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Taxonomic status of the barbastelles (Chiroptera: Vespertilionidae: *Barbastella*) from the Japanese archipelago and Kunashir Island

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Abstract

Until recently, almost all barbastelle populations inhabiting Asia, from Iran to Japan and Taiwan, were recognized as a single species, *Barbastella darjelingensis*. However, in recent years, it was shown that the taxonomic diversity within the genus *Barbastella* was underestimated, and *B. darjelingensis* sensu lato is highly variable, both morphologically and genetically. Transcaucasian and Central Asian populations were already raised to the species level as *B. caspica*. Populations from eastern and south-eastern Asia are still understudied and likely encompass undescribed species. Comparing results from mtDNA sequences, skull morphometry and baculum shape, we concluded that one of these putative species is represented by populations inhabiting Honshu, Hokkaido and Kunashir Islands, and provide a scientific description of this new species. Populations from Taiwan, southern China and Indochina represent a sister mitochondrial clade to this new species. However, available morphological material from these areas did not demonstrate a significant difference from the Himalayan *B. darjelingensis*. Therefore, we refuse to give this form any taxonomic status until further studies of more vast material are done.

Key words: *Barbastella*, new species, East Asia, Japan, Kuril Islands, taxonomy

Introduction

Though barbastelles overall are not especially rare or poorly known to scientists, this group, which has a highly infrequent distribution across Eurasia (Fig. 1), is poorly studied. Until recently, only one species of barbastelles—*Barbastella leucomelas* (Cretzschmar, 1830), Asian barbastelle—was recognized along the huge area from north-east Africa and the Caspian Sea to Japan and Taiwan (Tate 1942; Kuzyakin 1950; Ellerman & Morrison-Scott 1951; Wallin 1969; Corbet 1978; Yoshiyuki 1989; Koopman 1994; Rakhmatulina 2005; Simmons 2005). Moreover, in this whole area, very few named taxa have been suggested (Benda & Milikovsky 2008), of which only four could be considered valid: *darjelingensis* (Hodgson, 1855), *caspica* Satunin, 1908, *blanfordi* Bianchi, 1916 (emendation for *darjelingensis* Hodgson), and *walteri* Bianchi, 1916. Additional name, *formosanus* Lin, Zheng and Maeda, should be considered as *nomen nudum* (see Zhang e*t al*. 2007; Benda & Milikovsky 2008). For a long time even subspecies were almost not recognized for geographically distant populations, and within the whole range of the "Asian barbastelle" only two subspecies were accepted: the western, pale-coloured *B. l. leucomelas* and the eastern, dark-coloured *B. l. darjelingensis* (Wallin 1969; Bates & Harrison 1997). Later, it was suggested that the nominative *B. leucomelas* from Egypt may represent a subspecies of *B. barbastellus* and not belongs to the Asian species (Horáček *et al*. 2000).

Recently thanks to revisionary studies *Barbastella leucomelas* sensu stricto is now considered to be a separate species, endemic to north-east Africa and the Sinai Peninsula (Benda *et al*. 2008) and the name *B. darjelingensis* was adopted for all other Asian barbastelles, without recognizing any subspecies. A new species, *B. beijingensis*, was described from the Beijing municipality (Zhang *et al*. 2007), apart from the previously known distribution range of the *Barbastella* genus in Asia. Finally, on the basis of combined morphological and molecular data,

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Central Asian and Transcaucasian barbastelles were considered a distinct species under the name *B. caspica*, with *walteri* as its partial synonym (Kruskop 2015).

Meanwhile, *Barbastella* diversity in the easternmost part of the genus' distribution range was not investigated for decades. Yoshiyuki (1989) mentioned that the Japanese specimens differ from their mainland conspecifics, but did not discuss this subject thoroughly. Horáček *et al*. (2000) cited Yoshiyuki's point, and hypothesized that, similarly to many other Japanese mammals, the Japanese barbastelle population may represent a separate taxon. However, he had no argument to support this suggestion. Zhang *et al*. (2007), in a description of *B. beijingensis*, listed all the other Asian barbastelles as *B. leucomelas*. In the latter publication, specimens from Japan, Taiwan and Sichuan are shown to be well differentiated genetically from both *B. beijingensis* and *B. leucomelas* from Egypt; this was confirmed by Benda *et al*. (2008). However, Kruskop *et al*. (2012) noted that the specimen from Kunashir Island (which should be presumably closely related to the Japanese specimens) was quite different from the Nepalese *B. darjelingensis*. Finally, Kruskop (2015) suggested that there are two distinct mitochondrial lineages (presumably Japanese and south-east Asian), distant from all other named barbastelle forms; *B. beijingensis* was not included in this analysis, but, according to Zang *et al*. (2007), it is more closely related to *B. barbastellus* and *B. leucomelas*, therefore it should also be distant from that two lineages. Based on this, we hypothesized the presence of undescribed forms of barbastelles in the easternmost part of Asia, in particular in Japan. A study of old museum-housed and newly collected materials from Japan provided the opportunity to fulfill this gap, at least in part, and designate a new species-level taxon. However, our data is not sufficient to make final decisions on barbastelles from Indochina, Taiwan and the Sichuan (and probably other parts of China; the "Southeast Asian lineage"). Although genetically distinct from other lineages (including *B. beijingensis*), these barbastelles have uncertain morphological differences from *B. darjelingensis* sensu stricto. Therefore, in the text below, these barbastelles are referred to as *B.* cf. *darjelingensis*.

