



Unusual Pipistrelle: Taxonomic Position of the Malayan Noctule (*Pipistrellus stenopterus*; Vespertilionidae; Chiroptera)

Sergei V. Kruskop^{1,*}, Evgeniya N. Solovyeva¹, and Anna D. Kaznadzey²

¹Zoological Museum of Moscow State University, Moscow, Russia

²A.A. Kharkevich Institute for Information Transmission Problems, RAS, Moscow, Russia

(Received 13 July 2018; Accepted 20 November 2018; Published 17 December 2018; Communicated by Benny K.K. Chan)

Citation: Kruskop SV, Solovyeva EN, Kaznadzey AD. 2018. Unusual pipistrelle: taxonomic position of the Malayan noctule (*Pipistrellus stenopterus*; Vespertilionidae; Chiroptera). Zool Stud **57:**60. doi:10.6620/ZS.2018.57-60.

Sergei V. Kruskop, Evgeniya N. Solovyeva, and Anna D. Kaznadzey (2018) Despite huge progress in the systematics of bats and, in particular, of the Vespertilionidae family in latest years, the taxonomic position of a number of remarkable bat species has been uncertain until now, partly because of limits in acceptable comparative material. Researchers have previously placed the Malayan noctule, *Pipistrellus stenopterus*, into *Nyctalus*, because of similar body shape and proportions, or into *Pipistrellus*, based on karyological analysis. This study reassesses *Pipistrellus stenopterus* using available collection material and compares it to various members of *Nyctalus* and *Pipistrellus*, as well as with some other related and similar genera, based on respective morphological and molecular genetic features. This species demonstrates vast morphological peculiarities compared to other *Pipistrellus*-like bats. Nonetheless, both mitochondrial and nuclear genetic markers unequivocally place it close to other Asian pipistrelles, most probably in a sister position to the "*javanicus*" species group. We propose establishing *P. stenopterus* as a separate subgenus, *Alionoctula*. Our results also confirm that *Pipistrellus* is paraphyletic in its current state, and we suggest that further studies explore its internal taxonomy and limits.

Key words: Chiroptera, Taxonomy, Phylogeny, Vespertilioninae, Pipistrellus new subgenus.

BACKGROUND

Taxonomic status and position of many members of *Vespertilionidae*, the largest family within Chiroptera, remain unresolved. Some of them are "white spots" (groups or species with understudied taxonomic features due to lack of collected material) and some of them are known as "blind spots" (taxa for which, for various reasons, researchers have not paid enough attention) (Kruskop 2016). Fortunately, an increase in interest towards taxonomy studies in recent years has resulted in new collection materials, new revisions and species descriptions, and, finally, new perspectives on bat taxonomic diversity in general. The Malayan noctule (also known as "narrowwinged pipistrelle") was described by Dobson (1875) based on a specimen (No 42.8.19.14 in collection of the Natural History Museum, London) from Sarawak, Borneo (now – state of Malaysia). It was described as a species within the genus *Vesperugo* Keiserling and Blasius, 1839, which in Dobson's understanding included all the forms currently known as serotines and pipistrelles. This genus was divided by Dobson into several subgenera; the form *stenopterus* Dobson, 1875 was assigned to the nominotypical subgenus along with the 'true' noctules. It was also combined with other noctules by Miller (1907) in the genus *Pterigystes* Kaup, 1829. Tate (1942) combined

^{*}Correspondence: E-mail: selysius@mail.ru

pipistrellus-like species with shortened and robust rostrums (joffrei Thomas, 1915, stenopterus, anthonyi Tate, 1942 and brachypterus Temminck, 1840) into the "joffrei" species group within the genus Pipistrellus Kaup, 1829 and mentioned its similarity to Philetor Thomas, 1902. Hill (1966) also agreed with that opinion, although did not provide further taxonomic implications. Later he transferred Pipistrellus brachypterus into Philetor (Hill 1971), leaving other three forms, including *P. stenopterus*, in the previous species group. It was placed either within Nyctalus Bowditch, 1825, based on external similarities (Ellerman and Morrison-Scott 1966; Koopman 1994) and teeth morphology (Menu 1987), or within *Pipistrellus*, based on peculiarities of its skull and teeth (Koopman 1973; Pavlinov et al. 1995). Hill and Harrison (1987) allocated stenopterus into Hypsugo Kolenati, 1856 (as subgenus of Pipistrellus) based on its baculum morphology, and this placement was accepted by a number of other scientists (Corbet and Hill 1992; Nowak 1994). However, Horáček and Hanak (1985-86), while raising *Hypsugo* to the genus level, moved *joffrei* there, but left *stenopterus* in Pipistrellus because of its lower cheek teeth morphology. This point of view was supported by karyological studies (Volleth and Heler 1994), which unequivocally allocated stenopterus to the Pipistrellini tribe and Pipistrellus genus (close to South-East Asian P. mimus Wroughton, 1899). It is worth noting that Philetor brachypterus, according to respective data, should have been placed into the Vespertilionini tribe, close to Tylonycteris Peters, 1872; and this suggestion was recently supported by molecular studies (Ruedi et al. 2017). More recent works commonly place the Malayan noctule into Pipistrellus s. str. (e.g. Simmons 2005; Francis 2008), although results from respective material studies are not provided.

Specific shape, which definitely distinguishes *P. stenopterus* from all other pipistrelles, prompted us to analyze morphological traits of this species; we found that it is quite different from most of other known vespertilionine genera and probably could be assigned to a genus of its own (Kruskop 2003 2010). The uncertain status of this species demands further studies, in particular, a molecular genetics analysis.

