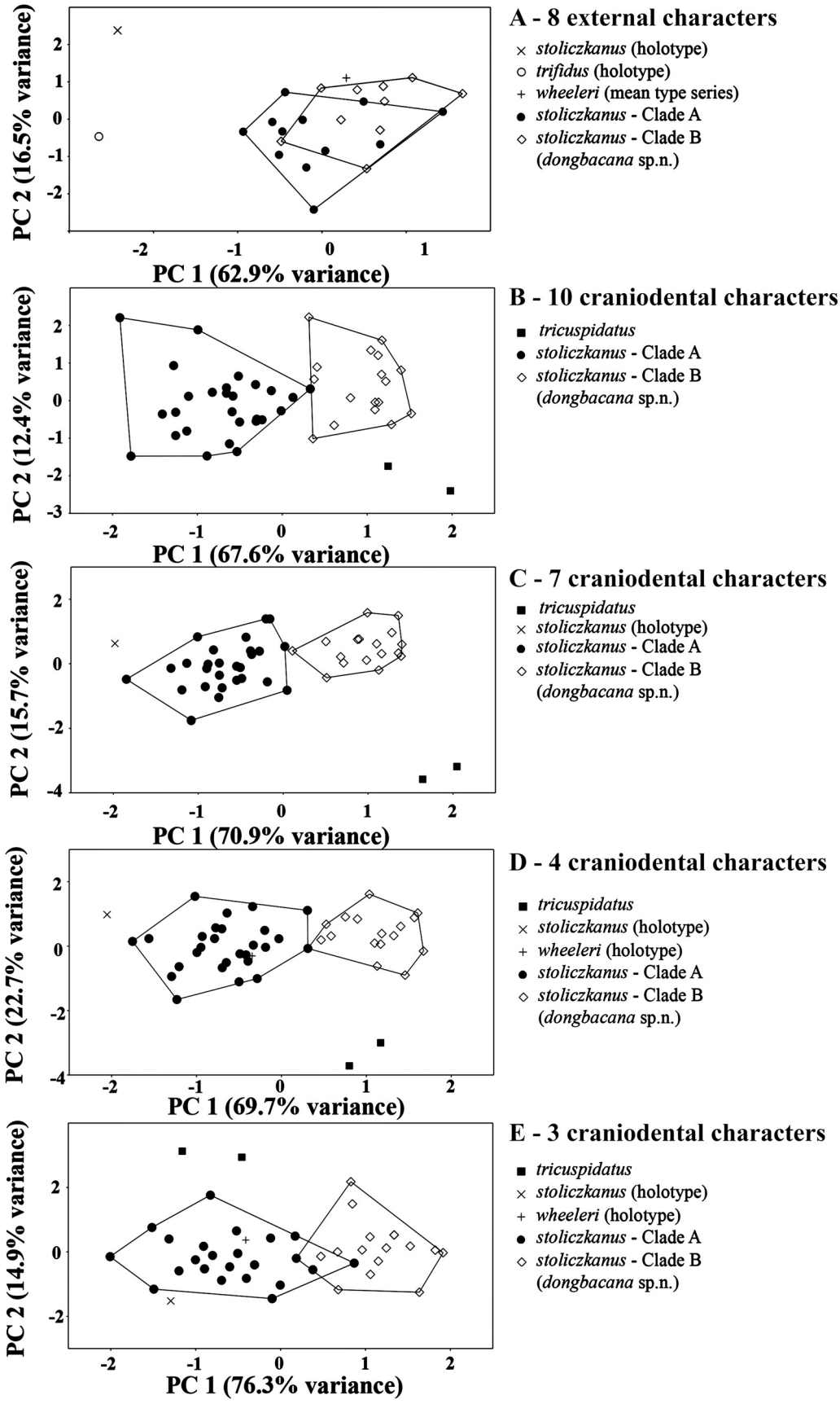

FIG. 4. Principal components analyses (PCA) of studied *Aselliscus* spp. A — PCA based on eight external characters; B–E — PCAs based on datasets of a reduction from 10 to three craniodental characters

(Fig. 4B–4E); and the holotypes of *A. stoliczkanus* / the bats of clade A (Fig. 4C–4E); whereas the holotype of *A. stoliczkanus* nested in clade A was found only in the analysis of three characters (Fig. 4E).

Discussion

Cryptic Diversity within A. stoliczkanus

Previously, Li *et al.* (2007) and Sun *et al.* (2009) found that the maximum genetic distance in Cyt*b* between different populations of Chinese *A. stoliczkanus* — corresponding to subclades A2 and A3 in our analyses (Fig. 2) — was relatively high (ca. 6.5%), but lower than the interspecific variation between *A. stoliczkanus* and *A. tricuspidatus* (14–16% in Li *et al.*, 2007). In addition, these populations were known to have similar echolocation call characteristics (Li *et al.*, 2007), as well as morphological and ecological features (Sun *et al.*, 2009). Thus, these authors suggested that the divergence in Cyt*b* sequences within Chinese *A. stoliczkanus* "may represent geographic races, rather than distinct species" (Li *et al.*, 2009: 741). More recently, by analyzing DNA barcodes (COI), Francis *et al.* (2010) suggested that bats of *A. stoliczkanus* can be divided into three deep lineages that may represent three different species. According to our COI analyses, these three lineages correspond to subclades A1+A4 and A5 and clade B (Fig. 2). However, phylogenetic inferences based solely on mitochondrial data can be misleading due to various processes, including mtDNA introgression between closely related species, incomplete lineage sorting of ancestral polymorphism, and male-biased dispersal associated with female philopatry (e.g. Kerth *et al.*, 2000; Rivers *et al.*, 2005; Berthier *et al.*, 2006; Pereira *et al.*, 2009; Mao *et al.*, 2010; Nesi *et al.*, 2011; Hassanin *et al.*, 2015).

