



# A taxonomic reassessment of *Rhinolophus rex* Allen, 1923 and its allies (Chiroptera: Rhinolophidae)

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## Abstract

This study integrates analyses of mitochondrial DNA sequences and morphological and acoustic data to re-evaluate the taxonomic status of *Rhinolophus rex*, *R. r. paradoxolophus* and *R. schnitzleri* throughout their distribution ranges. Based on a dense geographic sampling of specimens hitherto referred to these taxa and contrary to the current taxonomic view, our results indicate that all examined specimens of these taxa are representatives of a single, widely distributed and morphologically variable species, *R. rex*. The recognition of its geographic populations as different subspecies (*R. r. rex* and *R. r. paradoxolophus*) or distinct species (*R. schnitzleri*) based on morphological and acoustic data should be regarded as invalid. In the light of this revision, we also reassess the conservation status of *R. rex* against IUCN Red List criteria as Near Threatened.

## Keywords

Bats, conservation, cryptic species, integrative taxonomy, *Rhinolophus*, Southeast Asia

## Introduction

Horseshoe bats within the monotypic family Rhinolophidae are relatively small to medium-sized insectivores that occur throughout the Old World, including Africa, Europe, Asia, and Australasia. They have a unique nose-

leaf structure that includes an anterior leaf (horseshoe), an intermediate leaf (sella), a connecting process and a posterior leaf (lancet) and emit echolocation pulses that comprise a long constant-frequency (CF) component

preceded and followed by a frequency-modulated (FM) sweep (e.g., FM-CF-FM pulses). Since 2003, knowledge regarding the global diversity and distribution of rhinolophid bats has advanced greatly with the number of valid species increasing from 71 to 109. Most of the newly-recognised species were previously misidentified as representatives of widespread, polytypic taxa, whereas in some cases, two or more closely-related species formerly distinguished in morphological studies have now been subsumed into single taxa (Csorba et al. 2003; Simmons and Cirranello 2022b).

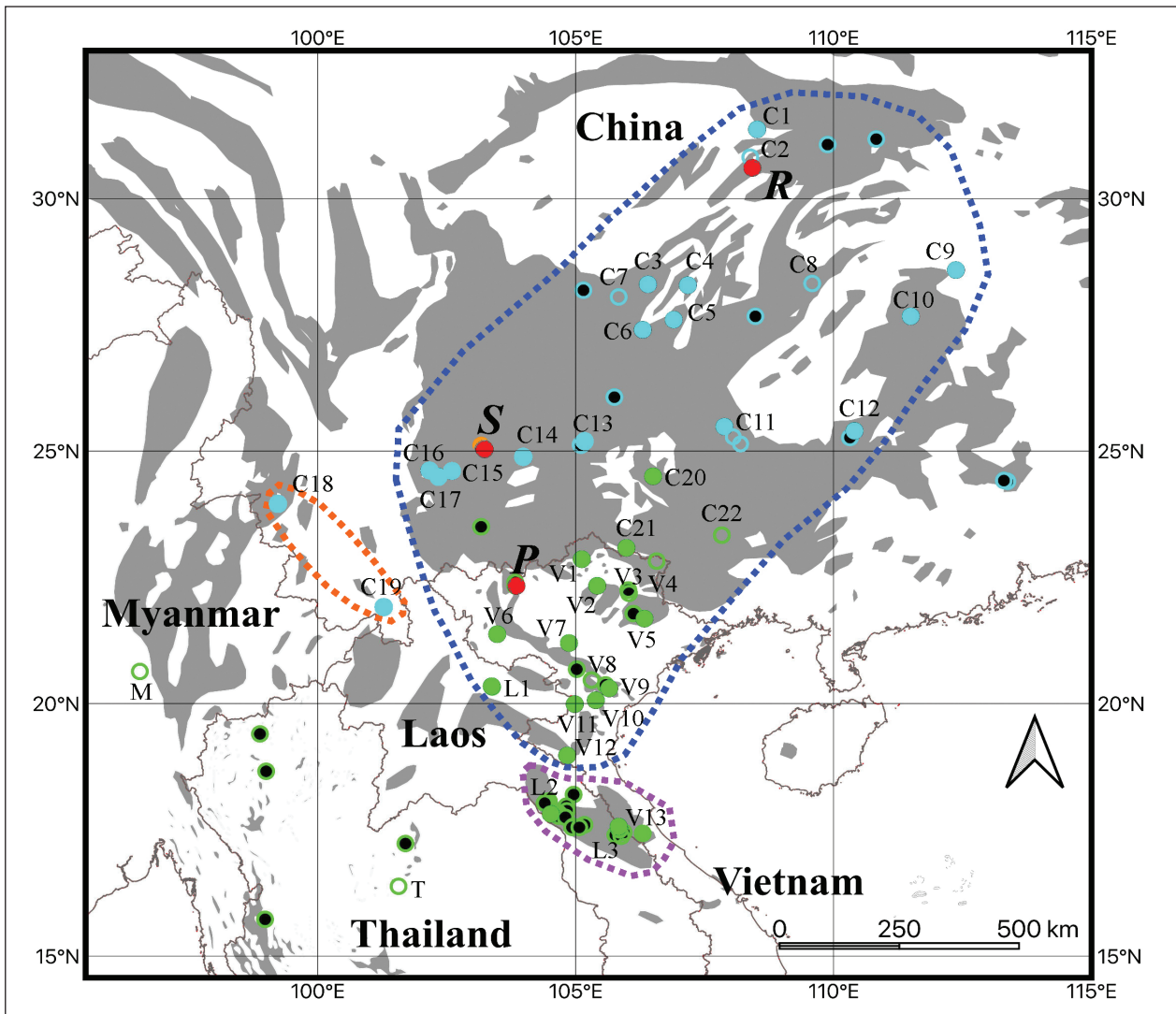
For many years, *Rhinolophus rex* Allen, 1923 (type locality: Wanhshien, Sichuan Province, China) and *R. paradoxolophus* (Bourret, 1951) (type locality: Sa Pa, Lao Cai, Vietnam), originally placed in *Rhinomegaphyllus*, were treated as distinct species within the “*R. philippinensis*” group which included other big-eared horseshoe bats (*R. macrotis* Blyth, 1844, *R. philippinensis* Waterhouse, 1843 and *R. marshalli* Thonglongya, 1973) (Hill 1972; Thonglongya 1973; Corbet and Hill 1992). However, since individuals of the two species were found to share strikingly similar external and cranial characteristics, despite the slight average differences in some measurements (e.g., the forearm and skull length of *R. rex* were 56.5–63.0 mm and 21.95–22.05 mm, respectively, whereas those for *R. paradoxolophus* were 50.5–57.0 mm and 20.1–20.88 mm) and seemingly disjunct distributions (e.g., the former species was regarded as endemic to south China, whereas the latter occurred in more southern localities from mainland Southeast Asia), their specific separation was long questioned (Hill 1972; Thonglongya 1973; Corbet and Hill 1992; Csorba et al. 2003; Simmons 2005). Further doubt was introduced when Zhang et al. (2009a) recorded specimens of two size classes, corresponding to *R. rex* and *R. paradoxolophus* (in Yunnan and Guangxi, China, respectively) that emitted similar frequency calls. As the authors also found a comparable situation for Chinese *R. rex* and *R. paradoxolophus* recorded in Laos, they suggested that small differences in morphological measurements did not warrant specific separation of the two forms and that *R. paradoxolophus* was best recognised as a race or subspecies of *R. rex* (Zhang et al. 2009a).