In this work, assessed the taxonomic position of *Pipistrellus stenopterus* with an integrated approach. We analyzed its phylogenetic position by comparing the sequences of two mitochondrial and one nuclear gene and also compared its qualitative morphological features, the proportions of skull and dental system, and the structure of baculum with other species of noctules and "pipistrelles". Allocation of the Malayan noctule to *Pipistrellus* s. lato seems doubtless, but its unique morphological features allowed us to suggest a separate subgenus within *Pipistrellus* for this species. We also demonstrated that the *Pipistrellus* genus itself is apparently paraphyletic.

MATERIAL AND METHODS

DNA extraction and analysis

Tissue sample of *Pipistrellus stenopterus* was taken from an ethanol preserved specimen (voucher number ZMMU S-103149; North Sumatra, Medan; February, 1972) from the collection of the Zoological Museum of Moscow State University.

DNA was extracted and purified using the QIAamp DNA MiniKit (Qiagen) including an overnight lysis step at 56°C and longer incubation with EB-buffer (5 min) at the purification step. We amplified two mitochondrial genes (cytb and COI) and one nuclear gene (RAG2). DNA was highly degraded, so only short fragments (100-200 bp) were obtained using the combination of internal primers designed for this study (Table S1). Primer pairs for cytb and RAG2 were developed manually using Bioedit (Hall 1999) and an alignment of candidate bat genomes from GenBank.

Primer sequences for the COI analysis were obtained using a bioinformatics pipeline developed ad hoc due to high variation of COI fragments in bats. This allowed us to select optimal oligonucleotides of a given length for PCR analysis or NGS target sequencing. In this study the length of candidate primers varied between 22 and 27 nucleotides. The candidate primer sequences were obtained from candidate bat genomes. These sequences were additionally modified according to the differences in the candidate genomes, which were aligned using the T-Coffee multiple alignment tool (Notredame et al. 2000). "Y" (C/T), "S" (C/G), "R" (A/G) and "K" (G/T), "W" (A/T), "D" (A/G/T) nucleotides were added within primer sequences to take account for the respective SNPs. The candidate primer sequences were then put through a pipeline, which employs the primer3 algorithm (Untergasser et al. 2012) with default parameters, including parameter for 3' stability of 9.0, GC content variation between 30 and 70% with an optimum of 50%, and melting temperature (Tm) between 57°C and 62°C with an optimum of 59°C. The pipeline allowed us to select the optimal primer sequences, which were used to amplify the query COI sequence, divided into overlapping amplicones not exceeding 180 bp. The pipeline ensured that primers were compatible so that none annealed to another, and that they were specific so no primers annealed to wrong genomic loci.

The PCR program that amplified short fragments included an initial denaturation at 95°C for 3 min, 45 cycles of 95°C for 30 s, annealing temperature (see Table S1) for 30 s and 72°C for 30 s, and a final extension of 72°C for 6 min. All stages of the extraction process included a negative control run in parallel. To avoid contamination, extraction and amplification of the DNA from the museum specimens were carried out in the ZMMU Laboratory of Historical DNA, exclusively equipped for work with museum DNA specimens, where no previous work on fresh tissues had been performed.

PCR products were visualized on a 1% agarose gel, then sequenced via Evrogen on ABI PRISM 3500xl sequencer. All sequences were deposited in GenBank under the following accession numbers: COI MH540193, cytb MH540194 and RAG2 MH540195.

Additional sequences were downloaded from GenBank (see Table S2).

Sequences were first aligned in BioEdit Sequence Alignment Editor 7.1.3.0 (Hall 1999) with default parameters. Subsequently, the alignment was checked and manually revised if necessary using Seqman 5.06 (Burland 1999). Genetic distances were calculated using MEGA 6.1 (Tamura et al. 2013). Because the sequences had different sources (*i.e.* different specimens of *Pipistrellus javanicus* as a source for COI and cytb sequences), we did not concatenate different genes and performed separate analyses for each alignment.

The optimum partitioning schemes for nuclear and mitochondrial alignments were identified with PartitionFinder (Lanfear et al. 2012) using the greedy search algorithm under AIC criterion. For COI there were HKY + G, SYM + G, F81 + I, for cytb – HKY + G, GTR + G and GTR + I + G; for RAG2 – GTR + G.

Phylogenetic trees were reconstructed under Bayesian criteria and the maximum likelihood (ML) method. Bayesian inference (BI) was performed in MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) with two simultaneous runs, each with four chains, for 5 million generations. We checked the convergence of the runs and that the effective page 3 of 15

sample sizes (ESS) were all above 200 by exploring the likelihood plots using TRACER v1.5 (Rambaut and Drummond 2007). The initial 10% of trees were discarded as burn-in. Confidence in tree topology was assessed by posterior probability (PP) (Huelsenbeck and Ronquist 2001). The ML trees were generated in IQ Tree (Nguyen et al. 2015) using ultrafast bootstrap of 10000 (UFBoot, Minh et al. 2013); models were selected using ModelFinder (Kalyaanamoorthy et al. 2017): TIM2 + I + G4 - COI, TIM2 + I + G4 - cytb, TIM3e + G4 -RAG2.