Although no biparentally inherited markers (nuDNA genes) have been sequenced for this study to examine current gene flow between isolated populations, our new data including Cyt*b* sequences of bats collected from Vietnam and morphological evidence have completed the gaps of previous studies. The comparison of our new Cyt*b* sequences with those published in previous studies (i.e., Li *et al.*, 2007; Sun *et al.*, 2009) confirms that genetic distances between clades A and B of *A. stoliczkanus* s.l. (10.0–10.9%) are comparable with the interspecific variation within the genus *Aselliscus* (12.8–13.1% of *A. stoliczkanus* s.l. versus *A. tricuspidatus*) or other genera of the families Hipposideridae and Rhinolophidae (Fig. 2 and Appendix III). Moreover, mtDNA divergences among subclades of clade A (4.1–6.3% in Cyt*b*, and 4.9–6.8% in COI) are significantly higher than their intraspecific variation and relatively comparable with the interspecific distances between many other bat taxa, i.e. between *Hipposideros armiger* and *H. larvatus* of the family Hipposideridae (8.5% in Cyt*b*, and 6.8% in COI; Fig. 2 and Appendix III); between *Murina shuipuensis* and *M. leucogaster* of the family Vespertilionidae (2.6% in COI — Eger and Lim, 2011); or between fruit bats of the tribe Scotonycterini (Hassanin *et al.*, 2015). In contrast to previous studies demonstrating a lack of morphological differences among geographical populations, our available data suggest that *A. stoliczkanus* s.l. might be divided into three separate morphological groups: (1) the holotypes of *A. stoliczkanus* and *A. trifidus*, (2) the bats of clade A and the holotype of *A. wheeleri*, and (3) those of clade B (Fig. 4). However, it should be noted that the affinity between the holotypes of *A. stoliczkanus* and *A. trifidus* is still uncertain since although our morphological analysis show they might be distinguishable from each other, their separation was not statistical+ly supported (Fig. 4); and that bats of clade A included in our morphological analyses were all representatives of subclade A1. Assuming that bats of *A. stoliczkanus* from Myanmar (subclade A5 in COI tree — Fig. 2) and the holotype of *A. trifidus* (without precise locality data) belong to the same taxon or a 'geographic race' sensu Li *et al.* (2007), there is a congruence between phylogenetic patterns, morphological differences and geographical distribution of different taxa previously allocated to *A. stoliczkanus*.

Morphological Differences Between 'Geographic Races' of A. stoliczkanus *s.l.: Observer Bias or Biological Phenomenon?*

In this study, type specimens of *A. stoliczkanus*, *A. trifidus*, and *A. wheeleri* were not available for direct assessment by the authors, because they are housed in different museums throughout the world. For this reason, the results obtained by our morphological comparison using morphometric measurements available in the literature may not be accurate due to the examined characters containing potential inter-observer variability (Lee, 1990; Yezerinac *et al*., 1992; Palmeirim, 1998). Indeed, the magnitude of differences between measurements taken by different and those taken by the same observers

are known to differ considerably from character to character (Lee, 1990; Palmeirim, 1998; Hayek and Heyer, 2005; Roitberg *et al*., 2011). For small sized bats, Palmeirim (1998) considered that the both the intra- and the inter-observer variability of measurements of several craniodental characters is adequate, and morphological comparisons using these characters from different sources can be performed with reasonable confidence.

To date, Sanborn (1952: 2) was the only author who directly examined type specimens of both *A. stoliczkanus* and *A. wheeleri* and considered that "the measurements of *stoliczkana* agree closely with those of *wheeleri* and sketches of parts of the skull agree in shape with *wheeleri*". However, most available measurements (in mm) presented by Sanborn (1952), for the holotype of *A. stoliczkanus* appeared to be smaller than those of type series of *A. wheeleri*, e.g. FA: 39.5 versus 40.0–43.8; Tib: 16.8 vs. 18.0–19.1; GLS: 14.4 vs. 14.8–15.0; condylo-basal length 12.5 vs. 12.8–13.0; ZB: 7.4 vs. 7.4–7.5; MB 7.0 vs. 7.0–7.2; CM^3: 4.9 vs. 5.1–5.1; and CM_3: 5.2 vs. 5.3–5.4. Our multivariate analyses of craniodental measurements with different simulated datasets that reduced the number of characters from 10 to three of our data or pooled with those from the literature indicate only marginal differences in revealing the significant differences in size between the holotype and other specimens of *A. stoliczkanus* s.l., as well as the significant separation among different morphological groups within this focal taxon. For example, the plots of PCs from a dataset reduced from seven to three characters always support the significant separation of the holotypes of *A. stoliczkanus* and *A. wheeleri* from clade B, and the strong affinity of the holotype of *A. wheeleri* and clade A. The separation of the holotype of *A. stoliczkanus* s.l. from bats of clade A is corroborated by the analyses of datasets reduced from seven to four characters (Table 3 and Fig. 4B–4E). Our cross-comparison of data from different observers (Osgood, 1932; Sanborn, 1952; this study) indicated that most measurements (GLS, ZB, MB, and CM^3) included in reduced datasets have adequate variance both between and within observers; whereas the strong affinity between the holotype (or type series) of *A. wheeleri* and our bats of clade A (Fig. 4D–E) coincides with their proximal distribution (Fig. 1). Based on this evidence, we suggest that significant differences in morphological characters among geographic races of *A. stoliczkanus* s.l. represent an actual biological phenomenon rather than a measurement artefact.