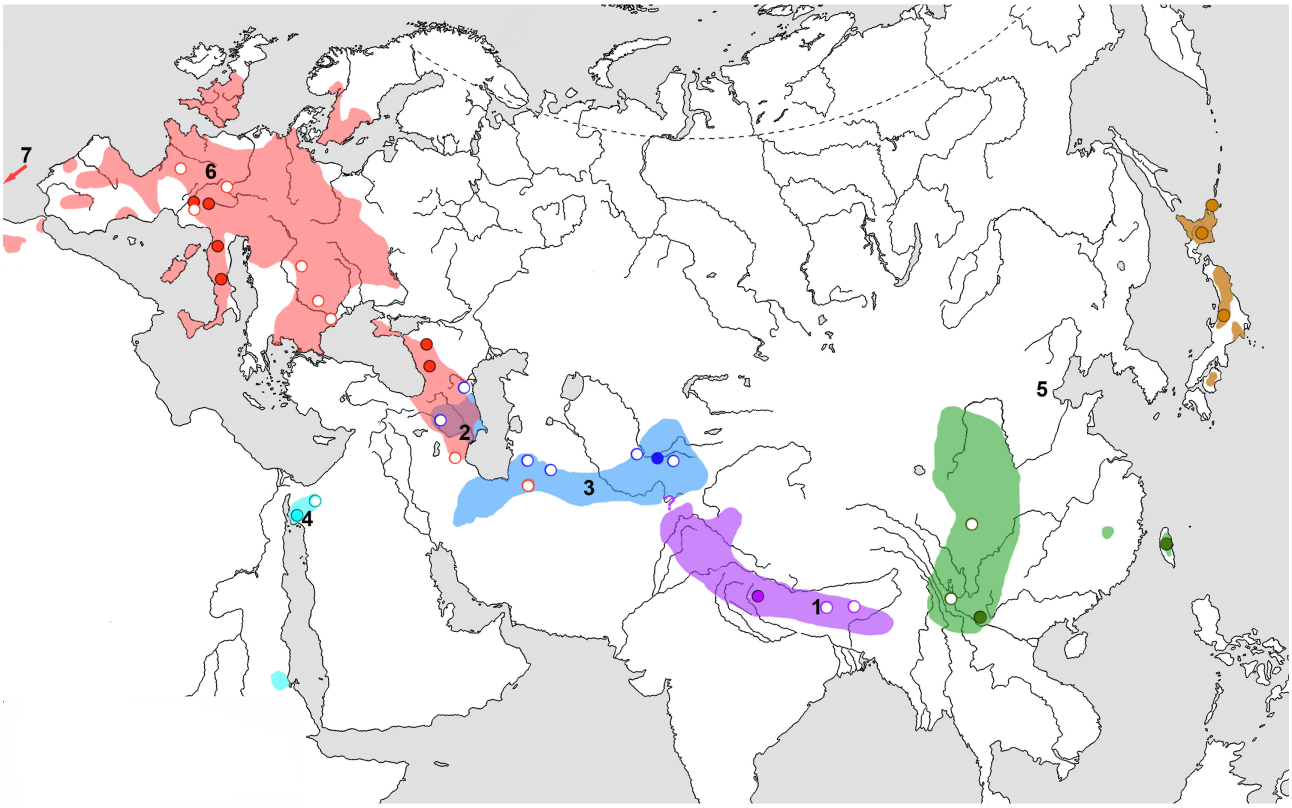

FIGURE 1. Distribution of barbastelles (adopted from the IUCN Red List (www.iucnredlist.org); with additions from Smith & Xie 2008; Benda *et al*. 2012; 2015; Fukui 2015). Red—*Barbastella barbastellus*, dark blue—*B. caspica*, light blue—*B. leucomelas*, magenta—*B. darjelingensis* sensu stricto, green—*B.* cf. *darjelingensis*, light brown—*B.* **n. sp.** Full symbols mark material genotyped by at least one gene; open symbols—specimens included into morphological study only. Type localities of named taxa: 1—*darjelingensis* Hodgson, 1855; 2—*caspica* Satunin, 1908; 3—*walteri* Bianchi, 1916; 4—*leucomelas* Cretzschmar, 1826; 5—*beijingensis* Zang et al., 2008; 6—*barbastellus* Schreber, 1774; 7—*guanchae* Trujillo, Ibáñez and Juste, 2002.

Material and methods

A total of 120 specimens of barbastelles (adults of both sexes, dry or alcohol preserved skins with extracted skulls) were studied, housed in the Berlin Zoological Museum (ZMB, Berlin), the Zoological Museum of Moscow University (ZMMU, Moscow), the National Museum of Science and Nature (NSMT, Tokyo), the Natural History Museum (BMNH, London), the Zoological Institute of the Russian Academy of Sciences (ZIN, St. Petersburg) the National Museum of Natural History (MNHN, Paris), the Natural History division of the National Museum (NMP, Prague), the Hungarian Natural History Museum (HNHM, Budapest), the Royal Ontario Museum (ROM, Toronto). Additionally, few specimens from private collections on Hokkaido were provided via Tokai University (marked in the list below by asterisks).

Barbastella darjelingensis: India: MNH54.9.1.13, sex unknown (type of *Synotus darjelingensis* Hodgson); MNH14.4.23.7, ♂; MNH17.4.23.8, ♂; MNH14.4.23.9, ♂; MNH17.4.23.10, ♀; MNH17.4.23.11, ♀; MNH17.4.23.12, ♀; ZMB4372, ♀; ZMB4862, ♂; Nepal: ZMMU S-164496, ♂; ROM MAM 74650, ♂; Bhutan: MNH16.7.29.8, ♀.

Barbastella cf. *darjelingensis*: China, Sichuan: MNH11.2.1.5, ♂; China, Yunnan: MNH23.3.7.1, ♂; Taiwan: HNHM 2000.9.6., ♀; Vietnam: ZMMU S-186684, ♀; ZMMU S-186685, ♂.

Barbastella leucomelas: Jordan: NMP PB4148, ♀; NMP PB4704, ♂; NMP PB4716, ♂; Sinai: NMP PB2883, ♂; NMP PB2884, ♂.

Barbastella barbastellus: Czech Republic: ZMMU S-74660, sex unknown; Slovakia: NMP 174/61, ♀; Ukraine: ZMMU S-49160, ♀; ZMMU S-49161, ♀; ZMMU S-49162, ♂; ZMMU S-84118, ♀; ZMMU S-151944, ♂; Moldova: ZMMU S-91357, ♂; ZIN 55684, ♂; France: MNHN1895-44, sex unknown; MNHN1932-4169, ♀; MNHN1932-4171, ♂; MNHN1932-4172, ♀; MNHN1932-4173, ♀; MNHN1932-4174, ♀; MNHN1932-4175, ♂; MNHN1932-4176, ♂; MNHN1950-416, ♂; MNHN1963-873, ♂; MNHN1963-878, ♂; MNHN1983-728, sex unknown; MNHN1981-1003, ♀; MNHN2003-224, ♀; MNHN2003-225, ♀; MNHN2004-1453, ♂; Russia, Krasnodar territory: ZMMU S-171543, sex unknown; ZMMU S-168252, ♀; ZIN 61672, ♀; ZIN 85774, ♂; ZIN 85775, ♀; ZIN 85776, ♂; ZIN 85778, ♂; ZIN 85779, ♂; ZIN 85780, ♀; Iran: NMP PB3243, ♀; NMP PB3244, ♀; NMP PB3245, ♀.

Barbastella caspica: Turkmenistan: ZMMU S-90966, ♂; ZMMU S-90967, ♀; ZMMU S-90968, ♂; ZMMU S-90969, ♀; ZMMU S-90970, ♂; ZMMU S-90971, ♀; ZMMU S-94696, ♂; ZMMU S-105053, ♀; ZIN 53997, sex unknown; ZIN 56633, ♂; ZIN 56634, ♀; ZIN 56635, ♂; ZIN 57945, ♂; Kyrgyzstan: ZIN 64609, ♂; ZIN 64610, sex unknown; ZIN 65452, ♂; ZIN 79612, ♂; ZMMU S-69214, ♂; NMP CT84/125; Uzbekistan: ZIN 57307, ♂; ZIN 57308, ♀; ZMMU S-74868, ♀; ZMMU S-94786, ♀; ZMMU S-135846, ♀; Tajikistan: ZMMU S-190370, ♀; ZMMU S-190371, ♂; ZIN 32255, sex unknown; ZIN 32256, sex unknown; ZIN 32257, sex unknown; ZIN 32258, sex unknown; ZIN 32260, sex unknown; ZIN 69063, ♂; ZIN 69064, ♀; Armenia: ZMMU S-108969, ♀; ZMMU S-173352, ♂; Russia, Dagestan: ZMMU S-28503, sex unknown; ZMMU S-28504, sex unknown.

Barbastella sp.: Hokkaido: NSMT M 14816, ♂; NSMT M 18559, ♂; NSMT M 18560, ♀; *92G001, ♀; *92G002, ♂; *DH0606, ♂; * KN0143, ♂; Honshu, Iwate: NSMT M 12470, ♀; NSMT M 16253, sex unknown; NSMT M 22936, ♂; NSMT M 22937, ♂; Honshu, Saitama: NSMT M 8774, ♀; NSMT M 16251, ♀; NSMT M 16252, ♂; Honshu, Nagano: NSMT M 52944, ♂; NSMT M 52945, ♀; NSMT M 52946, ♀; NSMT M 52947, ♀; NSMT M 52948, ♀; NSMT M 52949, ♂; NSMT M 52950, ♀; NSMT M 54617, ♂; Kunashir (Kunashiri) Island: BSI 168-07, ♂; NSMT M 61925, ♂.

