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Original Article

Molecular phylogenetic analysis of bats in the family Vespertilionidae (Order: Superfamily) in Mongolia

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ABSTRACT

Bat species in Mongolia have received a relatively low conservation priority compared to avian and other mammalian species. This might partially result from their understudied ecologies, distributions, and population sizes in Mongolia. Additionally, Mongolia hosts a relatively small assembly of bat species. In this study, we investigated the genetic characteristics of 14 species of bats ($n = 61$) from six genera (*Myotis, Vespertilio, Eptesicus, Hypsugo*, *Plecotus*, and *Murina*) using a 598 bp long mitochondrial DNA cytochrome c oxidase subunit 1 gene. We reconstructed Maximum likelihood and Bayesian-inference trees, together with estimated molecular divergence times on nodes of interest using an HKY substitution model. Our findings clearly showed four distinct clades (I–IV). Through the partial fragment mitochondrial DNA cytochrome c oxidase subunit 1 gene, 26 haplotypes ($Hd = 0.94 \pm 0.0001$) were identified overall from 61 individuals. We estimated a divergence time of 31.71–55.84 mya (95% highest posterior density (HPD)) on the node of clade I (genus *Myotis*) and the other three clades (other genera). Interestingly, in clade II, we no longer considered animals in haplotype 2 (H2) of *Hypsugo alaschanicus* as *H. alaschanicus*. Instead, this haplotype was recently described as *H. stubbei*. Additional molecular genetic studies are required to clarify intraspecific divergences of some vesper bats in Mongolia, particularly, M. *ikonnikovi, M. davidii, H. alaschanicus,* and *P. ognevi*.

Introduction

As part of the Mongolian Plateau, Mongolia hosts relatively unique biodiversity due to its location at the junction of the Central Asian deserts and Siberian taiga. In addition, Mongolia is a vast country, which covers diverse natural zones and landscapes. The geographical location of Mongolia results in harsh, continental climatic conditions, with temperatures dropping as low as −40°C in winter and rising as high as 35°C in summer, and relatively low precipitation characterizing most of the country (Jambaajamts 1989). This climate results in a relatively small number of registered species for particular groups of animals, especially bats and amphibians (Munkhbayar et al. 2020). For bat species, Mongolia hosts relatively few species compared to other warmer and more humid countries (Jargalsaikhan 2018). To date, Mongolian bat fauna remains only moderately known. Yet, bats are an important part of the ecosystems they inhabit, despite remaining understudied (Amick et al. 2021).

Historically, pioneering studies of Mongolian bat fauna surveys were conducted by several renowned naturalists, namely N.M. Przewalski, G. Radde, and P.K. Kozlov undertook expeditions to Central Asia and Mongolia in the 18th and 19th Centuries (Jargalsaikhan 2018; Koopman 1971). The findings and field notes from these historic expeditions were initially summarized in a book named the Mammals of Mongolia by Bannikov (1954). Bannikov (1954) included 8 species and 13 subspecies of bats in his book with species identification keys and distributions. More recently, reported 15 species from 6 genera, Batsaikhan (2014) published a guidebook book with 14 species, and Datzmann et al. (2012) included 17 species in Mongolia as part of the Eastern Palearctic. Most recently, Jargalsaikhan (2018) suggested that Mongolia has 19 species of bats, based on his own research studies and previous data.

All 19 species of bats in Mongolia are members of the family Vespertilionidae (also called vesper bats), the largest family of the

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order Chiroptera (Datzmann et al. 2012). Vesper bats are small and insectivorous, and approximately, 350 species of 44 genera of vesper bats are recognized (Datzmann et al. 2012; Nowak and Walker 1999). Of these, 40 species of vesper bats occur in the Eastern Palearctic region (Simmons 2005). Vesper bats are common and diverse in warmer, more humid parts of the world, with especially wide distributions for many species in the western and Eastern Palearctic (Datzmann et al. 2012; Koopman 1971). Because of their wide ranges, this group of bats is relatively well studied, particularly in the western Palearctic. Several previous studies explored the relationships of species within a particular genus and/or subfamily of Vespertilionidae using conventional morphological characteristics and molecular genetic data (Datzmann et al. 2012; Kawai et al. 2003; Kruskop et al. 2012, 2020; Pierson 1986; Simmons 2005). From these studies, Datzmann et al. (2012) and Kruskop et al. (2020) studied the phylogeny of some Mongolian vesper bats.