Taxonomy of Taxa within A. stoliczkanus *s.l.*

Previous taxonomic studies indicated that there is only a single trident bat species, *A. stoliczkanus* in the Southeast Asian mainland (Dobson, 1876; Tate, 1941; Sanborn, 1952; Simmons, 2005; Kruskop, 2013; Thomas *et al*., 2013). By contrast, our molecular and morphological analyses suggest that the taxonomic status of 'geographical races' (sensu Li *et al.*, 2007) within clade A of *A. stoliczkanus* should be revised. This clade includes (1) the holotype of *A. stoliczkanus,* (2) the bats of subclade A1 and A5 with *A. wheeleri* and *A. trifidus* as their namesake types*,* respectively and (3) specimens of the Chinese populations. This inference should be interpreted cautiously and can only be resolved if further investigations include DNA sequences of holotypes or topotypes of *A. stoliczkanus* and *A. trifidus*, as well as nuclear genes from specimens representing these geographical races. However, our combined molecular and morphological data clearly support the separation of the bats of clade B found in north-eastern Vietnam from all other recently identified 'geographical races' of *A. stoliczkanus* s.l. and from *A. tricuspidatus* at the species level; hence they are described here as a new species.

Systematic Description

Aselliscus dongbacana sp. n.
(Fig. 5B)

Holotype

IEBR-VN11-0143 (Field no.: Tu.230511.1, tissue code: VN11-0143), adult ♂, body in alcohol, skull and baculum removed, collected by V. T. Tu on 23 May 2011. Mass: 4.5 g. Measurements (in mm) are as follows: FA: 43.8; Head and body length: 40.5; Tail: 39.5; Ear length: 12.2; Tibia: 19.7; $3rd^{mt}$: 32.5; $3rd^{1}$: 15.7; $4th^{mt}$: 31.5; $4th^{1}$: 13.2, cartilage: bifurcate; and $5th^{mt}$: 27.9, $5th^{1}$: 13.1, cartilage: bifurcate. GLS: 14.94; CCL: 13.01; C^1C^1: 3.57; M^3M^3: 5.55; ZB: 7.61; MB: 7.29; BW: 6.05; CM^3: 5.28; ML: 9.42; CM_3: 5.66; UCL: 1.51; and LCL: 2.01. The sequence of COI has been deposited in the EMBL/GenBank/DDBJ nucleotide databases with accession no. KU161543.

Type locality

Na Phong cave, Ba Be National Park, Bac Kan province, Vietnam (22°23'N, 105°36'E; entrance altitude: 280 m a.s.l.).