Morphometric analysis. The following cranial measurements (abbreviations given in parentheses) were taken for morphometric analysis: condylobasal length (CBL), condylocanine length (CCL), occiput height (OH), mastoid width of skull at the level of the auditory bullae (MW), width of braincase (BCW), least postorbital width (POW), rostral width at the level of the infraorbital foramina (RW), rostral length from anterorbital foramina 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), molariform row length (PM), length of cingulum base of the upper canine (C), width of M2 (M2W), crown length of maxillary tooth row (cm), lower jaw length from alveolus of i1 to the glenoid process (MdLg), lower jaw length from alveoli of i1 to the angular process (MdLa), and lower jaw height to the tip of coronoid process (MdH). To assess the pattern of variation of quantitative characters, Principal Component (PC) and Discriminant Function (DF) analyses were performed for cranial measurements, using the Discriminant Function and Principal Component Analysis and Classification modules of STATISTICA for Windows version 7.0 (StatSoft, Inc., 2004).

Molecular analyses. Genomic DNA was isolated from 96% ethanol-preserved tissue samples of liver, kidney or muscle by proteinase K digestion, phenol-chloroform deproteinization and isopropanol precipitation (Blin & Stafford 1976; Sambrook *et al*. 1989).

The DNA from the tissues was extracted with a DNeasy Tissue Kit (QIAGEN K.K., Japan). The full sequences of the mitochondrial genes NADH dehydrogenase subunit 1 (*ND1*) and Cytochrome-b (*cytb*) were determined as described by Kawai *et al*. (2003). The partial sequences of the mitochondrial genes cytochrome c oxidase subunit I (*COI*) were amplified with primer set as described by Ivanova *et al*. (2006), VF1d 5'-TTCTCAAC-CAACCACAARGAYATYGG-3' and VR1d 5'- TAGACTTCTGGGTGGCCRAARAAYCA-3', for 5 specimens from Hokkaido, Kunashir (Kunasiri) Island and Taiwan (MSMT M52944, OCUM5377, DH0606, NSMT M 61925, OCUM7184). For other specimens, amplification of *COI* region was performed by the staff of the Canadian Centre for DNA Barcoding (CCDB), University of Guelph, Canada. The standard DNA barcode region—657 base pair 5' segment of the mitochondrial cytochrome oxidase subunit I (*COI*) gene was sequenced bidirectionally using standard DNA barcoding protocols for mammals (Clare *et al*. 2007; Borisenko *et al*. 2008; Ivanova *et al*. 2012). PCR amplification for the standard DNA barcode region was done using M13-tailed primer cocktails C_VF1LFt1 and C_VR1LRt1. The all of PCR products were sequenced using an ABI Prism BigDye Terminator v 3.1. Cycle Sequencing kit and analyzed on ABI 3730XL Genetic Analyzer or ABI 310 Genetic Analyzer.

For comparison with published sequences, the *ND1* sequences were trimmed from 957 bp to 929 bp, the *cytb* were trimmed from 1140 bp to 609 bp, the *COI* were trimmed from 657 bp to 523 bp. The sequences from each specimen were aligned with published subsequences in GenBank or published online datasets housed by the Barcode of Life Data Systems (BOLD; www.boldsystems.org). Alignments of sequence were done on MEGA7.0.26 software (Kumar *et al*. 2016). Phylogenetic relationships were estimated based on neighbor-joining and maximum likelihood. Three genes trees were constructed from the *ND1* (929bp), from *cytb* (609bp) and from *COI* (523bp). Genetic distance within genus and between genus for each gene were calculated on MEGA7.0.26

Neighbor-joining (NJ) trees were constructed using MEGA7.0.26. The NJ trees were based on the distance matrix estimated with Kimura's 2-parameter distances using transition + transversions and a uniform substitution rate. The Maximum Likelihood (ML) analysis was implemented in RAxML (Randomized Axelerated Maximum Likelihood) version 8.2.12 (Stamatakis 2014), based on the chosen GTR+I substitution models and probability values derived from 1000 replications. The most appropriate GTR (General Time Reversible) model with the lowest AIC for the ML analysis was evaluated using Model Selection option on MEGA 7.0.26.

In addition to the original sequences, 77 sequences were obtained from GenBank and BOLD. Specimens of *Plecotus* species—closest relatives of *Barbastella* genus—were used as outgroups to root the trees. List of all the specimens involved into molecular study is in the Appendix 1 (Tables A1, A2).

Results

Molecular data. Since we do not have the same sets of species and specimens for all three analyzed markers, we could not concatenate the partitions, and all the genes were analyzed independently. Although we did not obtain all three genes from each of the named and putative species, juxtaposition of the tree topologies, calculated for each individual gene, made it possible to evaluate the genetic structure of the genus and the relationships between their members.

In general, all three genes provided similar results, with an amendment for the presence and absence of certain species. Each named species was represented by well-defined branches, separated from the others with distances exceeding the in-group variation (Fig. 2; Tables 1, 2) The most closely related branches were represented by *B. leucomelas* and *B. caspica*, separated by only ca. 6–7% variation, which is only slightly more than the distance between *B. barbastellus* from Europe and the Canary Islands. Distances between other lineages were about twice as large. This was also true for *B*. sp. from Japan and *B*. cf. *darjelingensis* from Taiwan, Sichuan and Indochina. Both these lineages were well separated from all the named forms, as well as from each other, although according to *COI* data, they may have a sister position. Thereby, Japanese barbastelles seem to be well-separated at a high level (ca. 16–21%; Table 2) from all the other accepted or potential species, which suggests that they could represent a separate species.