Considering that the taxonomic conclusions of Zhang et al. (2009a) remained uncertain, Wu and Thong (2011) treated *R. rex* and *R. paradoxolophus* as geographically and morphologically distinct species when describing a new horseshoe bat species (*R. schnitzleri*) from a single specimen collected in Yunnan, China (type locality: Xiaodong cave, Gengjiaying commune, Yi-liang County, Kunming city, 25°02'N, 103°14'E, 1550 meters above sea level [m a.s.l.]). *Rhinolophus schnitzleri* was regarded as having a body and skull size intermediate between *R. paradoxolophus* in northern Vietnam and *R. rex* in different parts in China. It was also reported as being distinguished from the latter taxa by its nose-leaf structures (its sella being considerably narrower and longer than that of *R. rex* and *R. paradoxolophus*), certain craniodental characteristics (in *R. schnitzleri*, the first upper premolar (P2) is equally separated from the canine (C1) and second

premolar (P4), whereas in *R. rex* and *R. paradoxolophus* it is in contact with C1 and separated from P4) and baculum features (the baculum of *R. schnitzleri* is distinctly larger than that of *R. rex* and *R. paradoxolophus* in all respects). Following this view, Yu et al. (2016) identified two bats collected in a cave (N25°02.767', E103°13.455', 1632 m a.s.l.) near the type locality of *R. schnitzleri* as new records for the species. However, subsequent analyses of published morphological and acoustic data have shown that the phenetic disparities between *R. schnitzleri*, and *R. rex* / *R. paradoxolophus* complex are likely unreliable (Tables 1–2) and suggest that the specific status of the former taxon is questionable (Burgin et al. 2020).

A growing number of studies in recent years have integrated analyses of molecular, morphological and/or acoustic data to reassess the taxonomic systematics and evolutionary history of *R. rex* sensu lato (s. l.) and its allies (Francis et al. 2010; Tu et al. 2017; Zhang et al. 2018; Liu et al. 2019; Chornelia et al. 2022). For instance, Zhang et al. (2018) considered that all members apart from *R. philippinensis* within the formerly recognized “*R. philippinensis* group” should be transferred into the “*R. macrotis* group” and that specimens of *R. rex* from most parts of China and *R. paradoxolophus* from southern Guangxi, China and northern Vietnam were conspecific, with the latter taxon representing a subspecies of the former due to its smaller body size on average and higher echolocation frequencies. As the taxonomic treatments of Zhang et al. (2018) were consistent with those of Zhang et al. (2009a), the subspecies category has now been used for *R. rex* s. l. bats formerly assigned to *R. rex* and *R. paradoxolophus* as follows (Burgin et al. 2020): *R. r. rex* comprises bats restricted to Sichuan, Chongqing, Guizhou, Hunan, northern Guangxi and Guangdong provinces (China), whereas *R. r. paradoxolophus* includes individuals recorded in southern Guangxi and eastern Yunnan (China), eastern Myanmar, northern Thailand, northern and central Laos, and northern and central Vietnam (Fig. 1).

However, several lines of evidence suggest that current taxonomy of *R. rex* s. l. is debatable. For instance, it should be noted that while the phenetic disparity reported by Zhang et al. (2018) between *R. r. rex* and *R. r. paradoxolophus* was based on slight differences in morphometrics and acoustic frequencies, additional data from other studies suggests these taxa overlap greatly in both traits (Tables 1, 2). This means the subspecies identification of *R. rex* specimens by Zhang et al. (2018) may have been biased by insufficient taxonomic sampling (e.g., 22 *R. r. rex* sensu stricto [s. str.] specimens versus only 3 *R. r. paradoxolophus* s. str. congeners). In particular, in a recent analysis of mitochondrial cytochrome oxidase I (COI) gene fragment based on wider geographical samples of two recognised subspecies of *R. rex* sensu lato (s. l.) (sensu Burgin et al. 2020), Chornelia et al. (2022) found three reciprocally monophyletic lineages as follows: *rex1* – including only *R. r. rex* bats endemic to southwestern Yunnan, China; *rex2* – including only *R. r. paradoxolophus* individuals from the Khammoune region of central Laos; and *rex3* – including specimens



**Figure 1.** Distribution patterns of the three study taxa, *Rhinolophus rex rex* (cyan dots), *R. r. paradoxolophus* (green dots), and *R. r. schnitzleri* (dark orange dot) (sensu Burgin et al. 2020). Distribution of karst (shaded) in the region was modified from Ford and Williams (2007). Red dots with letters **R**, **P**, and **S** refer to the type localities of the three taxa *R. r. rex*, *R. r. paradoxolophus*, and *R. r. schnitzleri*, respectively. The distribution range of three potential cryptic species within *R. rex* sensu lato namely, *rex1*, *rex2*, and *rex3* (sensu Chornelia et al. 2022) are defined by dotted lines in dark orange, violet, and blue, respectively. Country codes include: **C** China, **L** Laos; and **V** Vietnam. Black symbols refer to literature records only of *R. r. rex* and *R. r. paradoxolophus*. Localities for specimens included in the genetic analyses are filled whereas those of individuals in morphological and/or acoustic analyses only are empty.

of both subspecies collected widely from all over China, Laos and Vietnam, excluding areas occupied by the two former taxa (Fig. 1). Since the interspecific COI divergences expressed by Kimura 2-parameter (K2P) distances between these three lineages were relatively higher than those between two sister species, *R. shameli* and *R. coelophyllus* ( $\geq 1.82\%$ ) and their splits were also supported by the multispecies coalescent analyses, Chornelia et al. (2022) suggested that they may represent potential cryptic species. This single locus species delimitation, however, requires further testing with additional genetic markers, geographically denser sampling and phenetic traits (Liu et al. 2019; Tu et al. 2021; Chornelia et al. 2022).