Morphological and morphometric study

Five Pipistrellus stenopterus specimens from North Sumatra (ZMMU S-103146-150; alcoholpreserved bodies, three with extracted skulls) housed in the Zoological Museum of Moscow State University were initially used for morphological studies of the species. An additional 322 specimens with extracted skulls, representing in total nine Eurasian genera (Arielulus, Glischropus, Falsistrellus, Hypsugo, Nyctalus, Phyletor, Pipistrellus, Scotozous, and Tylonycteris) and 40 species, most of which currently are or formerly were recognized as "pipistrelles" (see e.g. Ellerman and Morrison-Scott 1966; Hill and Harrison 1987; Corbett and Hill 1992; Koopman 1994), were used for the morphometric and morphological study. That group involved, in particular, 17 specimens of *Pipistrellus stenopterus*, including the holotype (NHM 42.8.19.14). The entire list of specimens used in the morphometric study is provided in appendix 1.

For the morphometric study, a set of 22 cranial and teeth measurements was taken to determine the inter-taxa variability: total length of the skull (TL), condylocanine length (CCL), condylobasal length (CBL), mastoid width of skull at the level of the auditory bullae (MW), width of braincase above mastoids (BCW), occiput height, measured from the lower margins of occiput condyles (OH), maximal width across zygomatic arches (ZW), least width of the postorbital constriction (POC). least width between eye sockets (IOW), rostral width at the level of the infraorbital foramina (RW), rostral length from anteorbital foramen to the alveolus of the inner incisor (RL), crown-measured width between the outer margins of upper canines (CC), crown-measured width between outer margins of M3 (MM), C-M3 length (CM), maxillary molariform row length (PM), length of the upper canine cingulum base (C), crown width and length

of the upper posterior molar (M3W and M3L), length of the hard palate from anterior margines of canines to the posterior palate emargination (Pal), crown length of mandibular tooth row (cm), lower jaw length from alveolus of i1 to the posterior extremity of glenoid process (MdL), and lower jaw height to the tip of coronoid process (MdH). Measurements were standardized in relation to the condylo-canine length, to avoid influence of the overall size. To assess the pattern of variation of quantitative characters, Principal Component (PC) and Discriminant Function (DF) analyses were performed using appropriate Analysis and Classification modules in STATISTICA for Windows version 8.0 (StatSoft, Inc., 2004).

To study the penial bone shape, baculum of the single adult male from the Sumatra group was prepared in a 6% solution of alkali (KOH) by standard procedure (White 1951).

RESULTS

Sequence characteristics

The complete matrix contained 41 samples of Vespertilioninae specimens for COI, 34 samples for cytb, and 33 samples for RAG2; *Myotis blythii* was taken as the outgroup in each alignment. Information on the length and variability of the fragments is provided in table 1 (all data shown for ingroup only). Nucleotide composition analysis showed similar proportions per nucleotide for RAG2 (21.3-29.0%), but an anti-G bias for COI (17.5%) and cytb (13.9%). MtDNA genes contained more phylogenetically informative positions (266 positions or 40.4% for COI and 444 positions or 38.9% for cytb) compared to nuclear gene RAG2 (184 positions or 20.8%).

Uncorrected mtDNA genetic distances are shown in tables 2-4 (below the diagonal). *p*-distances were high not only between groups (up to 22.28% for mtDNA and up to 14.31% for nuDNA), but also within groups (maximum 12.59% for mtDNA and 6.47% for nuDNA).

Phylogenetic analysis

The results of the phylogenetic analysis are presented in figures 1-3. BI and ML analyses yielded trees that demonstrated essentially similar topologies. COI phylogeny is less resolved than cytb and RAG2 phylogenies, but includes more specimens and species. Topology of the cytb phylogenetic tree is more consistent with RAG2 results than with COI; incongruities mainly concern the positions of *Hypsugo* and *Vansonia*.

We demonstrate that there are three main clades on the obtained phylogenetic trees.

Clade I includes *Arielulus*. The basal split occurs between *Arielulus* and a monophyletic clade, which comprises all other studied genera. *Arielulus* is rather distant from clade II + III, p = 17.66-22.28% for mtDNA genes and 8.35-14.31 for nuclear gene.

Clade II comprises three genera (*Tylonycteris* + *Philetor* + *Hypsugo*), two of them are monophyletic with a high support (1/97-1/100). *Hypsugo* is shown to be parafyletic on the COI tree: *H.* cf. *joffrei* remains within clade II, while *H. cadornae* forms a sister lineage to *Vansonia*. RAG2 and cytb reconstructions lack sequences of *H.* cf. *joffrei*, and *Hypsugo* is represented only by *H. cadornae*.

Clade III includes several lineages; relationships between them remain mostly unresolved:

- Vansonia - due to cytb and RAG2 data this genus represents the most differentiated lineage within Clade III; on the COI tree Vansonia forms a sister lineage to *Hypsugo*, but with a very low support (0.62/57).

- *Nyctalus* - "true" noctules form a monophyletic group with a relatively high support (from 0.95/82 to 1/100).

- Pipistrellus "East" - this complex represents

Locus	Length (b.p.)	Cons.	Var.	ParsInf.		Nucleotide fre	equencies (%)	
				-	T/U	С	А	G
COI	658	392	266	264	32.6	24.1	25.8	17.5
cytb	1140	648	492	444	31.4	25.9	28.8	13.9
RAG-2	1255	994	261	184	26.2	21.3	29.0	23.5

Table 1. Sequence characteristics

Cons.: conservative sites; Var.: variative sites. Pars.-Inf.: parsimony informative sites.