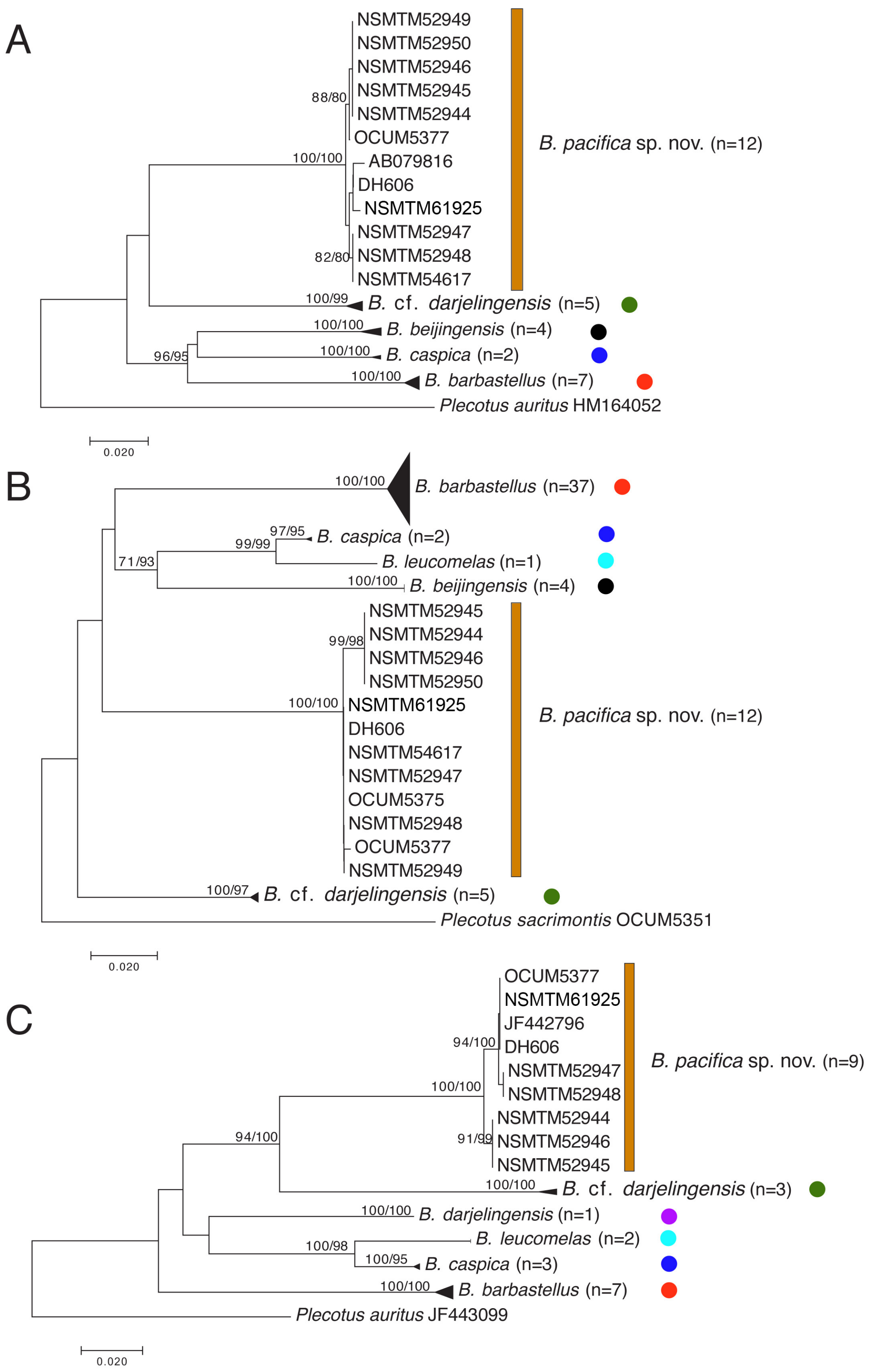

FIGURE 2. Phylogenetic relationships of *Barbastella* **sp. n.** based on A) *ND1* sequences (929bp), B) partial *cytb* (609bp) sequences and C) COI (523bp) sequences. The scale indicates genetic distance estimated by the Kimura 2-parameter method on the NJ tree. The bootstrap values derived from 1000 replication for NJ tree / maximum likelihood probabilities. Specimens of *Plecotus auritus* (ND1 HM164052; COI JF443099) or *P. sacrimontis* (*Cytb* OCUM5351) were used as an outgroup. The sample numbers correspond to those in the Appendix (Tables A1, A2).

TABLE 1. Within group mean distances (in %) of accepted and putative *Barbastella* species for three mitochondrial genes.

	Gene		
	COI	ND1	Cytb
Barbastella pacifica **n.sp.**	0.8 (n=9)	0.8 (n=12)	0.3 (n=12)
Barbastella cf. *darjelingensis*	0.5 (n=3)	0.4 (n=5)	0.4 (n=5)
Barbastella beijingensis	–	0.7 (n=4)	0.0 (n=4)
Barbastella barbastellus	0.8 (n=7)	0.8 (n=7)	0.9 (n=37)
Barbastella leucomelas	0.0 (n=2)	–	n/c (n=1)
Barbastella darjelingensis	n/c (n=1)	–	–
Barbastella caspica	0.3 (n=3)	0.6 (n=2)	0.3 (n=2)

TABLE 2. Between groups mean distances (in %) of accepted and putative *Barbastella* species for three mitochondrial genes.

	COI	ND1	Cytb	COI	ND1	Cytb	COI	ND1	Cytb
	B. pacifica **n. sp.**			*B.* cf. *darjelingensis*			*B. beijingensis*		
B. pacifica **n. sp.**	—								
B. cf. *darjelingensis*	16.1	14.5	12.8	—					
B. beijingensis	—	18.1	16.2	—	16.2	15.4	—		
B. barbastellus	25.1	17.3	17.0	20.6	18.1	15.9	—	14.7	17.9
B. leucomelas	21.2	—	15.7	19.2	—	14.7	—	—	13.2
B. darjelingensis	19.5	—	—	17.8	—	—	—	—	—
B. caspica	20.0	16.5	13.8	17.6	16.6	13.2	—	12.4	13.0

continued.

	COI	ND1	Cytb	COI	ND1	Cytb	COI	ND1	Cytb
	B. barbastellus			*B. leucomelas*			*B. darjelingensis*		
B. pacifica **n. sp.**									
B. cf. *darjelingensis*									
B. beijingensis									
B. barbastellus	—								
B. leucomelas	19.5	—		—					
B. darjelingensis	16.8	—	—	16.0	—	—	—		
B. caspica	16.8	14.2	15.0	5.9	—	17.1	12.7	—	—

Each branch corresponded to a species level and had high support in both the neighbor joining (NJ) and Maximum Likelihood (ML) reconstructions (Fig. 2). A combination of *B. leucomelas* and *B. caspica* had high support on *cytb* and *COI* trees (since we had no *ND1* sequences for *B. leucomelas*). A combination of *B. leucomelas* and *B. caspica* with *B. beijingensis* received support by ML analysis for the *cytb* gene (Fig. 2, B); a combination of *B. beijingensis*, *B. caspica* and *B. barbastellus* had relatively high support on the *ND1* tree (Fig. 2, A). These results corroborate with those of Zang *et al.* (2007). A combination of the Japanese *B.* sp. and *B.* cf. *darjelingensis* was well-supported only on the *COI* tree, but neither of these two species demonstrated similarity with any other species. The significance of the other basal nods of all the obtained trees does not allow us to make conclusions concerning interspecies relationships.

Skull morphometry. Barbastelles seem to be quite similar in cranial and dental size, and proportions. The named species can be arranged according to the average skull and teeth size; from smallest to largest, these are *B. leucomelas*, *B. barbastellus*, *B. darjelingensis* and *B. caspica*. However, all these species demonstrate a huge overlap in craniodental measurements, except for the canine length in *B. leucomelas*, which is shorter than in the other forms. As we know from its original description (Zhang *et al.* 2007), *B. beijingensis* has a large overall skull size, but a proportionally short toothrow.

In the Principal Component analysis, overall size similarity between most of the *Barbastella* species resulted in a high overlap between samples of accepted and putative species, when analyzed jointly. Only *B. leucomelas* tended to be well separated by the second PC (5.36% of total variance, best correlated with C and CC; first one covered 70.12%, correlated with overall size and lengths of tooth rows). However, although not segregated, other forms demonstrated a tendency to be separated from each other (e.g., *B. barbastellus*, *B. caspica* and the Japanese samples). The Indian sample (*B. darjelingensis* sensu stricto) was not separated by this particular analysis from either *B. barbastellus* or the Japanese specimens. Once we excluded the European barbastelle and well-differentiated *B. leucomelas*, we found that the other forms had similar individual size variability, which made the first PC less informative. However, we found combinations of PCs that provided not full but visible tendency for the separation between *B. darjelingensis*, *B. caspica* and the Japanese samples; to make this difference more obvious, we performed a few analyses for pairs of known or putative species. The most important result of this pairwise comparison was the suitable separation of the Japanese barbastelle from all other forms, including *B. darjelingensis* sensu stricto and the specimens from southern China, Taiwan and Indochina (Fig. 3).