In this study, we integrate analyses of two mitochondrial genes (COI and the complete cytochrome *b* [cyt *b*]) and morphological and acoustic data from available

specimens of *R. rex* s. l. and *R. schnitzleri* to re-evaluate the validity of taxonomic treatments proposed for these by previous authors and reassess their conservation status using the IUCN Red List Categories and Criteria version 15.1 (IUCN 2022).

## Methods

### Taxonomic assignments of study specimens

To assess the taxonomic status of taxa previously identified within *R. rex* s. l., our study material were hitherto assigned into two subspecies, *R. r. rex* and *R. r.*

*paradoxolophus* (sensu Burgin et al. 2020; Zhang et al. 2018) and to the three putative cryptic species, *rex1*, *rex2*, and *rex3* (sensu Chornelia et al. 2022) where relevant (Fig. 1).

## Genetic analyses

Eight new samples of Vietnamese *R. r. paradoxolophus* were collected from the chest muscles of voucher specimens, preserved in 95% ethanol and stored at  $-20^{\circ}\text{C}$  (Table S1).

Total DNA was extracted using the QIAGEN DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Two mitochondrial genes were sequenced for this study: *cyt b* (1,140 bp) and COI (705–1545 bp). Primer sets used for PCR amplification of *cyt b* were Mt-14724F/Cyb-15915R (Irwin et al. 1991), Cyb-14726F/Cyb-15909R (Arai et al. 2016), or Glu-CH/Thr-CH (Hassanin 2014) and for COI were UTyrLA/C1L705 (Hassanin et al. 2012) or MammMt-5533F/MammMt-7159R (Arai et al. 2019).

PCR amplifications of these mitochondrial genes were performed as detailed in Tu et al. (2021). PCR products were resolved by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light.

Both strands of PCR products were sequenced using Sanger sequencing on an ABI 3730 automatic sequencer at the Centre National de Séquençage (Genoscope) in Evry (France) and ABI 3730xl DNA Analyzer at the Infectious Disease Surveillance Center, Japan. The sequences were edited and assembled using CodonCode Aligner Version 3.7.1 (CodonCode Corporation) and Genetyx v11 software (Genetyx Corporation, Japan). Sequences generated for this study were deposited in the EMBL/DBJ/GenBank database under the accession numbers *OQ659403–OQ659410* and *OQ658596–OQ658598* (Table S1).

Our sequences were aligned with 83 COI and 37 *cyt b* sequences of *R. r. rex* and *R. r. paradoxolophus* acquired from GenBank using AliView 1.22 (Larsson 2014). The localities for bats sampled are shown in Fig. 1 and Table S1. No gaps and stop codons were found in the mitochondrial protein-coding sequences after translation into amino-acids. Our final COI (91 taxa, 657 bp) and *cyt b* (40 taxa, 1140 bp) alignments were analysed in PopART 1.5 (Leigh and Bryant 2015) to construct haplotype networks using the median joining method with equal weights for all mutations. Pairwise genetic distances between taxa were calculated with PAUP\* v. 4b10 (Swofford 2003) using the K2P distance.

## Morphological analyses

Thirty-one specimens (three released bats and 28 voucher specimens) of *R. rex* s.l. (*R. r. rex*:  $n = 13$  and *R. r. paradoxolophus*:  $n = 18$ ) were morphologically examined by the authors (Table S2). Specimens examined are held in the Institute of Ecology and Biological Resources

(IEBR, Hanoi, Vietnam), the American Museum of Natural History (AMNH, New York, USA), the Northeast Normal University (NENU, Jilin, China), the College of Life Sciences, Guangxi Normal University (GNU-CLS, Guilin, China) and the Faculty of Environmental Sciences, National University of Laos (FES-NUL, Vientiane, Laos). All specimens examined were adults, as confirmed by the presence of fully ossified metacarpal-phalangeal joints. The forearm length (FA) of all bats was taken to the nearest 0.1 mm. Craniodental measurements were taken to the nearest 0.01 mm using digital callipers under a stereomicroscope. These included: SL—the greatest length from the occiput to the front of canine; CCL—condylo-canine length, from the exoccipital condyle to the most anterior part of the canine; ZB—greatest width of the skull across the zygomatic arches; MB—greatest distance across the mastoid region;  $C^1C^1$ —greatest width across the upper canines between their buccal borders;  $M^3M^3$ —greatest width across the crowns of the last upper molars;  $CM^3$ —maxillary toothrow length, from the anterior of the upper canine to the posterior of the crown of the 3<sup>rd</sup> upper molar; ML—length of mandible, from the anterior rim of the alveolus of the first lower incisor to the most posterior part of the condyle; and  $CM_3$ —mandibular toothrow length, from the anterior of the lower canine to the posterior of the crown of the 3<sup>rd</sup> lower molar.

Bacula were extracted from two specimens of Vietnamese *R. r. paradoxolophus* and preserved following Friley (1947). Digital 2D images of these bacula were taken using the same acquisition parameters on a Leica M80 stereomicroscope (Leica Microsystems Ltd, Switzerland). The baculum of one intact specimen of Vietnamese *R. r. paradoxolophus* (VN11-0442) was also examined using an X-ray microtomography device (inspeXio SMX-90CT Plus, Shimadzu Corp., Kyoto, Japan) and its genitals were scanned with 18  $\mu\text{m}$  voxels and a 90 kv source voltage with 100 mA source current. An osseous 3D model of the baculum was manually segmented using Amira 5.2 software (Visage Imaging, San Diego, USA) (Sohn et al. 2021). This can be obtained from the Figshare repository (<https://doi.org/10.6084/m9.figshare.14784504.v1>). Four measurements of bacula were also taken to the nearest 0.01 mm: TBL—total bacular length, from the tip to the base; GWB—greatest width at the base of the baculum; GWM—greatest width at the mid-point of the shaft of the baculum; and GWT—greatest width at the tip of the baculum.