Mitochondrial DNA (mtDNA) markers are routinely used for phylogenetic studies of Vespertilionidae (Datzmann et al. 2012; Kawai et al. 2003; Kruskop et al. 2012, 2020; Nesi et al. 2011; Park et al. 2019; Pierson 1986; Simmons 2005; Stadelmann et al. 2004). The most frequently used mtDNA markers were NADH dehydrogenase subunit 1 (*ND1*), cytochrome *b* (*Cytb*), *12S rRNA*, and *16S rRNA*. As a result of previous molecular studies, the Palearctic bat fauna has undergone several taxonomic revisions (Kruskop et al. 2012).

In this study, we focused on the phylogeny of bat species that inhabit Mongolia. Thus, our study aims to reconstruct phylogenetic trees to clarify evolutionary relationships and evaluate genetic diversity parameters of Mongolia vesper bats (14 species) using the alternative mtDNA marker cytochrome c oxidase subunit 1 (*COI*).

Material and methods

Sample collection

We collected tissue samples (wing punches) from 61 individuals of 14 different Vesper bats from 31 localities across Mongolia (Figure 1). The sampling area covered major parts such as Lake Buir in the Eastern, Lake Uvs in the west, as well as areas between Darkhad Depression in the north, Gurvantes village in southern parts of Mongolia (Figure 1). These areas cover all natural zones that occur in Mongolia. Further information related to the studied species is summarized inSupplemental Table S1. For the collection of samples, we conducted 10 surveys between 2006 and 2017 (Table S1). During field surveys, we trapped bats using mist-nets near or at water points during the night (mainly between 2100 h–2350 h). During field surveys, we preserved tissue samples in tubes with 70% ethanol and deposited them within the collections of the Department of Biology of the Mongolian National University of Education prior to laboratory experiments. We applied for and obtained the necessary permits from the Ministry of Environment and Tourism to collect samples.

Laboratory experiments

We extracted total genomic DNA from tissue samples using a Qiagen DNeasy kit (QIAGEN Group) according to the manufacturer's protocol. We sent our extracted gDNA samples to the service provider company (Macrogen Inc.) for PCR amplification of *COI* in mtDNA and directed sequencing with a previously designed primer set: jgLCO1490 5′- TIT CIA CIA AYC AYA ARG AYA TTG G -3′ and

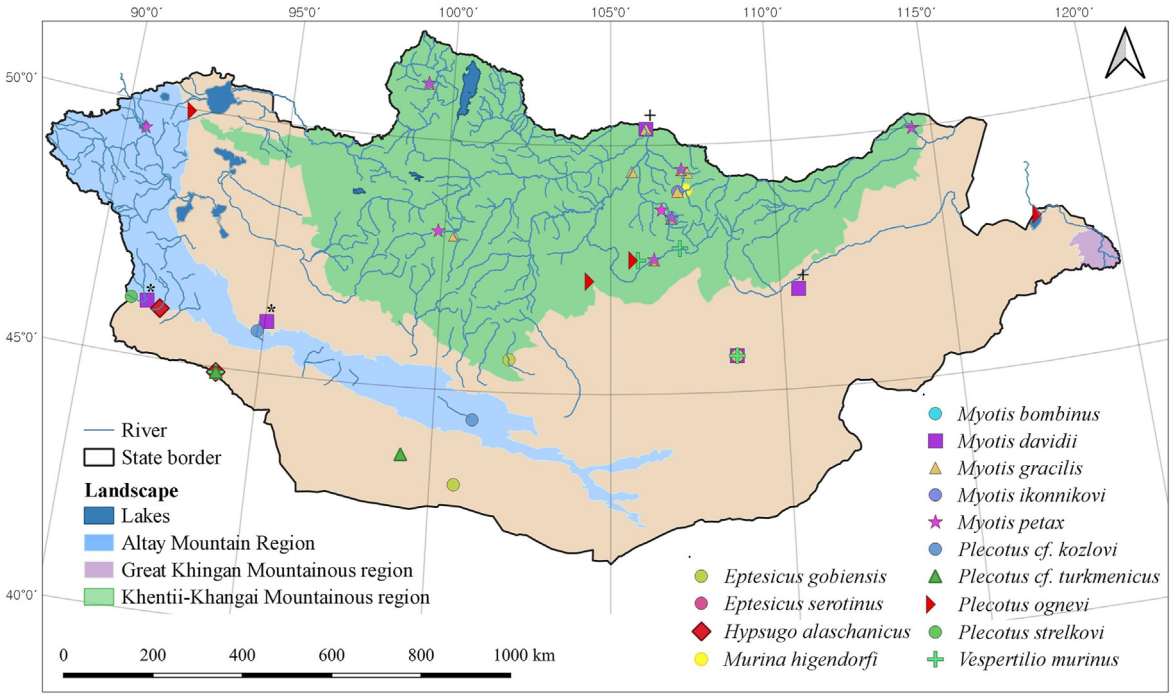

Figure 1. Sampling locations of the 14 species of Vesper bats in Mongolia. Each species was represented by a different shape. (*) are representing animals from H5 of *M. davidii*, while (+) representing animals from H4 of *M. davidii*.