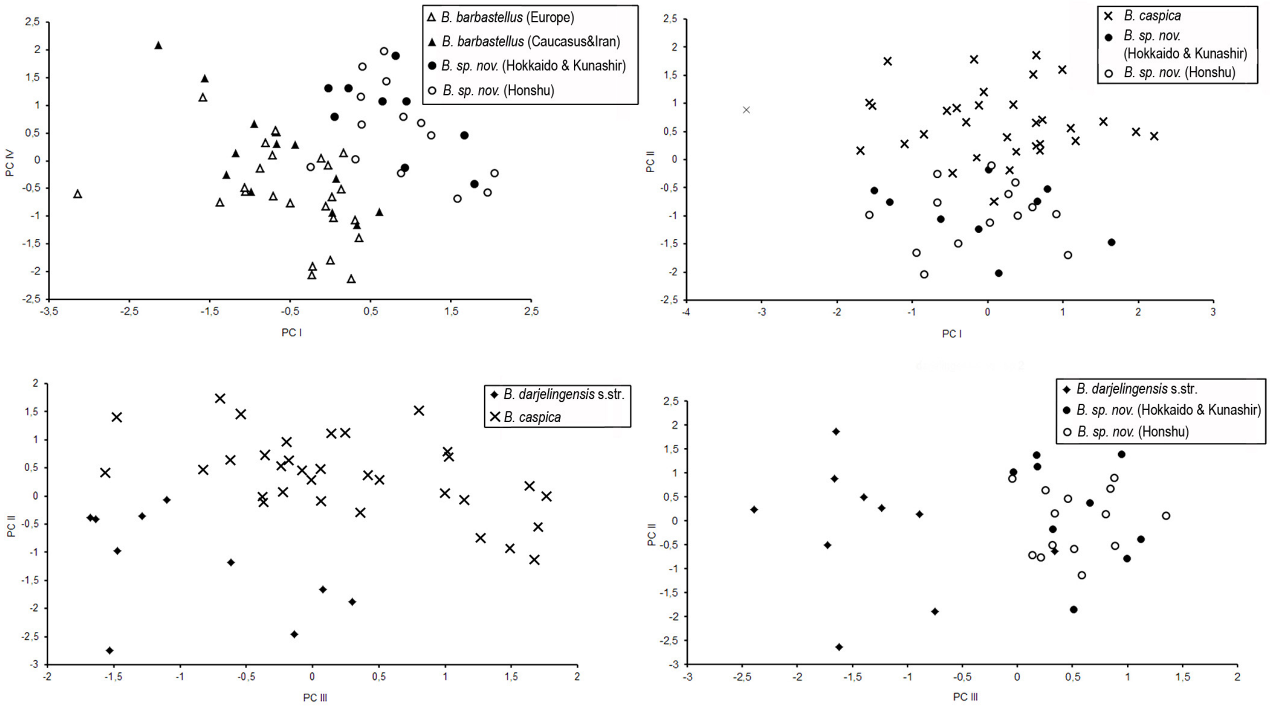

FIGURE 3. Bivariate scatter plots for the PC analyses of putative and accepted *Barbastella* species; Factors (Principal Components) are calculated from the 17 cranial and dental measurements in total for 117 specimens; analyzes were performed independently for each pair of species.

The Discriminant function analysis was conducted with the use of seven learning samples: *B. barbastellus*/Europe, *B. barbastellus*/Caucasus and Iran, *B. darjelingensis* sensu stricto, *B. caspica*, *B. leucomelas*, *B.* cf. *darjelingensis*/China & Vietnam and *B.* sp./Japan. For the two first canonical scores, all the specimens were grouped into three main clusters, one of which was composed of the smaller forms, *B. leucomelas* and *B. barbastellus*; *B. caspica* and *B.* sp. formed two other clusters, which emphasized the morphological originality of the Japanese race (Fig. 4). *Barbastella* cf. *darjelingensis* and, to some extent, *B. darjelingensis* took an intermediate position, between the first and third clusters. However, the third canonical score almost separated *B. darjelingensis* from the Japanese *B.* sp.; *B. leucomelas* was well segregated from all other forms by the fourth canonical score. In terms of distances, most analyzed samples were discriminated significantly ($p<0.01$; Table 3). Two geographically distant samples of *B. barbastellus* had an insignificant difference; a similar low difference could be seen between *B. darjelingensis* and the Chinese/Indochinese specimens, referred to as *B.* cf. *darjelingensis*, affirming that, despite differences in DNA, they are morphologically very similar.

TABLE 3. Distances between learning samples (below diagonal) and significance of difference between them (p-level, above the diagonal) calculated in forward stepwise Discriminant Function analysis.

	G_1:1	G_2:2	G_3:3	G_4:4	G_5:5	G_6:6	G_7:7
G_1:1 "*B. barbastellus*/Europe"		0.0566	0.0000	0.0000	0.0000	0.0000	0.0003
G_2:2 "*B. barbastellus*/Caucasus & Iran"	4.1410		0.0001	0.0000	0.0000	0.0000	0.0105
G_3:3 "*B. darjelingensis* sensu stricto"	14.7477	11.5865		0.0000	0.0000	0.0000	0.1454
G_4:4 "*B. caspica*"	31.1205	31.5732	27.4083		0.0000	0.0000	0.0000
G_5:5 "*B. leucomelas* sensu stricto"	25.9907	23.6446	32.7887	58.4385		0.0000	0.0000
G_6:6 "*B.* sp./Japan"	23.8317	20.2780	20.5073	16.4393	47.6675		0.0070
G_7:7 "*B.* cf. *darjelingensis*/China & Vietnam"	14.7170	12.2155	8.2600	24.6479	46.4444	11.0199	

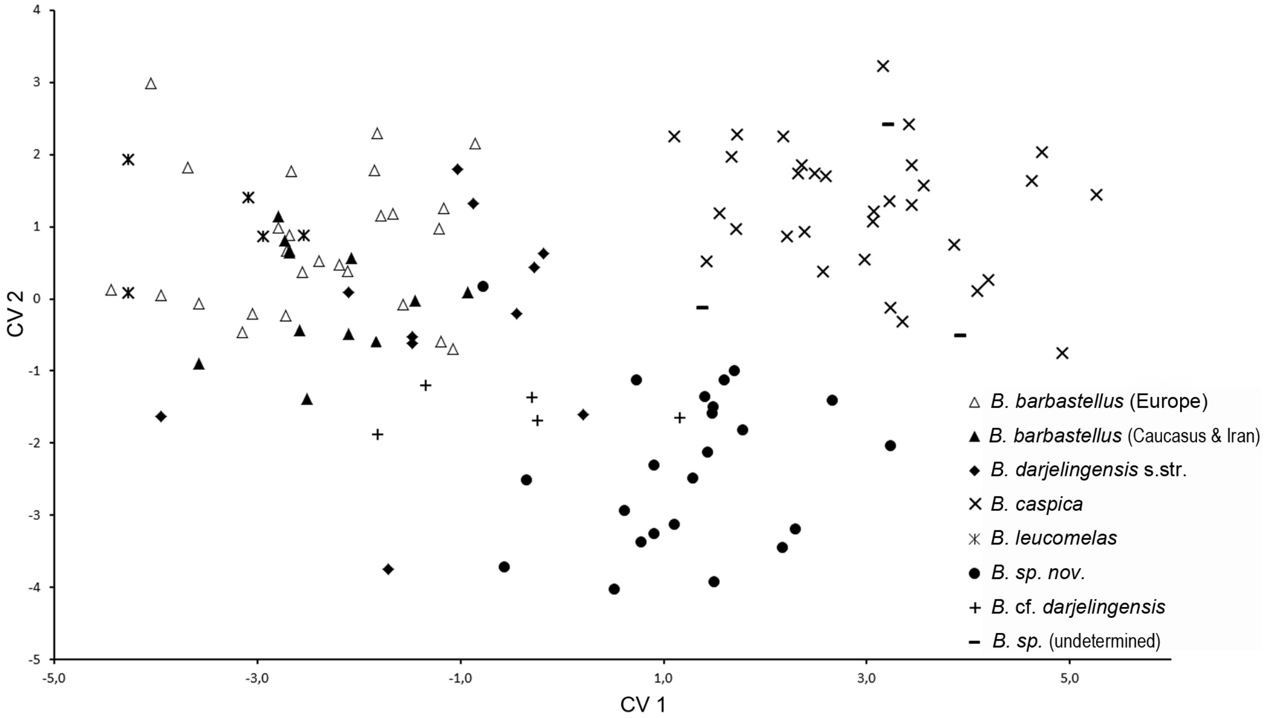

FIGURE 4. Bivariate scatter plot for the First and Second Canonical Variances calculated in Discriminant Function analysis for 18 cranial and dental measurements of 117 *Barbastella* specimens.