We undertook uni- and multi-variate analyses of morphometric data to test the phenetic affinities of *R. r. rex*, *R. r. paradoxolophus* and *R. schnitzleri* based on our material and specimens examined in previous studies (Allen 1923; Thonglongya 1973; Zhang et al. 2009b; Wu and Thong 2011; Yu et al. 2016). As these measurements are standard in bat research and vary little between observers, our comparisons can be performed with reasonable confidence (Palmeirim 1998; Tu et al. 2015). Prior to analysis, all data were scaled to the same precision of measurements obtained from the literature and data from both sexes was combined due to a lack of sexual dimorphism (T-test:  $p > 0.05$ ). Principal component

**Table 1.** Forearm length (FA) and frequency of maximum energy (FmaxE) of *Rhinolophus rex* s. l. and its allies recorded in the present and previous studies. Values are given as min–max and/or mean±SD; sample size. Acronyms and definitions for measurements are given in the text.

Taxon		Distribution	FA (mm)	FmaxE (kHz)	Ref.
A	B				
<i>R. r. rex</i>	<i>rex3</i>	China	53.1–60.0 57.4±2.1; 16	25.2–25.5 25.4±0.2; 2	[1]
		China	56.63±2.22; 21	24.83±0.51; 19	[2]
		China	55.4±1.1; 8	26.8±0.2; 18	[3]
		China	54.8–60.3; 16	—	[4]
		China	—	~ 25; 4	[5]
	<i>rex1</i>	China	—	~ 25; 2	[5]
<i>R. r. paradoxolophus</i>	<i>rex3</i>	Vietnam	49.4–55.4 53.0±2.3; 12	28.1–33.2 30.1±2.5; 5	[1]
		China / Vietnam	52.84±3.42; 11	31.02±0.51; 2	[2]
		Vietnam	48.0–57.6; 14	—	[4]
	<i>rex2</i>	Laos	54.1–55.9 55.1±0.9; 3	25.1–25.6 25.4±0.4; 2	[1]
		Laos	50.9–56.8; 17	~ 25	[6]
		Vietnam	56.8	25	[1]
	NA	Myanmar	54.3–56.0 55.2±1.2; 2	—	[7]
	NA	Thailand	54.0	—	[8]
<i>R. schnitzleri</i>	NA	China	54.3–57.7 56.2±1.7; 3	23.9–24.1 24.0±0.2; 2	[4,9]

Note: **A** sensu Burgin et al. (2020); **B** sensu Chornelia et al. (2022); **NA** Not Assessed, [1] this study; [2] Zhang et al. (2018); [3] Feng et al. (2001); [4] Wu and Thong (2011); [5] Chornelia et al. (2022); [6] Robinson and Webber (2000); [7] Oo et al. (2017); [8] Thonglongya (1973); [9] Yu et al. (2016).

analysis (PCA) was performed in PAST (Hammer et al. 2001) on nine log-transformed cranial measurements of our material and data from previous studies (in a few instances, missing data due to partially damaged skulls were replaced by mean values from the same taxon). The equalities of mean values of all morphological measurements and PC scores obtained from PCA between different taxa were tested using a one-way analysis of variance (ANOVA), followed by a Kruskal–Wallis test (Zar 1999).

## Acoustic analyses

In the field, echolocation calls of bats held in the hand or resting in a flight tent were recorded with Pettersson D240x, D980 and D1000X bat detectors (Pettersson Elektronik, Sweden) or an Echo Meter Touch detector (Wildlife Acoustics, USA). These call recordings were deposited in the bat call library of the ChiroVox project (Görföl et al. 2022) under the ChiroVoxIDs or accession numbers indicated in Table S2. All recordings were analysed using BatSound Pro 4.1 (Pettersson Elektronik, AB) and for each bat, the mean value of the frequency of maximum energy (FmaxE) was calculated from 5–10 calls. We also tabulated the same metrics reported for *R. r. rex*, *R. r. paradoxolophus* and *R. schnitzleri* in the literature to determine inter- and intraspecific variation in their echolocation calls.

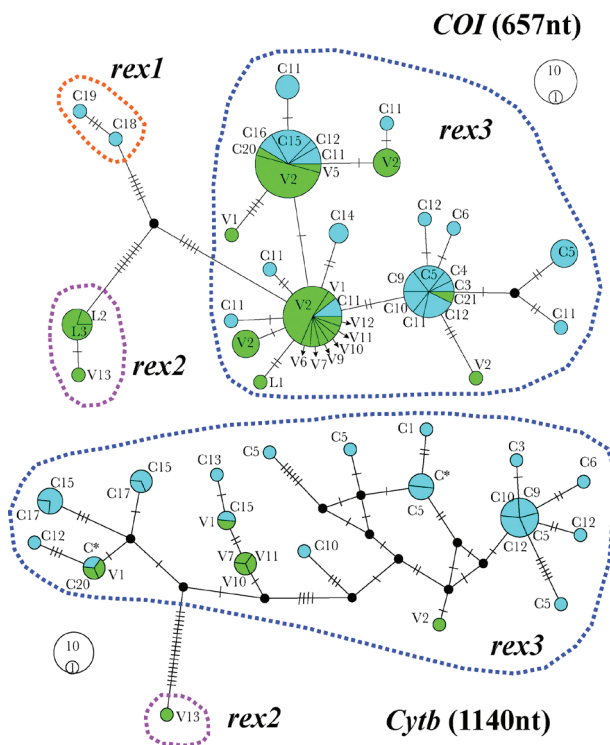
## Results

### Genetic analyses

Twenty-one haplotypes of the COI gene and 19 haplotypes of the *cyt b* gene differing by 0.2–3.8% and 0.1–2.3% K2P genetic distances (data not shown), respectively, were identified from our study material for *R. rex* s. l. (Fig. 2).