Table 2.	Uncorrected p-dista	ances (%) for sec	quences of COI	mtDNA gene for	groups (above	diagonal).
Values on	the diagonal corres	spond to average	uncorrected ing	group <i>p</i> -distances.	Standard error	estimates
are shown	above the diagonal					

	Arielulus	Tylonycteris	Philetor	Hypsugo joffrei	Hypsugo	Vansonia	Nyctalus	<i>Pipistrellus</i> East	Glishropus	<i>Pipistrellus</i> West
Arielulus	-	1.33	1.51	1.41	1.52	1.73	1.22	1.25	1.44	1.25
Tylonycteris	20.32	7.79	1.22	1.27	1.24	1.55	1.16	1.14	1.34	1.15
Philetor	19.44	17.30	0.46	1.31	1.39	1.62	1.27	1.18	1.43	1.19
Hypsugo joffrei	18.63	15.95	15.10	0.42	1.38	1.63	1.20	1.19	1.48	1.17
Hypsugo	21.08	16.82	17.30	15.69	0.46	1.46	1.20	1.10	1.34	1.14
Vansonia	19.77	18.75	17.42	16.83	14.29	-	1.36	1.26	1.52	1.25
Nyctalus	18.14	17.78	18.10	15.65	16.40	15.91	11.03	1.04	1.16	1.01
Pipistrellus East	19.56	18.61	17.76	17.21	16.91	16.19	17.01	12.59	1.05	1.00
Glishropus	17.66	18.09	17.23	16.61	15.45	15.75	15.51	15.21	0.30	1.14
Pipistrellus West	18.91	17.10	17.61	15.67	15.19	14.29	15.39	16.01	15.24	11.63

Table 3. Uncorrected p-distances (%) for sequences of cytb mtDNA gene for groups (above diagonal). Values on the diagonal correspond to average uncorrected ingroup p-distances. Standard error estimates are shown above the diagonal

	Arielulus	Tylonycteris	Philetor	Hypsugo	Vansonia	Nyctalus	<i>Pipistrellus</i> East	Glishropus	<i>Pipistrellus</i> West	P. kuhli
Arielulus	-	2.00	1.87	1.97	1.99	1.89	1.75	2.17	1.70	2.07
Tylonycteris	22.12	4.10	0.97	1.12	1.11	1.01	1.06	1.15	1.06	1.46
Philetor	20.79	16.69	10.79	1.02	1.01	0.94	0.89	0.97	0.91	1.33
Hypsugo	21.34	17.20	18.04	0.00	1.12	1.02	1.00	1.18	0.99	1.40
Vansonia	21.53	20.33	20.51	18.53	0.09	0.87	0.85	1.05	0.88	1.31
Nyctalus	21.97	18.70	20.20	19.43	17.69	10.64	0.81	0.88	0.84	1.18
Pipistrellus East	20.94	19.09	19.08	19.30	17.96	17.25	11.79	0.85	0.80	1.13
Glishropus	22.28	19.29	19.45	19.44	18.79	17.34	16.12	-	0.90	1.40
Pipistrellus West	20.20	18.67	19.55	19.04	17.40	16.80	17.07	17.14	11.18	1.05
P. kuhli	21.76	19.65	19.83	18.45	17.08	16.91	16.69	18.98	15.11	5.63

Table 4. Uncorrected p-distances (%) for sequences of RAG-2 nuDNA gene for groups (above diagonal). Values on the diagonal correspond to average uncorrected ingroup p-distances. Standard error estimates are shown above the diagonal

	Arielulus	Tylonycteris	Philetor	Hypsugo	Vansonia	Nyctalus	<i>Pipistrellus</i> East	Glishropus	<i>Pipistrellus</i> West	P. nathusii
Arielulus	6.47	1.05	1.83	1.30	1.40	1.24	1.03	1.52	0.81	7.43
Tylonycteris	11.89	4.56	0.88	0.90	0.92	0.82	0.80	0.93	0.71	0.71
Philetor	14.31	8.07	0.00	0.97	1.12	1.05	1.13	1.09	1.09	1.04
Hypsugo	9.43	8.14	8.28	0.00	0.91	0.84	0.95	0.90	0.83	0.80
Vansonia	9.78	8.96	10.46	5.43	0.13	0.54	0.72	0.64	0.58	0.52
Nyctalus	8.74	8.53	9.85	5.05	3.00	0.95	0.53	0.50	0.36	0.29
Pipistrellus East	10.34	9.92	11.18	7.31	5.14	3.43	1.33	0.56	0.50	0.44
Glishropus	10.02	8.98	10.17	6.16	3.56	2.65	3.46	-	0.59	0.51
Pipistrellus West	8.35	9.04	10.68	5.49	3.42	2.20	3.80	3.22	1.40	0.35
P. nathusii	0.78	8.19	9.66	4.60	2.45	1.34	3.37	2.42	2.19	-

a monophyletic group with medium or high support (0.83/87 - 1/100), and includes species from the eastern part of the genus range ("javanicus" species group: *P. coromandra*, *P. tenius*, *P.*

javanicus, *P. paterculus*, *P. abramus*; plus *P. stenopterus*). Relationships within *Pipistrellus* "East" are not resolved according to COI, but both cytb and RAG2 data place *P. stenopterus* as a



Fig. 1. Phylogenetic ML tree reconstructed from alignment of the mitochondrial gene COI. Numbers on tree nodes indicate bootstrap values (BS) and posterior probabilities (PP) for ML/BI, respectively.