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jgHCO2198 5′- TAI ACY TCI GGR TGI CCR AAR AAY CA -3′ (Geller et al. 2013). According to Macrogen, the reaction conditions for amplification consisted of initial denaturation at 94°C for 3 min, followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension for 1 min at 72°C, and final extension at 72°C for 7 min, with a total volume of 25 μl. Purified 598 bp PCR products were sequenced directly using the Sanger DNA sequencing approach (ABI PISM 3730XL Analyzer; Applied Biosystems). We ran extracted gDNA samples and PCR products on 1.5% agarose gel at a constant voltage of 150 V for 30 min in a 1 × TAE buffer to check molecular bands under UV light with negative controls. We deposited all novel haplotype sequences ($n = 26$) of the mtDNA *COI* gene from this study in the GenBank public database under accession numbers OM370809-OM370834.

Data analysis

Sequence editing and data assembling

All 61 *COI* gene sequences were edited manually using Chromas version 2.6.6 (Technelysium Pty., Ltd., South Brisbane, Australia), then aligned their positions in BioEdit version 7.2.5 (Clustal W tool; Thompson et al. 1994) with reference sequences, including the outgroup *Tadarida teniotis*. To conduct our phylogenetic analysis, we retrieved the same fragments of the *COI* gene ($n = 52$) from Genbank (National Center of Biotechnological Institute; NCBI). We summarized other related information for these reference sequences inSupplemental Table S2. Thus, we had two different datasets – Dataset I (61 sequences from this study) that we used for evaluating genetic diversity and differentiation analyses and Dataset II (113 sequences) that we used for phylogenetic analyses and estimating divergence times between species.

Genetic diversity and differentiation analysis

We used DNA Sequence Polymorphism version 6.12.03 (DnaSP; Rozas et al. 2017) to calculate the following diversity parameters: number of haplotypes (h), haplotype diversity (*Hd*), number of segregating sites (S), nucleotide diversity (π), and average number of nucleotide differences (K) from a 598 bp *COI* fragment in mtDNA. For diversity analyses, we included species with more than two samples. To quantify genetic distances between species, we calculated pairwise genetic differences (*Fst*) ($n = 14$; significant values were accepted when $P < 0.05$) in Arlequin version 3.5.2.2 (Excoffier and Lischer 2010). We also estimated the average number of pairwise differences within population/species (PiX) in Arlequin.

Phylogenetic and evolutionary dating

We reconstructed two types of phylogenetic trees, namely Maximum likelihood (ML) and Bayesian criteria (BI; Bayesian inference tree). The best fit substitution models for ML and BI trees in this study were determined by the Akaike Information Criterion (AIC; -lnL = 4481.2982; AIC = 9281.96), implemented in MEGA version 10.2.2 (Kumar et al. 2016). We constructed ML trees with 1000 bootstrap replicates and an HKY (Hasegawa-Kishino-Yano; Hasegawa et al. 1985) model in MEGA. From our ML analysis, we show a tree with the highest log likelihood (– 4502.71). Bayesian analyses also used the best fit HKY + I (proportion invariable site) model with site-specific gamma distribution (HKY +G+I; other parameters left as default). We calculated a single Bayesian run for Markov Chain Monte Carlo (MCMC) chains over 10,000,000 generations, with a sampled interval of 1000 generations, and we discarded the first 25% from the posterior distribution of trees using the burn-in command. We used the *sump* and *sumt* commands to summarize the remaining trees and other associated values in MrBayes environment. We implemented all of these Bayesian phylogenetic analyses in MrBayes v3.2.7 (Ronquist et al. 2012). We viewed the trace plots of clade posterior probabilities on Tracer version 1.7.1 (Drummond et al. 2012). Finally, we constructed a 50% majority-rule consensus tree in Figtree version 1.4.4 (Rambaut 2009) with posterior probabilities of nodes.