Considering the taxonomic treatments proposed for *R. rex* s. l. by Zhang et al. (2018) and Burgin et al. (2020), our analyses of the mtDNA sequences reveal a genetic admixture between geographic populations of two recognised subspecies, *R. r. rex* and *R. r. paradoxolophus* found in China, Laos and Vietnam. While mtDNA haplotypes derived from many specimens of both subspecies collected from spatially isolated locations were identical or intemixed, those of the same taxon found in sympatry were relatively divergent (Fig. 2). K2P genetic distances calculated from mtDNA sequences (COI/*cyt b*) within *R. r. rex* and *R. r. paradoxolophus* were 0.0–3.7% / 0.0–1.5% and 0.0–3.2% / 0.0–2.2%, respectively, while those between them were 0.0–3.8% / 0.0–2.3% (data not shown).

In agreement with the taxonomic classification of *R. rex* s. l. bats proposed by Chornelia et al. (2022), our phylogeographic analysis of COI sequences showed the existence of three haplogroups (separated by K2P dis-



**Figure 2.** MtDNA haplotypes of *Rhinolophus rex* ssp. specimens in the NJ networks. The colours of mtDNA haplotypes of study specimens match their corresponding locations in Fig. 1 (sensu Burgin et al. 2020; Zhang et al. 2018, Chornelia et al. 2022). MtDNA haplotypes of some Chinese specimens of *R. r. rex* whose collecting locations are uncertain are indicated with an asterisk (C\*) (Table S1).

tances of 2.0–3.9%), corresponding to *rex1*, *rex2* (but with a distribution range extending to central Vietnam), and *rex3* (Figs 1, 2; Table S3). Within *rex3*, however, the K2P genetic distances between the mtDNA haplotypes were up to 1.7% (Table S3). Similarly, in the *cyt b* haplotype network, the single haplotype available for the *rex2* was separated from *rex3* by a K2P genetic distance of 1.8–2.3%, while the maximum K2P distance between *rex3* individuals was 1.5% (Table S3).

### Morphological comparisons

#### External characteristics

Bats assigned to *R. r. rex* from China (= *rex3*) and *R. r. paradoxolophus* from Khammouan, Laos (= *rex2*) and Vietnam (= *rex2* and *rex3*) shared comparable noseleaf structures, including very wide internarial lappets, a tall and wide sella and a low and rounded lancet. Intra- and inter-specific variations in these features within and between these taxa were not distinguishable (Fig. 3).

Pairwise comparisons of data obtained from our work and previous studies (Thonglongya 1973; Robinson and Webbe 2000; Feng et al. 2001; Wu and Thong 2011; Yu et al. 2016; Oo et al. 2017; Zhang et al. 2018) showed a large overlap in forearm length among *R. rex* s. l. bats hitherto assigned to *R. r. rex* and *R. r. paradoxolophus*

**Table 2.** Craniodental measurements (mm) of *Rhinolophus rex* s. l. and its allies recorded by present and previous studies. Values are given as min–max, mean±SD; sample sizes differing from those reported under n are given in parentheses. Acronyms and definitions for measurements are given in the text.

Taxon	Location		n	SL	CCL	ZB	MB	CM3	C1C1	M3M3	ML	CM3	Ref.
	A	B											
<i>R. r. rex</i>	China	<i>rex3</i>	5	21.95–22.05 22.0±0.1 (3)	20.00 (1)	9.71–9.97 9.9±0.1 (4)	10.97–11.06 11.0±0.0 (4)	8.02–8.26 8.1±0.1	5.10 (1)	6.60 (1)	13.93–14.39 14.2±0.2	8.21–8.45 8.3±0.1	[1]
			1	22.1	20.0	9.9	11.2	7.8	5.1	6.6	14.0	8.0	[2]
			2	22.1–23.6 22.9±1.1	19.9–20.0 20.0±0.1	9.9–10.2 10.1±0.2	10.8–11.2 11.0±0.3	7.8–7.9 7.9±0.1	4.9–5.1 5.0±0.1	6.6–6.6 6.6±0.0	6.60 (1)	14.0–14.4 14.2±0.3	8.0–8.2 8.1±0.1
<i>R. r. paradoxolophus</i>	Vietnam	<i>rex3</i>	15	19.27–21.31 20.5±0.6	17.14–19.11 18.3±0.5	8.56–9.8 9.2±0.3	9.48–10.49 10.2±0.3	6.50–7.73 7.3±0.4	3.98–4.40 4.2±0.1	6.0–6.67 6.3±0.2	12.12–14.02 13.2±0.5	7.3–7.89 7.6±0.2	[1]
			1	21.96	18.35	9.63	10.45	7.3	4.58	6.73	13.48	7.41	[1]
			2	20.7–20.8 20.8±0.1	18.3–18.6 18.4±0.3	9.2–9.3 9.3±0.0	10.0–10.5 10.3±0.4	7.3–7.3 7.3±0.0	4.0–4.2 4.1±0.1	6.0–6.2 6.1±0.1	6.60 (1)	14.0–14.5 14.3±0.4	7.4–7.5 7.5±0.1
<i>R. r. schmitzleri</i>	Thailand	NA	1	21.1	18.2	9.7	10.5	7.1	4.5	6.4	13.2	7.4	[2]
			3	21.4–21.8 21.6±0.2	19.6–19.7 19.7±0.1	9.6–9.7 9.6±0.1	10.7–10.9 10.8±0.1	7.8–7.9 7.9±0.1	4.8–4.9 4.9±0.1	6.6–6.7 6.7±0.1	6.60 (1)	13.8–14.5 14.1±0.4	8.1–8.8 8.4±0.4

Note: **A** sensu Burgin et al. (2020); **B** sensu Chornelia et al. (2022); **NA** Not Assessed; [1] This study; [2] Thonglongya (1973); [3] Zhang et al. (2009); [4] Oo et al. (2017); [5] Wu and Thong (2011); [6] Yu et al. (2016).



**Figure 3.** Portrait and noseleaf morphology of selected male specimens of *Rhinolophus rex* ssp. found in allopatry. **A** *R. r. rex* (= *rex3*): NF.151109.4; C11 in Fig. 1; **B** *R. r. paradoxolophus* (= *rex3*): VN11-0442, V9 in Fig. 1; **C** *R. r. paradoxolophus* (= *rex2*): FES-CBC02618, L3 in Fig. 1. Not to scale.