Fig. 2. Phylogenetic ML tree reconstructed from alignment of the mitochondrial gene cytb. Numbers on tree nodes indicate bootstrap values (BS) and posterior probabilities (PP) for ML/BI, respectively.

sister lineage to the rest of the group with high support (see Figs. 1-3).

- *Glishropus* - this genus forms a sister lineage to *Pipistrellus* "East" with values of support from medium to high (from 0.85/84 to 0.99/97). This automatically turned *Pipistrellus* into a polyphyletic genus, which comprised two segments: *Pipistrellus* "East" and *Pipistrellus* "West".

- *Pipistrellus* "West" – this group either had a low support (55/0.66) or several species placed outside the group (*P. nathusii* or *P. pipistrellus*). *Pipistrellus* "West" includes species from the western part of the genus range (*P. kuhli, P. nathusii, P. pipistrellus*).

All these results support the division of *Pipistrellus* into *Pipistrellus* "East" and *Pipistrellus* "West" groups (*p*-distances between them are 16-17% for mtDNA and 3.8 % for nuDNA). According to the mtDNA, *p*-distances within these two groups are high, from 11.18 to 12.59%.

Morphometric characters

The data proved to be poorly factorized due to the high diversity of animals included in the

analysis, despite the size elimination: the first Principal Component covers less than 30% of the overall dispersion.

According to the results of the Principal Component analysis, *Pipistrellus stenopterus* forms a cluster of its own, only slightly overlapping with other taxa – less than the overlap between *Nyctalus* and "typical" *Pipistrellus* or between *Pipistrellus* and *Hypsugo*. In the space of the two first Principal Components (Fig. 4; PC I has heist correlations with CC and C, eigenvalue 5.994; PC II – with BCW, ZW and POC, 4.086), it only slightly overlaps with *Nyctalus* and *Arielulus* and does not overlap with *Pipistrellus* (only South-East Asian) or *Hypsugo*. In some other PC combinations, it slightly overlaps with the two latter genera (*Pipistrellus* – only South-East Asian). It has no overlap with *Philetor*.

Discriminant Function analysis was performed on the same dataset with ten learning samples: "stenopterus", "Pipistrellus (West)", "Pipistrellus (javanicus)" (smaller Asian species of Pipistrellus), "Pipistrellus (ceylonicus)", "Nyctalus (larger)", "Nyctalus (smaller)", "Hypsugo", "Hypsugo joffrei", "Arielulus" and "Tylonycteris". All the samples demonstrated significant differentiation



Fig. 3. Phylogenetic ML tree reconstructed from alignment of the nuclear gene RAG-2. Numbers on tree nodes indicate bootstrap values (BS) and posterior probabilities (PP) for ML/BI, respectively.

(*p* < 0.00001). As in previous case, *Pipistrellus* stenopterus ("stenopterus" sample) significantly differed from other analyzed samples, as well as from specimens put into the analysis as unidentified. According to the values of intergroup Squared Mahalanobis distances calculated during the analysis, they demonstrate a certain similarity to Asian pipistrelles (although not with West Palearctic species) and with Hypsugo joffrei (Table 5). It is worth noting that this group does not demonstrate the same similarity levels with the 'true' noctules or *Philetor*. Values of the intergroup Squared Mahalanobis distances exceed values of the intragroup distances about two- or threefold. Scotozous was included in the DF analysis as "undetermined" due to its small number of specimens; it was significantly different from all the samples, and also is guite distant from the "stenopterus" sample.

Overall, we may conclude that the skull proportions of *Pipistrellus stenopterus* are specific to the same extent, which is a characteristic of recognized Vespertilionin genera (involved in analysis).

Morphotypic characters

The Malayan noctule is a middle-size Vespertilionine bat (FA = 38-42 mm) of 'noctule' appearance: elongated and pointed wing (Aspect Ratio index about 2.36), rounded ears with wide and blunt tragi, and a well-developed calcar keel. Its skull (Fig. 5) is wide and high, with shortened and robust facial parts and rounded brain case, convex frontal profile, without basisphenoid pits. Its general shape is similar to that of Philetor brachypterus, Hypsugo joffrei and, in less degree, to small "true" Nyctalus. Lower molars are of nyctalodont type. Their talonids are somewhat longitudinally compressed, probably due to the overall shortening of the rostrum; therefore, postcristids on them go very close to the base of entoconides. Upper molars have pronounced hypocone and open trigon basin; upper small premolar exist, displaced from the tooth row; upper canines are long, with well-developed posterior blades and variably pronounced additional prongs; upper outer incisors are relatively large, situated close to appropriate canines.



Fig. 4. Scatter plot of the two first Principal Components, calculated for 43 species of *Pipistrellus, Nyctalus, Glischropus, Scotozous, Philetor, Hypsugo, Tylonycteris, Falsistrellus* and *Arielulus* (322 specimens, including 17 *P. stenopterus*) based on 22 skull measurements. PC I (28.54% of total variance) have high correlations with C and CC; PC II (19.46%) – with BCW, ZW and POC. Genotyped specimen of *P. stenopterus* is marked by asterisk.

Penial bone (baculum) of the Malayan noctule, prepared from one adult specimen from Sumatra, is about 3.5 mm in length, with widened basal end and gradually narrowed main shaft. It has a narrow and deep basal notch and a shallow but definite urethral groove (Fig. 6). Its distal end is bifurcated, with tips turned downwards.