Furthermore, we estimated evolutionary divergence times between species from different genera in dataset II using BEAST v2.5.1 (Bouckaert et al. 2014) with the same substitution models as ML and BI analyses. We used a default Strict Molecular Clock model to estimate rates of evolution pattern on each node of interest. To estimate the molecular clock on the nodes of interest, we calibrated two nodes: the node of *Plecotus ognevi* and *P. kozlovi* and the node of *P. turkmenicus* and *P. strelkovi* using a Calibrated Yule tree prior function. Calibration prior was centered at the median age of selected nodes as reported in a previous study (Datzmann et al. 2012; the median age for 1st node was 4.0 mya, 2.0–7.0 mya 95% HPD (highest posterior density), for 2nd node was 10.0 mya, 6.0-15.0 mya 95% HPD). We ran the analysis by sampling every 10,000 generations for a total of 10,000,000 generations. We examined output values of BEAST in Tracer version 1.7.1 (Rambaut et al. 2018). We used TreeAnnotator version 2.6.3 (Bouckaert et al. 2014) to discard 10% of the total constructed trees and summarize an appropriate maximum clade credibility tree. After summarization, we generated a maximum clade credibility tree that we visualized in Figtree version 1.4.4 (MCMC.tree file), with node 95% HPD.

We drew a median-joining (MJ) network to unravel genetic relationships among identified haplotypes for particular bat species in Network version 5.0.0.1 (Bandelt et al. 1999). We generated a Roehl network data (*.rdf) file to construct an MJ network in DnaSP.

Results

Genetic diversities and differentiation

We examined 9 out of 14 species with genetic diversity parameters (Table 1), five species were excluded due to a limited number of samples (less than two). Through the partial sequences of the mitochondrial *COI* gene, were identified 26 haplotypes (*Hd* = 0.94 ± 0.0001) overall from 61 individuals of 14 species. At the species level, we observed the highest *Hd* and π values for several species, namely *Vespertilio murinus* (h = 4; *Hd* = 1.0 ± 0.00) and *Myotis davidii* (h = 4; *Hd* = 0.81 ± 0.01), and *Hypsugo alaschanicus* (S = 58; π = 0.064) and, *Eptesicus gobiensis* (S = 7; π = 0.0078), respectively (Table 1). The overall number of segregating sites (S) for a dataset I was 233.

The pairwise genetic *Fst* distance values between species from different genera are summarized in Table 2. We observed the

Table 1. Genetic diversity indices of the *COI* mtDNA gene for 14 species of Vesper bats in Mongolia. *n* = number of samples, h = number of haplotypes, *Hd* = haplotype diversity, S = number segregating sites, π = nucleotide diversity, K = average number of pairwise differences.

Species	*n*	h	*Hd*	S	π	K	Haplotypes
M. davidii	7	2	0.41 ± 0.01	3	0.004	2.81	H4, H5
M. gracilis	12	3	0.31 ± 0.02	2	0.0005	0.33	H6–H8
M. petax	18	4	0.62 ± 0.003	2	0.0012	1.04	H9–H12
Plecotus ognevi	5	1	0.00 ± 0.00	0	0.00	0.00	H22
P. kozlovi	2	2	1.0 ± 0.00	1	0.0016	1.00	H23, H24
P. gobiensis	2	1	0.00 ± 0.00	0	0.00	0.00	H25
P. strelkovi	1	1	–	–	–	–	H26
Eptesicus serotinus	1	1	–	–	–	–	H13
E. gobiensis	3	2	0.66 ± 0.003	7	0.0078	4.66	H14, H15
Hypsugo alaschanicus	3	2	0.66 ± 0.003	58	0.064	38.6	H16, H17
Vespertilio murinus	4	4	1.0 ± 0.00	3	0.0025	1.50	H18–H21
Murina hilgendorfi	1	1	–	–	–	–	H1
Myotis bombinus	1	1	–	–	–	–	H2
M. ikonnikovi	1	1	–	–	–	–	H3
Overall	**61**	**26**	**0.94 ± 0.0001**	**233**	**0.139**	**83.59**	**H1–H26**