(sensu Burgin et al. 2020) and to two putative cryptic species *rex2* and *rex3* (sensu Chornelia et al. 2022) (Table 1; Fig. 4A). The same pattern in forearm length was also found between *R. rex* s. l. and *R. schnitzleri* (Table 1; Fig. 4A).

### Craniodental characteristics

As detailed in previous studies (Hill 1972; Thonglongya 1973; Csorba et al. 2003), our specimens of *R. r. rex* and *R. r. paradoxolophus* shared similar cranial features including elongated and narrow skulls with prominent and elliptical median nasal swellings.

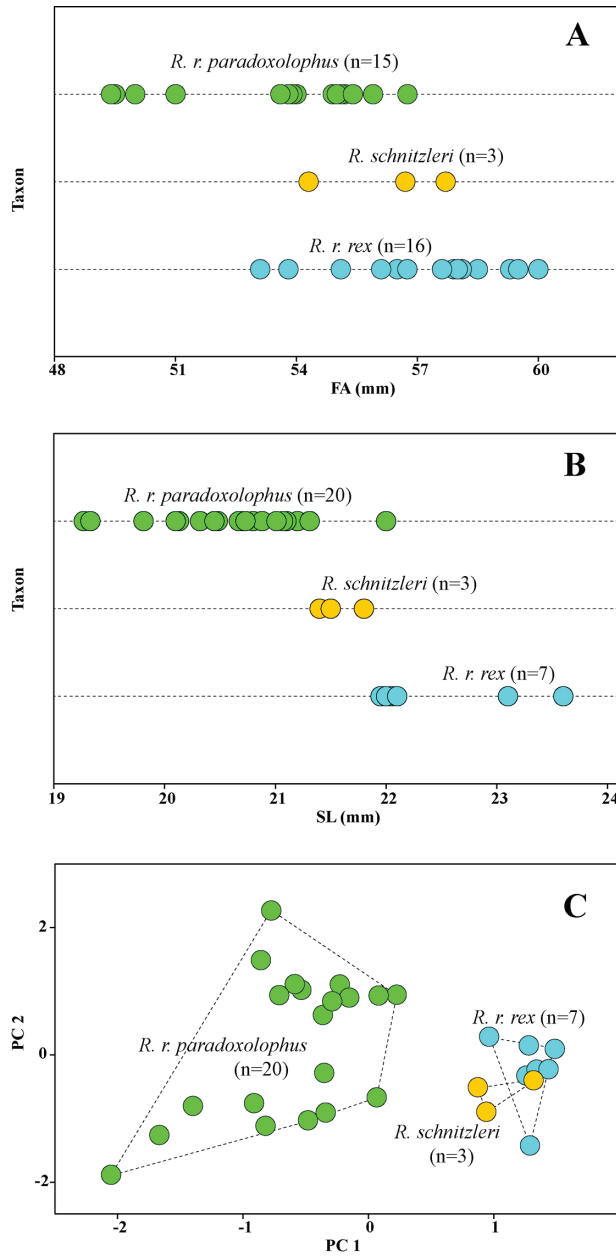
In terms of the craniodental dimensions, *R. r. rex* specimens from northern China (e.g., C2, C7, C8 in Fig. 1) were significantly larger than those of *R. r. paradoxolophus* from Laos, Myanmar, Thailand, and Vietnam (Table 2; Fig. 4B) (ANOVA;  $P \leq 0.05$ ). In relation to these two taxa, the three known specimens of *R. schnitzleri* were slightly smaller than *R. r. rex* but averaged larger than *R. r. paradoxolophus* in most respects, although these differences were not statistically significant (Table 2) (ANOVA;  $P > 0.05$ ). In contrast, in our PCA of nine cranial measurements, specimens of *R. r. rex* and *R. schnitzleri* specimens were grouped in a separate cluster

from specimens including *R. r. paradoxolophus* on the size axis of PC1 (Fig. 4C; Table S4).

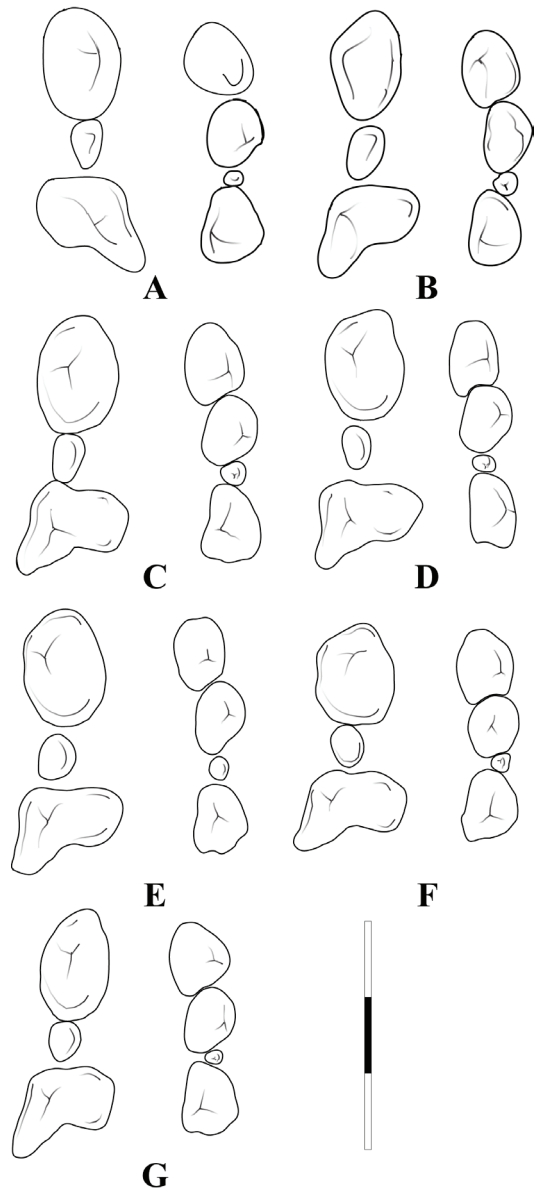
Our examinations also revealed high individual variation in the anterior dental features of *R. r. paradoxolophus* specimens occurring in sympatry and allopatry in Vietnam. For instance, the first upper premolar ( $P^2$ ) was either equally separated from or in contact with the upper canine ( $C^1$ ) and the second upper and lower premolar ( $P^4$  and  $p_3$  respectively) were usually situated in the toothrow, although sometimes displaced externally, and the crowns of these teeth also varied in size and shape (Fig. 5). As such, the differences reported by previous studies (Wu and Thong 2011; Yu et al. 2016) in the dental morphology of *R. r. rex*, *R. r. paradoxolophus* and *R. schnitzleri* are unlikely to be reliable for the purposes of distinguishing specific or subspecific variation.