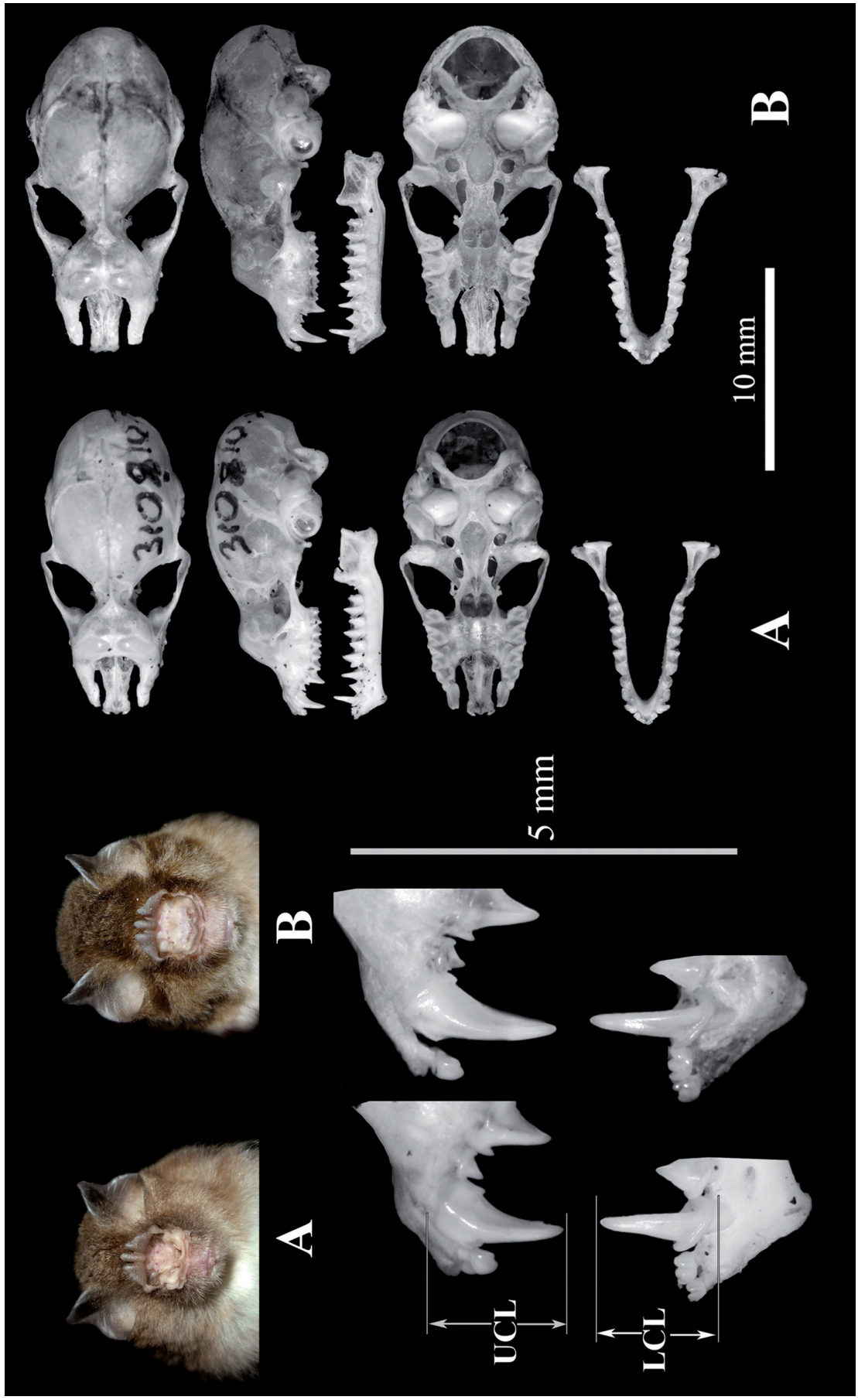

FIG. 5. Portraits and skull photographs of *A. stoliczkanus* s.l. A — *A. stoliczkanus* (IEBR-T5024, ♂) and B — *A. dongbacana* sp.n. (holotype IEBR-VN11-0143, ♂)

Paratypes

IEBR-VN11-0124 (Field no.: Tu.20.05.11.2; adult ♂; accession no. of COI sequence: KU161541); IEBR-VN11-0125 (Field no.: Tu.20.05.11.3; adult ♂; accession no. of COI sequence: KU161542); IEBR-VN11-0146 (Field no.: Tu.23.05.11.4; adult ♂; accession no. of COI sequence: KU161545); bodies in ethanol, skulls extracted; IEBR-VN11-0115 (Field no.: Tu.19.05.11.2; adult ♀; accession no. of COI sequence: KU161539), IEBR-VN11-0118 (Field no.: Tu.19.05.11.5, adult ♀; accession no. of COI sequence: KU161540), IEBR-VN11-0144 (Field no.: Tu.23.05.11.2; adult ♂; accession no. of COI sequence: KU161544), bodies in ethanol, collected from same location as holotype. HNHM 2007.27.9., adult ♂, body in ethanol, skull removed, accession no. of COI sequence: KU161556, collected in Ba Be National Park by N. M. Furey and G. Csorba on 02 May 2007.

Referred material

A series of other specimens identified as clade B collected from Na Hang Nature Reserve, Tuyen Quang province, Vietnam, Khau Ca Nature Reserve, Ha Giang province, and Phia Oac-Phia Den Nature Reserve, Cao Bang province, Vietnam are also referred to this species (Appendix I). All of these specimens are deposited in the IEBR and in the HNHM. Bats identified as *A. stoliczkanus* were previously recorded at Kim Hy Nature Reserve, Bac Kan province (Furey *et al.*, 2009, 2010, 2011); these specimens should be allocated to *A. dongbacana* because this area is situated in the distribution range and just ca. 50 km away from the type locality (Ba Be National Park) of the new species.

Etymology

The specific epithet refers to the restricted distribution range of the new species, called 'Đông Bắc' in Vietnamese. Its proposed English name is 'Dong Bac's trident bat' and Vietnamese name is 'Dơi mũi ba lá Đông Bắc'.

Diagnosis

A member of the *A. stoliczkanus* complex comprising all specimens found in northeastern Vietnam (Fig. 1) with a FA of ca. 42.8 mm, a GLS of ca. 15.2 mm (Table 1). The noseleaf is characterized by an upper margin divided into three points, and three lateral leaflets (Fig. 5). The pelage is characterized by long and soft hairs, brown or reddish brown on the dorsum and grey or white-grey on the belly. The ears are small and pointed (Fig. 5). The rostrum is sloping and elongated. The sagittal crest is relatively developed. The upper toothrows are convergent anteriorly. The upper incisors are bilobed. The upper and lower canines have low posterior cusps and are relatively robust with a length of ca. 1.95 mm and ca. 1.51 mm, respectively. The upper anterior premolar (PM^2) is compressed. The M^3 is scarcely reduced (Fig. 5). COI and Cyt*b* sequences differ from the other species of the genus *Aselliscus* by > 10%.

Description

Externally, this is a small species with a FA of ca. 42.8 mm. The upperparts are buffy brown to greyish-brown; the underparts are pale to buffy white. The noseleaf structure is characterized by an upper margin divided into three points, and three lateral leaflets. The ears are small and pointed. (Fig. 5). The cartilage of the fourth and fifth metacarpal is bifurcate.

The skull of the new species is small with a GLS of ca. 15.2 mm. The rostrum is sloping and elongated. The sagittal crest is relatively developed. The anteriors of the zygoma have a well-developed jugal projection. The upper toothrows are convergent anteriorly. The upper incisors are bilobed. The upper and lower canines have low posterior cusps; the upper anterior premolar (PM^2) is compressed. The M^3 is scarcely reduced (Fig. 5).

The baculum of the new species is bow-shaped or relatively straight in lateral view. The basal portion is widened with two lateral lobes. The shaft tapers slightly from the basal portion to the blunt tip (Fig. 3).