DISCUSSION

The general topology of the obtained trees corroborates results of previous authors (*e.g.* Hoofer and van Den Bussche 2003; Roehrs et al. 2010). *Arielulus* (which was described as a part of *Pipistrellus* (Hill and Harrison 1987), but later allocated to the Eptesicini tribe (Voleth and Heller 1994; Roehrs et al. 2010) takes the most basal position, opposite to all the other studied taxa.

Philetor, Tylonycteris and *Hypsugo* (members of the "Hypsugine group" sensu Roehrs et al. 2010) took a stable sister position to all typical Pipistrellines.

Pipistrellus stenopterus, in its turn, is placed within the Pipistrellines and could be confidently treated as a member of this clade. It is very close to Asian pipistrelles from the "*javanicus*" species group and has no close relations to *Philetor* or to *Hypsugo joffrei*. These results correspond well both with published karyological data (Volleth and Heller 1994) and with our previous morphological studies (Kruskop 2003).

In both external and cranial shape *P. stenopterus* resembles *Nyctalus* and, to greater extant, *Hypsugo joffrei*, which explains why both species were allocated to same species group by several authors (Tate 1942; Koopman 1994). However, our phylogenetic reconstructions

Table 5. Significance of intergroup difference (above diagonal), Squared Mahalanobis distances between groups (below diagonal) calculated for ten learning samples established for DF analysis, and Squared Mahalanobis distances between group centroids and 17 *P. stenopterus* specimens, used in the analysis (one that genotyped is in bold)

Names of learning samples	Sample No	1	2	3	4	5	6	7	8	9	10	11
stenopterus	1		0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Pipistrellus (West)	2	95,898		0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Pipistrellus (javanicus)	3	56,933	16,172		0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Pipistrellus (ceylonicus)	4	44,272	30,577	13,686		0,0000	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Nyctalus (larger)	5	65,121	71,794	57,359	60,831		0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Nyctalus (smaller)	6	78,891	47,842	49,861	59,927	11,729		0,0000	0,0000	0,0000	0,0000	0,0000
Hypsugo	7	99,577	18,092	25,708	36,744	73,750	52,535		0,0000	0,0000	0,0000	0,0000
Hypsugo joffrei	8	43,753	103,135	70,832	59,633	97,198	93,759	77,013		0,0000	0,0000	0,0000
Philetor	9	77,503	167,118	119,393	128,394	125,437	123,125	129,975	26,355		0,0000	0,0000
Arielulus	10	94,540	62,991	56,432	38,062	108,711	100,561	50,819	88,305	164,070		0,0000
Tylonycteris	11	171,655	173,470	163,979	203,538	175,195	150,117	174,374	152,497	116,451	243,548	
ROM MAM 36135		10,126	89,962	47,954	42,935	63,176	75,811	88,580	47,196	78,857	95,544	160,443
ROM MAM 36136		9,867	101,659	61,559	47,265	65,306	76,070	98,664	47,616	79,022	89,272	163,782
ROM MAM 36137		15,392	108,110	69,968	61,326	92,810	100,258	113,712	54,389	76,425	116,393	152,749
ROM MAM 36138		7,291	93,523	51,870	44,719	73,999	91,191	90,102	41,553	72,577	88,458	175,282
ROM MAM 36139		17,313	105,765	67,842	60,787	79,526	94,996	105,967	62,817	86,937	105,401	148,832
ROM MAM 36140		16,218	154,768	108,846	90,126	97,931	118,407	167,592	68,977	101,738	163,315	219,194
ROM MAM 41434		10,878	102,156	60,101	51,380	71,963	80,164	102,822	58,373	88,177	101,854	196,841
ROM MAM 41435		15,524	120,540	82,188	67,198	75,830	90,774	129,643	84,877	119,655	129,502	213,416
ROM MAM 41436		18,511	74,535	38,495	34,772	53,080	64,671	76,574	52,477	74,327	79,954	148,484
ZMMU S-103148		22,405	67,382	43,095	41,257	68,624	67,719	77,012	61,656	104,022	74,355	166,820
ZMMU S-103149		16,296	142,357	95,999	80,610	82,137	101,234	149,788	63,397	94,193	145,212	199,613
ZMMU S-103150		15,253	114,209	84,834	70,200	74,693	90,094	126,233	61,176	102,535	117,441	214,844
BMNH42.8.19.14 (type)		14,587	89,268	61,840	48,373	80,465	86,135	87,206	36,805	76,611	90,329	177,280
NMW 40264		15,359	153,987	102,875	74,902	90,571	114,299	149,597	58,560	92,284	127,677	217,676
NMW 40265		16,622	85,438	54,988	41,974	75,547	89,866	95,166	60,420	107,558	82,161	198,656
NMW 40269		29,971	154,646	112,617	91,211	135,858	146,003	154,097	88,597	125,211	139,587	184,291
NMW 40277		16,045	139,613	90,446	71,241	93,198	121,109	147,711	62,578	105,083	128,374	247,593

clearly demonstrate that this similarity is of a convergent nature, and does not reflects true relationships between the respective species. It can be assumed that the external similarity (a wide shortened muzzle, narrow pointed wings, smooth fur) of *Pipistrellus stenopterus* to *Philetor* and *Nyctalus* species is associated with adaptation to a similar way of foraging (Kruskop 1999), although published information on the ecology of this species is rather poor (Kingston et al. 2008).