Overall, we conclude that Japanese specimens possess a significant difference in cranial morphometry from the other named forms of *Barbastella*. The only possible exception is *B. beijingensis*, for which we have no original measurements. Uncertainty with the Chinese and Vietnamese samples may be due to the very limited number of these specimens and possible heterogeneity of the whole sample, which may distort the analysis results.

Baculum shape. Barbastelles have penial bones (*baculum*) with a parallel-sided or slowly narrowing main shaft with a blunt tip, straight or somewhat concaved lateral profile, and widened basal third, which forms a variably pronounced urethral groove (Strelkov 1989; Benda *et al.* 2008; Fig. 5). In European barbastelles, the basal third is less wide, does not form lateral lobes and, therefore, also lacks a basal notch (which can be very small or entirely absent); the whole bone is characterized as "bottle-shaped". In Asian barbastelles, as well as in *B. leucomelas*, lateral basal lobes are better developed, with a distinct basal notch formed between them; the general shape can be approximated as triangular. In *B. leucomelas*, latero-basal lobes are very wide and short, with minute median notches (Benda *et al.* 2008). In *B. caspica*, basal lobes are somewhat narrower and the medial notch is more pronounced. Bacula of Nepalese specimens of *B. darjelingensis* appear somewhat more delicate, having a narrower main shaft that is parallel-sided and not gradually tapering to the epiphysis; proportionally, its basal lobes are similar to those of *B. caspica*, but with a less-developed median notch. In all four species, penial bones are similar in size, being ca. 0.7–0.9 mm in length. Unfortunately, nothing is known about the bacula of Chinese *B. beijingensis*, thus, we cannot make any comparison with that form. Baculum of the Vietnamese *Barbastella* cf. darjelingensis is similar in size to that of *B. barbastellus* and *B. caspica*, but with distinctly longer and narrower basal lobs (Fig. 5H) that turn downwards and are divided by a deep and wide medial notch. In contrast, Japanese specimens have penial bones with very wide basal lobes and a very small and shallow medial notch, as in *B. caspica* and *B. darjelingensis*. However, they have a proportionally wide main shaft, which is somewhat widened in distal epiphysis—a feature not seen in other barbastelles. The penial bone of the Hokkaido specimen described by Yoshiyuki (1989), is similar in size to penial bones of other barbastelles (0.73 mm in length), but specimens from Honshu, prepared by us, have penial bones about twice larger, ca. 1.4 mm in length (Fig. 5 i-k).

Deeply divergent mitochondrial lineages in East Asia suggest a regional presence of an underestimated cryptic diversity within the *Barbastella* genus. For one of these lineages, valuable genetic divergence is supported by skull proportions and baculum shapes. Here, we describe this form as a new species.

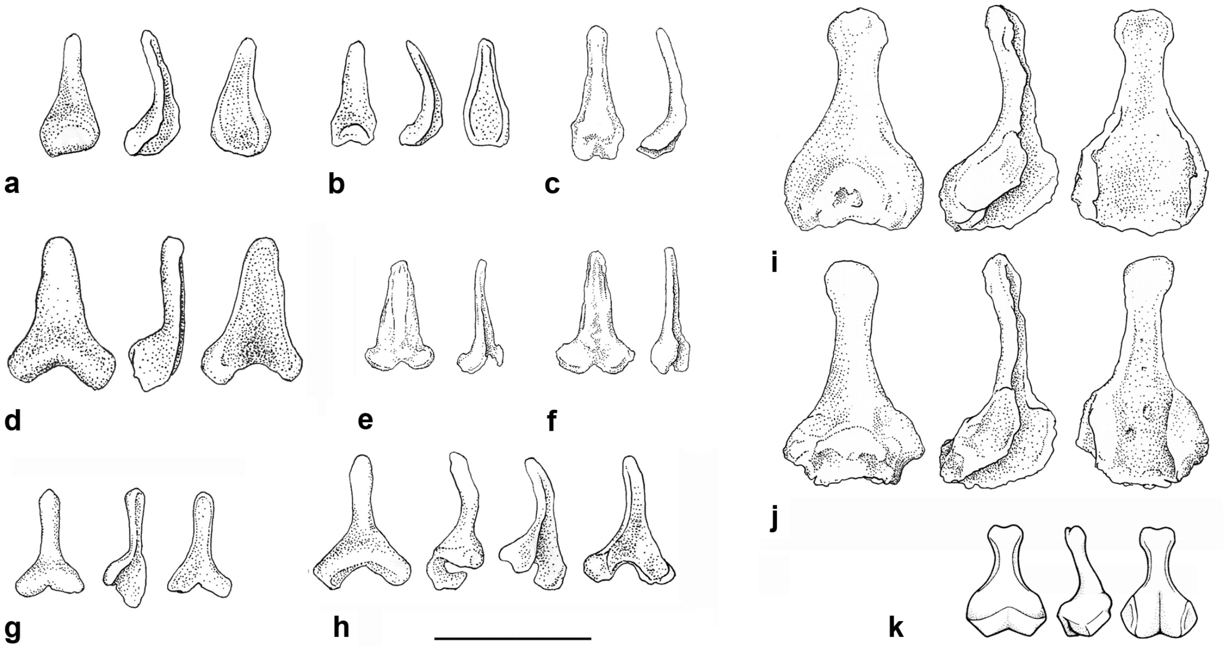

FIGURE 5. Penial bones of barbastelles: a–c—*B. barbastellus* (a, b—Russia, Krasnodar territory, ZMMU S-169241, S-169265; c—Czech Republic, n/n); d–e—*B. caspica*, (d—Tajikistan, ZMMU S-169301; e—Kirghizstan, CUP CT84/253); f—*B. leucomelas*, Egypt, Sinai, NMP 90521; g—*B. darjelingensis*, Nepal, Annapurna Himalaya, ZMMU S-164496; h—*B.* cf. *darjelingensis*, Vietnam, Lao Cai province, ZMMU S-186685; i–k—*B. pacifica* **sp. nov.** (i, j—Japan, Nagano prefecture, NSMT M-52949, 52944; k—Japan, Hokkaido, NSMT M-18559); c, e and f adopted from Benda *et al.* 2008; k—from Yoshiyuki 1989; others—original. Scale bar 1 mm.

Systematics

Order Chiroptera Blumenbach, 1779

Family Vespertilionidae Gray, 1821

Subfamily Vespertilioninae sensu stricto

Genus Barbastella Gray, 1821

Barbastella pacifica sp. nov.

urn:lsid:zoobank.org:act:AFD93791-1867-4971-ACDE-64F113BBC7AE

Holotype. BSI 168-07, adult male, carcass in alcohol and skull, captured on July, 2006. Collected by Selezneva T.A., Tiunov M.P.

Type locality. Kunashir (Kunashiri) Island, Filatova (Ruyabetsu) stream, ca. 1 km from the mouth (Selezneva & Tiunov 2007); N 44.190°, E 146.023°.

Paratypes. Kunashir Island: NSMT M 61925, male, carcass in alcohol and skull; Hokkaido: NSMT M 14816, male, dry skin and skull; NSMT M 18559, male, skull; NSMT M 18560, female, dry skin and skull.

Other referred material. 19 specimens from Hokkaido and Honshu; see list above.

Etymology and common name. The name '*pacifica*' applies to the species restriction for islands in the North-West Pacific. 'Japanese barbastelle' can be suggested as a common name for this species since most part of its range cover Japanese islands Hokkaido and Honshu.

Diagnosis. Middle size bat with typical *Barbastella* appearance. Large species within the genus (condylo-basal length of skull more than 13.6 mm and forearm length no less than 39 mm), with proportionally high brain case, moderately sloped facial profile and long dark-brown fur.