Comparisons with other species

In its morphological characters, *A. dongbacana* differs significantly from *A. tricuspidatus* by external, craniodental, and baculum features as well as its disjunct geographical distribution. As compared to *A. stoliczkanus* s.l., the new species is significantly different in size from the holotypes of *A. stoliczkanus* and *A. trifidus* (Table 1, Figs. 1, 4A, and 4C–4E). The external and bacular characters of *A. dongbacana* greatly overlap with those of *A. stoliczkanus* s.l. found in Indochina and Southern Thailand (including the type series of *A. wheleeri*), but the average of most craniodental measurements of the new species are significantly larger than those of the latter. The upper and lower canines of *A. dongbacana* are also significantly longer and more robust than those of the others (Table 1 and Fig. 5).

As for the acoustic characters, Furey *et al.* (2009) reported that the echolocation calls of *A. dongbacana*

found at Kim Hy Nature Reserve, Bac Kan province are characterized by a typical constant frequency followed by frequency modulated (CF/FM) signal, with a frequency of maximum energy (FmaxE) of 127.5±2.6 kHz (n = 5). Li *et al.* (2007) found that Chinese *A. stoliczkanus* s.l. emits calls with a relatively low FmaxE, e.g. in Sichuan and Guizhou the average frequency is 120.3±0.3 kHz (n = 10) and the range of values in Yunnan is 118.4–119.3 kHz. In Myanmar, Khin (2012) recorded an FmaxE of 126.68 ± 4.36 kHz for *A. stoliczkanus* s.l., whereas the FmaxE of the *A. tricuspidatus* ssp. collected in YUS Conservation Area, Papua New Guinea is around 115 kHz (Robson *et al.*, 2012).

Genetics

The Cyt*b* and COI sequences of *A. dongbacana* sp. n. differ from those of *A. stoliczkanus* s.l. and *A. tricuspidatus* by > 10.0% (Fig. 2 and Appendix III).

Distribution

The species is currently known only from karst areas in Northeastern Vietnam (Fig. 1).

Ecology and habitat

Like other *Aselliscus* species, *A. dongbacana* sp. n. is also associated with karst areas, and use caves as roosts both in heavily degraded and intact limestone habitats. So far, nothing is known on the diet of *A. dongbacana* sp. n., but they might forage on small nocturnal insects in dense environments like *A. stoliczkanus* sensu stricto (s.s.) does (Li *et al.*, 2007). However, the differences in skull size and especially in canine length suggest that their food sources may be different. Further studies on the diet of the two taxa is essential for a better understanding of whether food sources are important factors in their diversification. During our surveys, several pregnant females of *A. dongbacana* sp. n. were captured in May, while lactating females were found in June. These observations confirm that March–July is the primary reproductive period for the new species and also for other insectivorous bats in North Vietnam (Furey *et al.*, 2011).

Conservation status

To date, *A. stoliczkanus* s.l. has been classified as Least Concern in the IUCN Red List (Bates *et al.*, 2008). However, *A. dongbacana* sp. n. is endemic to northeast Vietnam and little is known about the current population trends of the species. Unfortunately, like many other regional plants and animals, *A. dongbacana* sp. n. might be at risk due to various types of roost and habitat destruction, i.e. mining, timber harvesting or cave tourism (Day and Urich, 2000; Clements *et al.*, 2006; Furey *et al.*, 2010). Further studies are needed to assess the impacts of habitat changes on *A. dongbacana* sp. n. to identify high priority conservation areas to protect the species (Hutson *et al.*, 2001; Furey *et al.*, 2010; Kingston, 2010).

The speciation of Aselliscus *in mainland Southeast Asia: when and how?*

Our molecular dating based on Cyt*b* sequences indicates that the separation between *A. dongbacana* sp. n. and *A. stoliczkanus* s.s. took place during the late Miocene (ca. 7.2 Mya), much earlier than the diversification among subclades of *A. stoliczkanus* s.s. around the Plio-Pleistocene boundary (ca. 2.8–2.4 Mya — Fig. 2 and Appendix IV). The period of interspecific divergence seems therefore to coincide with the hypothetic climatic and associated vegetation changes in the region during the late Miocene. Indeed, at the beginning of the late Miocene (ca. 10–8 Mya or more recently), the extent and uplift of the Himalayan mountains and the Tibetan Plateau, linked to the development of the Northern Hemispheric ice sheets played an important role in driving the Asian aridification (An, 2000; An *et al*., 2001; Zhang Y. G. *et al*., 2009). As a consequence, the cool, dry climate caused the vegetation to change from mixed coniferous and broad-leaved forests to grasslands in Asia, and rainforests of the region were thought to be compressed into different refugia (Morley, 2000; An *et al*., 2001). At the end of the late Miocene and until the early Pliocene epochs, Southeast Asia was a single block of rainforest, as a consequence of the warm and humid climatic conditions. However, the uplift of Himalaya-Tibetan plateau about 3.6–2.6 Mya and the onset of extensive glaciations on the Northern Hemisphere during the late Pliocene and Pleistocene epochs, led to the development of more open vegetation types and the contraction of the rainforest into several isolated refugia (Morley, 2000; An *et al*., 2001; Meijaard and Groves, 2006). With this in mind, the current distribution of *Aselliscus* spp. in Mainland Southeast Asia (Fig. 1) suggests that their separation probably occurred in different glacial refugia across the region during two major phases of aridification in Asia since the late Miocene. *Aselliscus* bats are very small (body mass ca. 5 g), fly at low speeds and are usually associated with karst areas and forage in cluttered habitats (Li *et al.*, 2007; Francis, 2008). These morphological and ecological features

indicate that they might have poor dispersal capacities and high natal philopatry that could prevent gene flow among different isolated populations and facilitate speciation events. Despite their long separation, these taxa were found to have similar morphology and echolocation call features; whereas previous studies indicated that different species of hipposiderid bats are usually recognizable by their call features (i.e., Kingston *et al.*, 2001; Thong *et al.*, 2012). However, given that *Aselliscus* spp. are associated with karst areas, we hypothesize that their ecological evolution might be under stabilizing selection imposed by the special environmental conditions of karst habitats (i.e., forests and caves) (Bickford *et al.*, 2007) and consequently reduces morphological and acoustic variation between different taxa.