General shape and proportions of teeth – in particular, relative size of the small premolar, shape and size of the outer upper incisor and its position in relation to the inner one, the shape of indentation on the canine, visible presence of the hypocone, and nyctalodont lower molar – confirm the relationship between *P. stenopterus* and other *Pipistrellus*. Nyctalodont lower molars also put *P. stenopterus* apart from any *Hypsugo* of *Falsistrellus* (Horáček and Hanak 1985-86; Menu 1987). However, its tooth rows carry traces of longitudinal compression, which, apparently, is a consequence of shortening and widening of the rostrum. Overall skull shape is much more robust than in other *Pipistrellus*, which is a common feature of the fast-flying aerial foragers (Kruskop 1999), although nothing in its structure contradicts the kinship between *P. stenopterus* and other *Pipistrellus*.

Similar can be said about the penial bone, although without the adaptive implications. Baculum in Pipistrellini s. str. (sensu Hoofer and



Fig. 5. Skull features of *Pipistrellus stenopterus*: (A) general skull shape; (B) mandible shape in lateral view of *P. stenopterus* (ZMMU S-103149; B1) and *Nyctalus* (*N. plancyi* ZMMU S-164496; B2); (C) upper incisors and canine in lateral view of (C1) *Philetor brachypterus* (ROM MAM 113087) and (C2) *P. stenopterus* (ROM MAM 41436).

van Den Bussche 2003) is guite conservative in its general shape and is similar among most representatives (Hill and Harrison 1987). It usually has a narrow and elongated main shaft, broadened sideward and downward at the basal end; with shallow urethral groove, which is commonly seen at least in basal third; it usually has a distinct basal notch. Distal tip usually demonstrates more or less pronounced bifurcation (especially distinct in Oriental Pipistrellus species and in Gischropus). Formally, baculum of the Malayan noctule possess all the mentioned features, but it is quite thick and robust and in general proportions differs from Nyctalus, Pipistrellus, and Glischropus more than they differ from each other. At the same time it clearly looks unlike any baculum of Hypsugo, or serotine. or Philetor.

We conclude that, although morphological features of *P. stenopterus* do not contradict the results of the phylogenetic reconstruction, this species has morphological peculiarities that prominently distinguish it from other *Pipistrellus* species and related taxa. Its taxonomic propinquity

to *Pipistrellus* is almost undoubtable. Features that make *P. stenopterus* similar to *Nyctalus*, *Philetor* and *Hypsugo joffrei* should be treated only as a result of the adaptation to the same life patterns. At the same time, *P. stenopterus* is morphologically and morphometrically unique. Analysis of the phylogenetic data together with morphological studies allowed us to establish a new subgenus for the Malayan noctule within the *Pipistrellus* genus. Since no generic names were previously suggested for *P. stenopterus*, this subgenus requires a formal description.

It is also necessary to mention that the *Pipistrellus* genus in its current understanding, based on previously published molecular studies (Hoofer and van Den Bussche 2003; Roerhs et al. 2010; Heaney et al. 2012; Koubinova et al. 2013; Benda et al. 2016), is most likely paraphyletic. Along with the results of our studies of the cytb gene, *Nyctalus* frequently forms a common clade with the "Western" *Pipistrellus* branch, while *Glischropus* does the same with the "Eastern" one; or, in other cases, all three genera form a



Fig. 6. Penial bones (baculum) of selected Vespertilionine species: 1, *Pipistrellus abramus* (ZMMU n/n, Vietnam). 2, *Glischropus bucephalus* (ZMMU S-184658). 3, *Pipistrellus nathusii* (ZMMU S-183034). 4, *Hypsugo joffrei* (ZMMU S-186691). 5, *P. stenopterus* (ZMMU S-103149). 6, *P. coromandra* (ZMMU S-184690). 7, *Nyctalus noctula* (ZMMU S-180228). 8, *Philetor brachypterus*. 9, *Scotozous dormeri*. 10, *H. pulveratus*. 1-7, original drawings; dorsal, lateral and ventral views. 8-10, after Hill & Harrison, 1987, dorsal and lateral views. Scale bar = 3 mm.

common group with an unresolved basal topology. The most remote *Pipistrellus* lineage, *P. rueppeli*, which frequently falls into basal position to all other Pipistrellus + Nyctalus + Glischropus, was already considered a separate genus Vansonia (Koubinova et al. 2013). Two other major Pipistrellus lineages – "Western" and "Eastern" – although morphologically similar, seem to be divided genetically at the same level as Nyctalus and Glischropus. However, the absence of significant morphological differences between "Western" and "Eastern" pipistrelles and the relatively small number of analyzed taxa keep us from making a final decision on this issue. We suggest further studies with more species and a more robust molecular data set

SYSTEMATIC

Family Vespertilionidae Gray, 1821 Subfamily Vespertilioninae s. str. Tribe Pipistrellini Tate, 1942 Genus *Pipistrellus* Kaup, 1829

Alionoctula subgenus nov. urn:lsid:zoobank.org:act:6866D047-8C2D-4440-9704-1C652B14D91F

Type species: Vesperugo stenopterus Dobson, 1875

Distribution: South-East Asia: Malayan Peninsula (south of Kra), Riau Islands, Sumatra, northern Borneo and Mindanao Island (see: Corbet and Hill 1992; Kingston et al. 2008).

Content: Only type species.

Etymology: From Latin *alio* – another, and *noctula* – noctule bat (derived from *nox* – night); to reflect external similarity of *P. stenopterus* with the "true" noctules and the convergent nature of this similarity. The name has female gender.