Description. A large species of barbastelle (CBL 13.65–14.45 mm, CM 4.75–5.01 mm, HB 51–61 mm, FA 39.0–43.0 mm), demonstrating all the typical traits of the genus. Ears are broadly triangular, connected at the frontal point, and relatively long (A 14.1–19.0), without an additional lobe on the outer margin (as opposed to *B. barbastellus* and *B. beijingensis*). The tragus is long (ca. 8.5 mm) and more or less triangular; its posterior margin is slightly concave in the upper third and convex at the widest part of the tragus. Ears and naked facial parts are brown.

The fur is long, thick and soft, with somewhat wavy underfur hairs. Hairs are about 10.0–10.5 mm in length on the back and about 8.0–8.5 mm long on the belly (almost as long as in *B. caspica*, and longer than in *B. darjelingensis* and in specimens from Vietnam). The underfur is dark brown with a slight mixture of pale grayish-brown hairs; guard hairs are dark brown, with pale terminal parts. In adult animals, lengths of the paler parts vary from ca. 1.8 mm on the sides of the back to ca. 4.5 mm in the middle, and can be nearly absent on the nape and head. The overall appearance is deep brown, shadowed with light brown and definitely less glossy than in *B. caspica* and *B. leucomelas*. Belly colouration in general is similar to that of the back. Pale tips appear more grayish and are about 1.5–2.0 mm in length. On the sides and in the groin area, the pale parts represent about half of the hair lengths, forming a grayish-coloured zone. On both the back and belly, the transition from dark to pale parts of the hairs is gradual, without any conspicuous border. As a whole, the tone of the fur colouration and the degree of pale shading are most similar to that of *B. barbastellus*. In immature animals, the pale tips of hairs are less developed, which results in a darker overall appearance.

In the context of the genus *Barbastella*, the skull of the new species has a moderately high and rounded braincase (Fig. 6). Its frontal part is slightly higher than the temporal part, but less than in *B. barbastellus*, *B. leucomelas* or *B. beijingensis*, and a shallow depression can usually be seen on the level of the coronal suture. The sagittal ridge is not developed and the occipital ridge is weak. The facial part is wide, with a shallow but prominent depression, somewhat less pronounced than in *B. darjelingensis*; in the lateral view, the facial profile slopes almost evenly, and concaves only slightly. The anterorbital foramen is large, rounded and situated over the roots of P4; the suborbital channel is ca. 1.2–1.3 mm. The lacrimal foramen is exposed, turned backward and situated above the level of the anterorbital foramen. On the lower jaw, the coronoid process is vertical and short, as in other barbastelles. The angular process is slightly projected backward beyond the level of the articular process, more than in *B. barbastellus*, but less than in *B. darjelingensis*, and its tip is blunter than in the latter species.

Teeth are similar to that of other barbastelles. In the upper teeth, the inner incisor is definitely bicuspidate, with a subequal height of both cusps and sometimes with a minute indentation on the posterior edge, about 2/5 in height of the appropriate canine. The outer incisor is small, but with a commonly definite posterior cusp (having a sharp rectangular indentation shape); it is similar or slightly smaller than the inner one in the crown area and one third to half of its height. The upper canine is without additional cusps or indentations, and its base is wide-oval on a cross-section. The upper small premolar is highly reduced; in some individuals, it is absent in one or both sides of the jaw. The first and second upper molars have greatly reduced hypocones and basins opened at the base of the metacone. The lower canine has a moderate secondary cusp, which is higher than the outer incisor. The outer incisor crown is somewhat simplified: the posterior supplementary cusp is usually not developed, and in some individuals, one of three main cusps is also rudimentary.

The baculum, as mentioned above, has a specific feature in the shape of the main shaft, which is proportionally wide with a widened and very blunt distal epiphysis. In comparison to the baculum of *B. darjelingensis*, it is wider and massive.

Overall, the new species, besides genetic differences, can be separated from all other species by its fur colouration: darker, especially on the belly, than in *B. leucomelas* and *B. caspica*, more brown than in *B. barbastellus* and *B. darjelingensis*, and, from the latter, it also has longer and less contrasting pale hair tips. It differs from *B. leucomelas* and *B. barbastellus* by its larger overall size, and from the latter and *B. beijingensis*, by its ear shape. It differs from *B. darjelingensis* by its shorter and blunter angular process of the mandible, and from *B. beijingensis* by a less inflated braincase. The baculum shape easily distinguishes the new species from *B. barbastellus*, *B. leucomelas*, *B. caspica* and barbastelles from Southeast Asia.

Variation. We did not find any variation in the haplotypes between the northern and southern populations of this species, suggesting that the divergence of these populations is very recent. Morphologically, as shown above, these two samples were also indistinguishable; we only found that animals from the Honshu population more frequently lacked upper small premolars. The difference in bacula size between our specimens and an individual depicted by Yoshiyuki (1989) may be a result of age or individual variability, which requires further study.

Distribution. The newly described species is restricted in distribution to the Japanese and Southern Kuril Islands and widely separated from the nearest populations of congeneric species living on mainland Asia. It occurs on Kunashir Island, and on most parts of Hokkaido (except the south-west), and has a sporadic distribution on Honshu (recorded in the Iwate, Fukushima, Tokyo, Kanagawa, Nagano, Gifu, Shizuoka prefectures) and on Sikoku (Fukui 2015).

Ecological remarks. The natural history is poorly known (Fukui 2015). Most probably, this species is forest dwelling, inhabiting temperate mixed and broad-leafed forests. Hibernation sites and day roosts are in caves, artificial underground shelters and rock crevices. The population number and natural density is not known; the status of the whole population, and especially the isolated southern sub-populations, requires further in-depth study.