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Appendix I

Studied specimens of *Aselliscus* spp.

Museum	Taxon	Clade	Sex	DNA N°	Field N°/ Specimen N°	GenBank accession Nº		Morphology			Country	Province	Locality
						COI*	Cyt*b*	External	Skull	Baculum			
HNHM	*A. stoliczkanus*	A	M		2005.82.50.				X		Laos	Khammouane	
VNU	*A. stoliczkanus*	A			MA269				X		Laos	Luang Phrabang	Gotte de Thump Cap
HNHM	*A. stoliczkanus*	A	M		2000.111.2.				X		Thailand	Kanchanaburi	
HNHM	*A. stoliczkanus*	A	F		88.49.1.				X		Vietnam	Ninh Binh	Cuc Phuong NP
HNHM	*A. stoliczkanus*	A	F		88.50.1.				X		Vietnam	Ninh Binh	Cuc Phuong NP
HNHM	*A. stoliczkanus*	A	F		88.50.2.				X		Vietnam	Ninh Binh	Cuc Phuong NP
HNHM	*A. stoliczkanus*	A	F		88.50.3.				X		Vietnam	Ninh Binh	Cuc Phuong NP
HNHM	*A. stoliczkanus*	A	M		88.50.4.				X		Vietnam	Ninh Binh	Cuc Phuong NP
IEBR	*A. stoliczkanus*	A	M	IEBR-M-4053	IEBR-M-4053	KU161550						Phu Tho	Xuan Son NP
IEBR	*A. stoliczkanus*	A	F	IEBR-M-4078	IEBR-M-4078	KU161551						Phu Tho	Xuan Son NP
IEBR	*A. stoliczkanus*	A	M	VN2013XS21			KU161559				Vietnam	Phu Tho	Xuan Son NP
IEBR	*A. stoliczkanus*	A	F	VN1987B9			KU161558				Vietnam	Phu Tho	Xuan Son NP
IEBR	*A. stoliczkanus*	A	F	IEBR-M-3457	IEBR-M-3457	KU161547					Vietnam	Quang Binh	Phong Nha-Ke Bang NP
IEBR	*A. stoliczkanus*	A	M	IEBR-M-3474	IEBR-M-3474	KU161548					Vietnam	Quang Binh	Phong Nha-Ke Bang NP
IEBR	*A. stoliczkanus*	A	F	IEBR-M-3482	IEBR-M-3482	KU161549					Vietnam	Quang Binh	Phong Nha-Ke Bang NP
HNHM	*A. stoliczkanus*	A	M	21922	21922	KU161546					Vietnam	Quang Tri	Dakrong NR
HNHM	*A. stoliczkanus*	A	M	22724	22724	KU161555					Vietnam	Quang Tri	Dakrong NR
HNHM	*A. stoliczkanus*	A	M		2007.50.26.				X		Vietnam	Quang Tri	Dakrong NR
IEBR	*A. stoliczkanus*	A	F	T5025	Tu.30.08.10.10	KU161553			X		Vietnam	Quang Tri	Dakrong NR
IEBR	*A. stoliczkanus*	A	M	T5024	Tu.31.08.10.7	KU161552			X	X	Vietnam	Quang Tri	Dakrong NR
IEBR	*A. stoliczkanus*	A	F	VN2835B9	B250813.2		KU161560				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	M	VN2834B8	B250813.1		KU161561				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	M	VN2836B10	B250813.3		KU161562				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	M	VN2850B24	B250813.17		KU161563				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	F	VN2851B25	B250813.18		KU161564				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	F	VN2875B49	B250813.42		KU161565				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	F	VN2876B50	B250813.43		KU161566				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	M	VN2883B57	B250813.50		KU161567				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	F	VN2884B58	B250813.51		KU161568				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	F	VN2885B59	B250813.52		KU161569				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	F	VN2913B72	B280813.2		KU161570				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	M	VN2940B98	B280813.10		KU161571				Vietnam	Quang Tri	Bac Huong Hoa
IEBR	*A. stoliczkanus*	A	M		B20140419.1			X	X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	M		B20140419.2				X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	F		B20140419.10			X	X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	F		B20140419.14				X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	M		B20140419.15				X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	F		B20140419.16			X	X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	F		B20140419.17			X	X		Vietnam	Thanh Hoa	Xuan Lien NR