### Bacular morphology

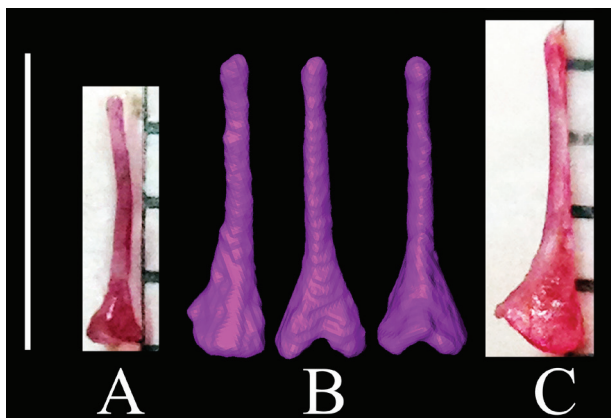
Examination of three *R. r. paradoxolophus* specimens from northern Vietnam (= *rex3*) showed that their baculum size and shape varied individually (Fig. 6). For example, the total baculum length of these specimens ranged between 3.5–4.5 mm and largely overlapped with measurements for *R. r. rex* (3.1–3.2 mm,  $n = 2$ ) and *R. schnitzleri* (4.2–



**Figure 4.** Scatter plots generated from quantitative morphological analyses of *Rhinolophus r. rex*, *R. r. paradoxolophus*, and *R. schmitzleri*. **A** and **B** Ranges of FA and SL measurements of specimens within each taxon. **C** Plot of PC1 against PC2 from PCA on log-transformed craniodental measurements of specimens.



**Figure 5.** Occlusal views of upper (left) and lower (right) anterior dentition of *Rhinolophus rex* ssp. specimens. **A** *R. rex rex* (AMNH 56890, holotype, ♀) from China; *R. rex paradoxolophus* from Vietnam: **B** MNHN 1948.358, holotype (♀, Sapa, Lao Cai), **C** NF.120606.3 (♂, V3 in Fig. 1), **D** NF.120606.7 (♀, V3 in Fig. 1), **E** VTTu15.006 (♂, V4 in Fig. 1); **F** GT1269 (♂, V11 in Fig. 1), and **G** GT1706 (♀, V8 in Fig. 1). Scale = 3 mm.



**Figure 6.** Bacula of *Rhinolophus r. paradoxolophus* specimens found in allopatry in northern Vietnam. **A** VN11-0057 (V6 in Fig. 1); **B** VN11-0442 (V9 in Fig. 1); and **C** VTTu15.006 (V4 in Fig. 1). Scale = 4 mm.

**Table 3.** Bacular measurements of *Rhinolophus rex* s. l. and its allies recorded in the present and previous studies. Values are given as min–max and/or mean±SD; sample size. Acronyms and definitions for measurements are given in the text.

Taxon*	Distribution	TBL	GWB	GWM	GWT	Ref.
<i>R. r. rex</i> (= <i>rex3</i> )	China	3.1–3.2; 2	0.5–0.6; 2	0.2–0.2; 2	0.2–0.2; 2	[1]
<i>R. r. paradoxolophus</i> (= <i>rex3</i> )	Vietnam	3.5–4.5 4.0±0.6; 3	0.8–1.5 1.2±0.4; 3	0.2–0.4 0.3±0.1; 3	0.2–0.4 0.3±0.1; 3	[2]
	Vietnam	4.2; 1	1.2; 1	0.3; 1	0.3; 1	[1]
<i>R. schnitzleri</i>	China	4.2–4.7	1.0–1.3	0.3–0.5	0.3–0.4	[1,3]
		4.5±0.3; 3	1.2±0.2; 3	0.4±0.1; 3	0.2±0.1; 3	

Note: \*sensu Burgin et al. (2020) = sensu Chornelia et al. (2022); [1] Wu and Thong (2011); [2] this study; and [3] Yu et al. (2016).

4.7 mm, n = 3) from China, as reported by Wu and Thong (2011) and Yu et al. (2016) (Table 3; Fig. 6).

### Acoustic comparisons

Our pairwise comparisons of data recorded by different authors (present study; Chornelia et al. 2022; Feng et al. 2001; Furey et al. 2009; Robinson and Webbe 2000; Tu et al. 2017; Zhang et al. 2018) revealed a large overlap in echolocation call frequency (FmaxE) between geographic populations hitherto assigned to two subspecies, *R. r. rex* and *R. r. paradoxolophus* (sensu Burgin et al. 2020) and to the three putative cryptic species, *rex1*, *rex2* and *rex3* (sensu Chornelia et al. 2022), as well as between *R. rex* s. l. and *R. schnitzleri*. For instance, the greatest difference between populations examined, without taking into account the effects of sexual dimorphism and/or geographical distance, was less than 10 kHz, e.g. 24 kHz vs. 33 kHz (Table 1).

## Discussion

### How many taxa are in *R. rex* s. l.?

Our mtDNA analyses of *R. rex* s. l. from China, Laos, and Vietnam are mostly consistent with previous studies (Tu et al. 2017; Zhang et al. 2018; Chornelia et al. 2022) in showing: (1) a complex and admixed genetic structure for bats assigned to *R. r. rex* and *R. r. paradoxolophus* (sensu Zhang et al. 2018; Burgin et al. 2020), and (2) the existence of three relatively divergent haplogroups uniting bats of the three putative cryptic species, *rex1*, *rex2* and *rex3* recognised by Chornelia et al. (2022), respectively (Fig. 2). In the latter case, however, the gaps between intra- and interspecific divergences for both COI and *cyt b* genes within and between these taxa are only small e.g., between 0–1.7% vs. 2.0–3.9% and between 0–1.5% vs. 1.8–2.3%, respectively (Table S3). These findings suggest that the use of any values within the known ranges of mtDNA K2P distances amongst studied *R. rex* s. l. bats as standard thresholds for cryptic species delimitation (e.g., a COI K2P value of 1.82%) by Chornelia et al. (2022) in the absence of additional data is unjustified (Francis et al. 2010; Tobe et al. 2010). In addition, as the current

distribution of the three a priori taxa, *rex1*, *rex2* and *rex3*, of *R. rex* s. l. is confined to three relatively disjunct karst blocks in mainland Asia (Fig. 1), their interspecific genetic variations could be due to female philopatry and/or other evolutionary processes (e.g., range contractions, fragmentations and expansions of their ancestral populations during the Pleistocene) (Tu et al. 2017; Zhang et al. 2018; Liu et al. 2019). As these explanations were not considered by Chornelia et al. (2022), their hypothesis that several cryptic species may be present in *R. rex* s. l. should be ruled out, although further investigation using additional samples from unstudied areas and nuDNA sequences is needed to confirm this.