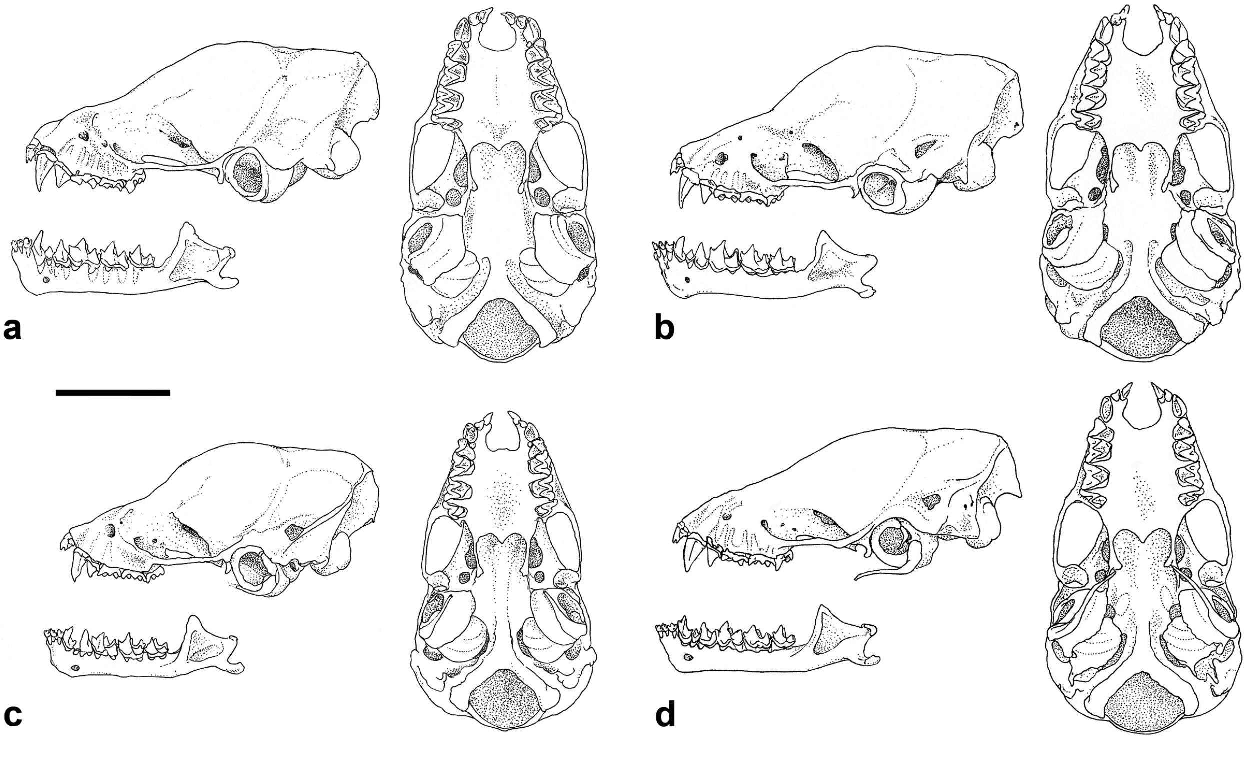

FIGURE 6. Skulls of the newly describing *Barbastella* species: a—*Barbastella pacifica* **sp. nov.**, holotype BSI 168-07, adult male, Kunashir Island; b—*Barbastella pacifica* **sp. nov.**, NSMT M-8774, female, Honshu, Saitama; c—*B. leucomelas*, NMP PB2883, adult male, Egypt, Sinai; d—*B.* cf. *darjelingensis*, ZMMU S-186685, adult male, Vietnam, Lao Cai. Scale bar 5 mm.

Discussion

The taxonomic situation observed in the *Barbastella* genus likely reflects the common problems of the "morphological" era in taxonomy. Barbastelles are unique among other bats, and their identification in any part of the genus distribution area causes no difficulties. All the currently known species are allopatric, or at least parapatric; only in the Caucasus region and Iran two morphologically distinct species occur sympatrically, though inhabiting different landscapes (Benda *et al*. 2011, 2012). Therefore, these two species were accepted by most specialists, with all the other forms considered subspecies or even just local populations without taxonomic rank (e.g. Koopman 1994; Simmons 2005). This was exacerbated by the fact that, within the enormous territory from Turkestan to eastern China, barbastelles are quite rarely captured, often inhabiting hard-to-reach areas and represented by very few specimens in zoological collections, which hinders their study and adequate comparisons by specialists. The only probable exception may be Japan, where barbastelles are not rare (Yoshiyuki 1989; Fukui 2015). However, there are also very few Japanese specimens in European collections, and specimens from the western Palearctic and especially from Central Asia and the Himalayas were hardly accessible to Japanese scientists. Altogether, this apparently decreased reseachers' interest in barbastelle taxonomy.

The widespread use of molecular genetic techniques brought about change. Among other things, they allowed researchers from different countries to compare samples without having specimens in hand, by exchanging genetic sequences or placing them into public databases. Such comparisons are usually not adequate for final taxonomic decisions, but they at least allow the realization of the existence of certain problems and outline their possible solutions.

Insular isolation of the Japanese barbastelle and its remoteness from the rest of the *Barbastella* genus distribution range, on the whole, made it possible to prescribe its special status, which was confirmed by both morphology and molecular genetics. Meanwhile, there is still very little material available from Taiwan, mainland China and the northern parts of Southeast Asia. The genetic data suggests the existence in this territory of at least one more taxon on the same divergence level as the Japanese barbastelle. However, the morphological data contradict this. The fact that currently existing data are highly scattered does not allow us to determine whether there is only one such taxon in the entire vast territory, and where its possible distribution and taxonomic boundaries are with respect to already known species (*B. darjelingensis* and *B. beijingensis*). Differences in mitochondrial DNA alone cannot serve as a basis for describing a new species, because of the possibility of the spread of alien DNA through a population, obtained from ancient hybridisation (as is known for some other bats; see e.g. Artiushin *et al.* 2009; Juste *et al.* 2013; Furman *et al.* 2014; Vallo *et al.* 2014; Kuo *et al.* 2015). These and other examples clearly demonstrate the potential role of reticulated processes in bat speciation, and the requirement to integrate different methods. A clear understanding of *Barbastella* taxonomic diversity in the eastern part of Asia requires an integrative approach, including further study of representative samples from different parts of the range, as well as the analysis of nuclear markers.

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APPENDIX. Material used in molecular study.

TABLE A1. List of GenBank sequences obtained in this study

	specimen number	COI	ND1	Cytb	Locality
B. pacifica **sp. nov.**	NSMT M52944	LC456136	LC456160	LC456145	Nagano Pref., Japan
	NSMT M52945	LC456137	LC456161	LC456146	Nagano Pref., Japan
	NSMT M52946	LC456138	LC456162	LC456147	Nagano Pref., Japan
	NSMT M52947	LC456139	LC456163	LC456148	Nagano Pref., Japan
	NSMT M52948	LC456140	LC456164	LC456149	Nagano Pref., Japan
	NSMT M52949	-	LC456165	LC456150	Nagano Pref., Japan
	NSMT M52950	-	LC456166	LC456151	Nagano Pref., Japan
	NSMT M54617	-	LC456167	LC456152	Nagano Pref., Japan
	OCUM5375	-	-	LC456153	Hokkaido, Japan
	OCUM5377	LC456141	LC456168	LC456154	Hokkaido, Japan
	DH0606	LC456142	LC456169	LC456155	Hokkaido, Japan
	NSMT M61925	LC456143	LC456170	LC456156	Kunashir (Kunashiri) Is
B. cf. *darjelingensis*	OCUM7184	LC456144	LC456171	LC456157	Tsuifeng, Nantou Hsien, Taiwan
B. caspica	ZMMU S-194788	-	LC456172	LC456158	Sughd Region, Tajikistan
	ZMMU S-194789	-	LC456173	LC456159	Sughd Region, Tajikistan

TABLE A2. Accession numbers of sequences obtained from GenBank (*ND1* and *cytb* genes) and BOLD (COI gene).

	ND1	cytb	COI
B. pacifica **sp. nov.**	AB079816		
B. leucomelas		EU743795	NMP90521
			NMP90522
B. darjelingensis			BM647-05|ZMMU S-164497
B. cf. *darjelingensis*	EF534771	EF534763	ABBSI270-11|ZMMU S-186684
	EF534772	EF534764	ABBSI271-11|ZMMU S-186685
	EF534773	EF534765	
	EF534774	EF534766	
B. beijingensis	EF534767	EF534759	
	EF534768	EF534760	
	EF534769	EF534761	
	EF534770	EF534762	
B. caspica			SKMZM1184-13|ZMMU_SVK_13-060
			SKMZM1069-12|ZMMU_S-190370
			SKMZM1185-13|ZMMU_SVK_13-061
B. barbastellus	KJ948255	JQ683176, JQ683177,	JF442793|ZMMU_S-174675
	AF401376	JQ683178, JQ683179,	JF442794|ZMMU_S-175325
	DQ915031	JQ683180, JQ683181,	ABBWP099-06|MHNG1916.059
	DQ915032	JQ683182, JQ683183,	ABBWP012-06|MHNG1804.094
	KF218431	JQ683184, JQ683185,	FR856638
	KF218432	JQ683186, JQ683187,	FR856639
	KF218433	JQ683188, JQ683189,	FR856640
		JQ683190, JQ683191,	
		JQ683192, JQ683193,	
		JQ683194, JQ683195,	
		JQ683196, JQ683197,	
		JQ683198, JQ683199,	
		JQ683200, JQ683201,	
		JQ683202, JQ683203,	
		JQ683204, JQ683205,	
		JQ683206, JQ683207,	
		JQ683208, JQ683209,	
		JQ683210, JQ683211,	
		JQ683212	