APPENDIX I. Continued

Museum	Taxon	Clade	Sex	DNA N°	Field N°/ Specimen N°	GenBank accession Nº		Morphology			Country	Province	Locality
						COI*	Cyt*b*	External	Skull	Baculum			
IEBR	*A. stoliczkanus*	A	F		B20140419.19			X	X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	F		B20140419.20				X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	F		B20140419.21			X	X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	F		B20140419.24			X	X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	F		B20140419.25			X	X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	F		B20140419.31			X	X	X	Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	M		B20140419.4				X	X	Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	M		B20140419.5				X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	F		B20140419.54			X	X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	F		B20140419.8			X	X		Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	M	25001	GT1251	KU161557*		X	X	X	Vietnam	Thanh Hoa	Xuan Lien NR
IEBR	*A. stoliczkanus*	A	M	VN11-0417	VN11-0417	KU161554			X		Vietnam	Thanh Hoa	Ngoc Lac Town
IEBR	*A. stoliczkanus*	B	M	VN11-0115	VN11-0115	KU161539					Vietnam	Bac Kan	Ba Be NP
IEBR	*A. stoliczkanus*	B	M	VN11-0118	VN11-0118	KU161540			X		Vietnam	Bac Kan	Ba Be NP
IEBR	*A. stoliczkanus*	B	M	VN11-0124	VN11-0124	KU161541		X	X		Vietnam	Bac Kan	Ba Be NP
IEBR	*A. stoliczkanus*	B	M	VN11-0125	VN11-0125	KU161542		X	X	X	Vietnam	Bac Kan	Ba Be NP
IEBR	*A. stoliczkanus*	B	M	VN11-0143	VN11-0143	KU161543		X	X		Vietnam	Bac Kan	Ba Be NP
IEBR	*A. stoliczkanus*	B	M	VN11-0144	VN11-0144	KU161544			X		Vietnam	Bac Kan	Ba Be NP
IEBR	*A. stoliczkanus*	B	M	VN11-0146	VN11-0146	KU161545		X	X	X	Vietnam	Bac Kan	Ba Be NP
HNHM	*A. stoliczkanus*	B	F	21907	2007.27.9.	KU161556*					Vietnam	Bac Kan	Ba Be NP
IEBR	*A. stoliczkanus*	B	M		VTTu-0173				X		Vietnam	Cao Bang	Phia Oac-Phia Den NR
IEBR	*A. stoliczkanus*	B	M		VTTu-0170			X	X	X	Vietnam	Cao Bang	Phia Oac-Phia Den NR
IEBR	*A. stoliczkanus*	B	F		VTTu-0174				X		Vietnam	Cao Bang	Phia Oac-Phia Den NR
IEBR	*A. stoliczkanus*	B		KF2581	B290613-5		KU161574				Vietnam	Ha Giang	Khau Ca NR
IEBR	*A. stoliczkanus*	B	M	KF2602	B300613-9		KU161575				Vietnam	Ha Giang	Khau Ca NR
HNHM	*A. stoliczkanus*	B	F		98.3.5.				X		Vietnam	Tuyen Quang	Na Hang NR
HNHM	*A. stoliczkanus*	B	F		98.90.13.				X		Vietnam	Tuyen Quang	Na Hang NR
IEBR	*A. stoliczkanus*	B	M		B200514.12			X	X		Vietnam	Tuyen Quang	Na Hang NR
IEBR	*A. stoliczkanus*	B	M	IEBR.M.1919	B200514.3	KU161538			X		Vietnam	Tuyen Quang	Na Hang NR
IEBR	*A. stoliczkanus*	B	M	VN3431B1	B220514.1		KU161572	X	X		Vietnam	Tuyen Quang	Na Hang NR
IEBR	*A. stoliczkanus*	B	M	VN3432B2	B220514.2		KU161573	X	X		Vietnam	Tuyen Quang	Na Hang NR
IEBR	*A. stoliczkanus*	B	F	B250514.4	B250514.4				X		Vietnam	Tuyen Quang	Na Hang NR
IEBR	*A. stoliczkanus*	B	F	B250514.7	B250514.7				X		Vietnam	Tuyen Quang	Na Hang NR
IEBR	*A. stoliczkanus*	B	M	B250514.8	B250514.8			X	X		Vietnam	Tuyen Quang	Na Hang NR
IEBR	*A. stoliczkanus*	B	M	B300514.1	B300514.1			X	X		Vietnam	Tuyen Quang	Na Hang NR
HNHM	*A. tricuspidatus*		M	2397.7					X	X	Papua New Guinea		
HNHM	*A. tricuspidatus*		F	2466.12					X		Papua New Guinea		Morobe

* — COI sequences were also done in Hungary

Appendix II

GenBank accession nos. of specimens included in the phylogenetic analyses

Original name	Clade	COI	Cyt*b*	Country	Province	Locality
Pteropus scapulatus		NC_002619	NC_002619			
Rousettus leschenaultii		HM541872	DQ888669			
Megaderma lyra		HM540834	DQ888678			
Rhinolophus luctus		HM541591	DQ297596			
R. hipposideros		JF443130	DQ297586			
R. affinis		HM541411	DQ297582			
R. ferrumequinum		JF443129	DQ297575			
R. pearsonii		HM541681	DQ297587			
R. pusillus		HM541458	DQ297583			
Hipposideros pomona		JF443930	DQ888671			
H. pratti		HM540611	DQ297584			
H. armiger		HM540326	DQ297585			
H. larvatus		JF443896	DQ888672			
Coelops frithii		HQ918409	DQ888674			
Aselliscus tricuspidatus			DQ888675	Vanuatu		Espiritu Santo
A. tricuspidatus			DQ888679	Vanuatu		Espiritu Santo
A. stoliczkanus	A		DQ888670	China	Yunnan	
A. stoliczkanus	A		DQ888668	China	Yunnan	
A. stoliczkanus	A		EU434953	China	Yunnan	
A. stoliczkanus	A		DQ888676	China	Guizhou	
A. stoliczkanus	A		DQ888677	China	Guizhou	
A. stoliczkanus	A		DQ888673	China	Sichuan	
A. stoliczkanus	A		EU434954	China	Yunnan	
A. stoliczkanus	A	HM540134		Myanmar		
A. stoliczkanus	A	HM540133		Myanmar		
A. stoliczkanus	A	HM540130		Myanmar		
A. stoliczkanus	A	HM540159		Laos	Louang Namtha	
A. stoliczkanus	A	JF443870		China	Guizhou	Libo
A. stoliczkanus	A	JQ600013		China	Guizhou	Libo
A. stoliczkanus	A	HM540163		Vietnam	Sapa	Ta Phin
A. stoliczkanus	A	HM540168		Vietnam	Sapa	Ta Phin
A. stoliczkanus	A	HM540169		Vietnam	Sapa	Ta Phin
A. stoliczkanus	A	HM540128		Laos	Attapeu	Ban Keng Bit
A. stoliczkanus	A	JF443872		Laos	Vientiane	Phou Khao Khouay
A. stoliczkanus	A	HM540129		Laos		Namet
A. stoliczkanus	A	HM540161		Laos		Namet
A. stoliczkanus	A	HM540172		Laos		Ban Phon Song
A. stoliczkanus	A	HM540127		Laos		Ban Xam Kang
A. stoliczkanus	A	HM540146		Laos		Xe Bang Fai
A. stoliczkanus	B	HM540152		Vietnam	Tuyen Quang	Na Hang NR
A. stoliczkanus	B	JF443865		Vietnam	Tuyen Quang	Na Hang NR
A. stoliczkanus	B	HM540158		Vietnam	Lang Son	Huu Lien NR