Diagnosis: Middle-size vespertilionine bats with the forearm length about 40 mm and body weight about 15-20 g. Fur is short and thick, reddish-brown or brown, slightly paler on ventral part, does not spread to the wing membrane. Tail is shorter than body length. Calcar lobe is welldeveloped. Ears are widely rounded, tragi are short and wide, very slightly bent forward. Muzzle is wide and fleshy. Wing proportions are similar to that of *Nyctalus*, with an elongated third finger and a shortened fifth one.

Skull is wide and robust, with rounded brain

case and wide and short rostrum. Basisphaenoid pits are absent. Upper canine is long, with welldeveloped posterior blade and usually with indentation on it. Anterior upper premolar is well developed, but strongly displaced inward from the tooth row. Anterior upper incisor is bicuspidate and about twice as large as the posterior one in both height and crown area. Gap between incisors and canine base is very short. Upper molars are robust, rectangular, with visible hypocone and halfclosed basin. Lower molars are of nyctalodont type, posterior ones not reduced. Mandible is massive, with steep symphysis and almost vertical coronoid process.

Baculum is about 3.5 mm in length, much more massive than in other *Pipistrellus*, and gradually narrows from base to distal end. The latter is slightly widened and bifurcated, with tips turned downward. Base is with deep and narrow notch.

Comparison: Main differences of Alionoctula from other pipistrelles and noctules are stated in the "Results" section. In general, Alionoctula is similar to other Pipistrellus (especially from the "javanicus" species group) in some dental features, for example, in the proportions and position of the small premolar and shape of upper incisors and upper molars. Its differences include larger size, robust skull, wide and fleshy muzzle, and distinctly narrower wings. On lower molars, trigonids are somewhat compressed longitudinally, and therefore postcristid goes very close to the base of entoconide (as opposing to other Pipistrellus, where they are widely separated). Alionoctula is well separated from Nyctalus because of larger anterior premolars, thicker zygomatic arches, absence of basispheanoid pits, and more abrupt symphysis of mandible. From Philetor, Alionoctula could be distinguished by larger size and different shape and position of upper incisors. Thick baculum with downward curved bifurcation on distal end sets Alionoctula apart from all other members of the Pipistrellini tribe. Karyotype (N = 32, NF = 50; Volleth et al. 2001), while falling into the ranks of the *Pipistrellus* variability, is not seen in any other members of this genus.

CONCLUSIONS

Phylogenetic reconstruction based on molecular genetic data unequivocally place *Pipistrellus stenopterus* within the Pipistrellini tribe, close to the Oriental branch of *Pipistrellus*

and guite far from *Philetor* and *Hypsugo*. This may finalize the discussion about the kinship of this unusual species. At the same time, its morphological peculiarities distinctly allocate it among other Pipistrellus, which led us to the decision to suggest a new subgenus for it, Alionoctula subgen. nov. During this study, the Pipistrellus genus in its current understanding was again shown to be paraphyletic, in accordance with previously published data. Such an undesirable taxonomic situation requires further analysis with many more genetic markers and more taxonomic units. In general, the existence of a previously unrecognized superspecific taxon of bats, as well as the existence of a paraphyletic taxonomic unit. indicates insufficient knowledge of the respective biodiversity structure, and fully justifies further research in this field.

Acknowledgment: This work and the new subgenus name have been registered with ZooBank under urn:lsid:zoobank. org:pub:6866D047-8C2D-4440-9704-1C652B14D91F. Laboratory study of the collection materials was done in the Zoological Museum of Moscow University, using the collection facilities, with the support from its director, Dr. M.V. Kalyakin. Molecular analysis was also performed on the facilities of ZMMU. Additional collection materials were studied in the Royal Ontario Museum. Canada, with the kind permission of Dr. J. L. Eger and Dr. B. Lim; in the Museum of Natural History, Great Britain, under the support of Mr. R. Portela Miguez; in the Hungarian Natural History Museum, Hungary, under the support of Dr. G. Csorba and Dr. T. Görföl; in the National Museum of Science and Nature, Japan, under the support of Dr. S. Kawada; in the Berlin Zoological Museum, Germany, with permission from and under the support of Dr. F. Mayer and Mrs. N. Lange; in the National Museum of Natural History, France, under the support from Dr. J.-M. Pons; in the collection of the National Museum of Prague, Czech Republic, with kind permission from Dr. P. Benda; and in the Zoological Institute of RAS, Russia, with permission from Dr. A. Abramov and Dr. F. Golenishchev. We would like to express our gratitude to Vladimir Lebedev (ZMMU) for his help and advice. Study of collections was supported by the Russian Foundation for Basic Research (grant No 17-04-00689a); DNA extraction and analysis was supported by the Russian Science Foundation under grant 14-50-00029; bioinformatics analysis was supported by the Russian Science Foundation

under grant 14-50-00150. The study was performed in line with State theme of scientific work of the ZMMU (AAAA-A16-116021660077-3).

Author contributions: SVK, ENS and ADK designed the study and wrote the manuscript. SVK studied and measured the specimens and performed the statistical analyses, and prepared appropriate figures. ADK designed primers for the molecular analysis. ENS analyzed the molecular data, contributed reagents/materials/analysis tools, and prepared appropriate figures and tables. All authors participated in revising the manuscript. All authors read and approved the final manuscript.

Competing interests: The authors declare that they have no competing interests.

Availability of data and materials: All studied specimens are available in museum collections (see Appendix 1). All analyzed sequences are available in GenBank. Statistical data available upon request.

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable.

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Supplementary Materials

Appendix 1. List of specimens used for morphological comparison and in morphometric study. (download)

Table S1. Primers used in this study. (download)

Table S2.Sequences used in the analysis.(download)