In agreement with our genetic analyses, our morphological and acoustic analyses indicate that geographic populations of *R. rex* s. l. in China and nearby countries previously assigned to either *R. r. rex* or *R. r. paradoxolophus* (sensu Zhang et al. 2018; Burgin et al. 2020), or to three putative cryptic species, *rex1*, *rex2* and *rex3* (sensu Chornelia et al. 2022) overlap greatly in most independent characters, excepting craniodental dimensions (Figs 3–6; Tables 1–3). However, it should be noted that differences in skull size were found only between a handful of *R. r. rex* specimens from northern China and *R. r. paradoxolophus* specimens from southern Guangxi, China and nearby countries (Laos, Myanmar, Thailand, and Vietnam), such phenotypic disparity among allopatric populations of *R. rex* s. l. may reflect adaptive plasticity of these traits in response to differing environments (Tu et al. 2021) or may be biased by incomplete sampling. In support of the latter inference, our pairwise comparisons of morphometrics and acoustic data failed to reveal any phenetic disparities between *R. schnitzleri* and *R. rex* s. l. specimens (Fig. 4; Tables 1–3). Although none of the available specimens of *R. schnitzleri* have been examined genetically, the proximity of their collection localities and those of *R. rex* s. l. (both from the same karst block in Yunnan, China: Fig. 1) suggests that the likelihood of cryptic speciation events having occurred without geographic isolation in this species complex is low. As a consequence, we conclude that the description of *R. schnitzleri* was based on a misinterpretation of morphological variation within *R. rex* (due to the limited information available at the time: Wu and Thong 2011; Yu et al. 2016) and that *R. schnitzleri* should be regarded as a junior subjective synonym of *R. rex*. In line with this, from a biogeographical viewpoint and based on our

univariate analyses of craniodental measurements (Figs 1, 4B; Table 2), bats previously identified as *R. schnitzleri* may resemble *R. r. paradoxolophus* s. str. rather than *R. r. rex* s. str. and as such, the differences in skull size between geographic populations of the two a priori subspecies of *R. rex* s. l. consequently disappear (Fig. 3C).

Taken together, our results indicate that *R. rex* is a single, widespread and morphologically variable species and that previous classifications of its geographic populations as different subspecies (e.g., *R. r. rex* and *R. r. paradoxolophus*) or even distinct species (e.g., *R. schnitzleri*) should be regarded as invalid.

## Reassessment of the conservation status of *R. rex*

*Rhinolophus rex*, *R. paradoxolophus* and *R. schnitzleri* are currently included as valid species on the IUCN Red List of Threatened Species, whereby *R. rex* is listed as Endangered and endemic to China, *R. paradoxolophus* as Least Concern and occurring from southern China to Laos, Thailand and Vietnam, and *R. schnitzleri* as Data Deficient and endemic to the type locality in Yunnan, China (Bates et al. 2008; Sun 2019, 2020). In view of our finding that these taxa actually represent a single, widely distributed and morphologically variable species (*R. rex*), reassessment of its conservation status (IUCN 2022) is warranted. In this context, while the current extent of occurrence of *R. rex* is >20,000 km<sup>2</sup> (Fig. 1), most of its populations occur at low density (Oo et al. 2017; Sun 2020; this study) and have likely declined due to increased modification and destruction of their roosting and foraging habitats (i.e., cave systems and forests) in recent decades (Clements et al. 2006; Furey et al. 2010; Furey and Racey 2016; Hughes 2017). As such, *R. rex* qualifies as Near Threatened because its global population has likely reduced by 25–30% over the past 10 years and will likely continue to decline in coming years (nearly qualifying as Vulnerable under criterion A4c).

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## Supplementary Material 1

### Table S1

**Authors:** Tu VT, Furey NM, Görföl T, Hassanin A, Arai S, Koyabu D, Douangboubpha B, Csorba G (2023)

**Data type:** .pdf

**Explanation note:** Table S1. GenBank Accession numbers of samples included in genetic analyses of this study.

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**Link:** <https://doi.org/10.3897/vz.73.e101487.suppl1>

## Supplementary Material 1

### Table S2

**Authors:** Tu VT, Furey NM, Görföl T, Hassanin A, Arai S, Koyabu D, Douangboubpha B, Csorba G (2023)

**Data type:** .pdf

**Explanation note:** Table S2. List of specimens that were morphologically and/or acoustically examined by the authors in this study.

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**Link:** <https://doi.org/10.3897/vz.73.e101487.suppl2>

## Supplementary Material 3

### Table S3

**Authors:** Tu VT, Furey NM, Görföl T, Hassanin A, Arai S, Koyabu D, Douangboubpha B, Csorba G (2023)

**Data type:** .pdf

**Explanation note:** Table S3. Range (min–max) of K2P distances (%) based on COI / *cyt b* datasets between study specimens of the three putative cryptic species within *Rhinolophus rex* s. l. (sensu Chornelia et al. 2022).

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**Link:** <https://doi.org/10.3897/vz.73.e101487.suppl3>

## Supplementary Material 4

### Table S4

**Authors:** Tu VT, Furey NM, Görföl T, Hassanin A, Arai S, Koyabu D, Douangboubpha B, Csorba G (2023)

**Data type:** .pdf

**Explanation note:** Table S4. Factor loading for PCs obtained from PCA of cranial characters.

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**Link:** <https://doi.org/10.3897/vz.73.e101487.suppl4>