Appendix III

Average nucleotide distances (%) based on the Kimura 2-parameter (K2P) model between *Aselliscus* spp., and associated outgroups based on complete mitochondrial Cyt*b* (1,140 bp, below the diagonal) and COI (657 bp, above the diagonal) gene sequences

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	16.6	19.0	21.0	20.7	20.1	19.6	21.8	20.4	21.8	21.9	19.9	20.0	18.6	NA	20.2	20.1	
2	15.4		19.3	21.8	19.8	20.7	19.9	20.2	19.8	19.5	19.0	19.3	19.0	20.9	NA	20.2	19.8
3	20.1	20.0		18.3	18.1	19.6	17.7	20.1	18.9	19.8	18.4	20.9	18.4	19.8	NA	19.0	19.4
4	18.7	20.6	18.1		15.4	15.4	13.7	14.5	13.7	18.9	18.4	17.4	16.8	15.7	NA	15.4	17.0
5	18.6	20.7	18.9	10.9		11.6	15.2	16.1	13.9	17.7	18.1	17.0	19.2	16.3	NA	15.4	16.5
6	19.5	21.2	18.5	12.5	11.3		14.2	15.5	15.5	17.8	18.1	17.4	18.7	17.8	NA	16.7	16.8
7	19.4	20.7	18.8	12.1	11.4	11.7		16.3	14.6	19.5	18.6	17.5	19.7	18.3	NA	16.7	18.9
8	18.7	20.0	18.3	10.9	11.1	11.8	11.8		16.1	19.0	19.5	18.4	19.8	19.9	NA	18.0	17.8
9	18.2	20.8	17.8	10.7	10.8	12.5	12.3	10.2		17.4	18.0	16.7	18.3	17.5	NA	17.0	18.3
10	17.8	20.4	19.8	16.4	16.4	16.8	16.7	17.2	16.7		6.8	15.5	14.5	16.4	NA	17.7	17.6
11	18.2	20.4	19.8	16.1	16.1	16.7	17.2	17.4	16.7	8.5		15.8	13.1	16.6	NA	16.4	16.9
12	18.6	20.8	20.0	16.6	17.4	16.4	16.2	17.5	16.6	13.8	15.0		16.2	16.3	NA	16.6	16.1
13	17.1	19.0	18.7	17.0	16.2	16.8	17.5	16.6	15.2	11.0	10.4	13.7		16.9	NA	15.5	16.2
14	18.7	19.2	20.0	15.8	15.7	15.7	16.3	16.6	15.3	15.2	15.1	14.5	15.2		NA	14.9	17.0
15	18.2	20.9	18.8	16.3	16.1	15.9	15.4	15.9	15.6	14.6	15.6	14.4	13.6	14.0	0.4 / NA	NA	NA
16	19.4	21.0	19.4	16.9	16.1	17.6	16.2	17.1	15.8	15.9	16.6	15.3	15.0	14.1	13.1	6.3/6.8	11.6[b]
17	19.5	19.7	19.7	15.9	15.9	16.6	15.6	16.5	15.8	15.1	14.8	15.7	13.8	13.5	12.8	10.3[a]	0.2/2.0

Taxon: 1 — *Pteropus scapulatus*; 2 — *Rousettus leschenaultii*; 3 — *Megaderma lyra*; 4 — *Rhinolophus affinis*; 5 — *R. ferrumequinum*; 6 — *R. hipposideros*; 7 — *R. luctus*; 8 — *R. pearsonii*; 9 — *R. pusillus*; 10 — *Hipposideros armiger*; 11 — *H. larvatus*; 12 — *H. pomona*; 13 — *H. pratti*; 14 — *Coelops frithii*; 15 — *Aselliscus tricuspidatus*; 16 — *A. stoliczkanus* clade A; and 17 — *A. stoliczkanus* clade B
NA — not applicable; [a, b] — the range (min–max) of K2P distances calculated from Cyt*b* sequences (10.0–10.9) and COI sequences (10.7–13.5), respectively

Appendix IV

Chronogram reconstructed from the Cyt*b* dataset for *Aselliscus* spp. and associated outgroups. Mean divergence values (expressed as million year ago, Mya) are given at each node and horizontal bars represent the 95% highest posterior density ranges. Clade names of *A. stoliczkanus* s.l. correspond to those given in Fig. 2