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Table 2. Estimated pairwise *Fst* genetic differences (below), and average number of pairwise differences within population (PiX) (diagonal, *italic*).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
M.hilgendorfi	*0.000*													
My.bombinus	1.000	*0.000*												
My.ikonnikovi	1.000	1.000	*0.000*											
My.davidii	0.981	0.973	0.959	*2.280*										
My.gracilis	0.997	0.997	0.996	0.988	*0.333*									
My.petax	0.984	0.976	0.976	0.976	0.987	*1.820*								
E.serotinus	1.000	1.000	1.000	0.977	0.997	0.984	*0.000*							
E.gobiensis	0.965	0.961	0.953	0.972	0.991	0.981	0.900	*4.666*						
H.alaschanicus	1.000	1.000	1.000	0.983	0.997	0.984	1.000	0.968	*0.000*					
V.murinus	0.987	0.986	0.985	0.982	0.995	0.983	0.986	0.974	0.986	*1.500*				
P.ognevi	1.000	1.000	1.000	0.986	0.997	0.984	1.000	0.986	1.000	0.994	*0.000*			
P.kozlovi	0.992	0.991	0.990	0.978	0.996	0.981	0.990	0.968	0.995	0.986	0.985	*1.000*		
P.gobiensis	1.000	1.000	1.000	0.982	0.997	0.985	1.000	0.973	1.000	0.989	1.000	0.993	*0.000*	
P.strelkovi	1.000	1.000	1.000	0.978	0.997	0.983	1.000	0.957	1.000	0.986	1.000	0.984	1.000	*0.000*

lowest mean *F*st value in the genus *Eptesicus* (*F*st = 0.90, *E.serotinus* and *E.gobiensis*), followed by the genus *Myotis* (*F*st = 0.98; within the genus, a comparison of *M.davii* and *M. ikonnikovi* accounted for the lowest value, at *F*st = 0.95) (Table 2). Additionally, we calculated an average number of pairwise differences within population/species for these nine species (Table 2). We recorded the highest PiX value for *E. gobiensis* (PiX = 4.666).

Phylogenetic relationships

For simplicity, we used 78 sequences or taxa (which consisted of 26 haplotypes from this study and 52 reference sequences) for our phylogenetic analysis, instead of all 113 sequences. The phylogenetic statuses of Mongolian vesper bats were represented by ML and BI trees (Figure 2). Because of very similar topologies in both the BI and ML trees, we show only the single 50% majority-rule consensus tree with supporting values of Bayesian posterior probability (BI; BPP) and bootstrap (ML; BS). A 50% majority-rule consensus tree clearly supported four clades (I–IV). Five species from the genus *Myotis* occurred in clade I. Within clade I, *M. petax* and *M. bombinus* (BPP = 0.52) clustered close to each other, while *M. davidii* was located as a clade outgroup (Figure 2). A second clade (II) accounted for three genera, including *Eptesicus, Vespertilio, and Hypsugo,* in two subclades. In the first subclade, the taxa *E. gobiensis* and *E. serotinus* clustered together (BPP = 0.98; BS = 99) (Figure 2). Whereas, the second subclade consisted of two genera, namely *Vespertilio* and *Hypsugo*, both with a single species (BPP = 0.99; BS = 100). Moreover, four haplotypes of *V. murinus* clustered together with reference sequences from a wide range of samples in the Palearctic (Figure 2). Interestingly, *H. alaschanicus* ($n = 3$) split into two haplotypes that showed relatively high divergence (Figure 2) (BPP=0.97; BS = 97). Four species of genus *Plecotus* clustered as Clade III; a group which showed that *P. ognevi* and *P. kozlovi* are relatively close sister species (BPP = 0.66; BS = 66). The remaining two species, *P. gobiensis* and *P. strelkovi,* fell out as a distinct group in this clade (Figure 2). The last clade (IV) consisted of only *Murina hilgendorfi*, a species that clustered as an outgroup to other Mongolian Vesper bats, along with *Tadarida teniotis* (Figure 2A). In addition, phylogenetic relationship patterns are also supported by an MJ network reconstruction (Figure 2).

Subsequently, we dated the evolutionary divergence time for our five main clades, and estimated divergence times (95% HDP) provided at the nodes of Figure 2. We constructed separated a maximum clade credibility tree with estimated times. We manually added these times to the nodes of our phylogenetic tree (due to similar topologies). The analysis showed that genus *Myotis* in Clade I diverged from other genera between 31.37–55.84 mya (95% HPD), and Clades II and III diverged between 26.21–46.43 mya. The estimated divergence time for the subclade of Clade II was 21.86–39.50 mya (Figure 2).

Discussion

We investigated the genetic characteristics of vesper bats (Vespertilionidae) in Mongolia based on a 598 bp fragment of the *COI* gene. Before our study, Datzmann et al. (2012) examined phylogenetic relationships of this group of bats, including some Mongolian samples, using an alternative, the mtDNA's *ND1* gene. Hopefully, our findings contribute to knowledge gaps in Mongolia.

We attempted to estimate the level of genetic diversity within and among species in this study. The overall genetic diversity parameters came out with a high level of diversity ($Hd = 0.94 \pm 0.0001$; $\pi = 0.139$), at the species level, it has moderate to lower diversity (mean $Hd = 0.435$; $\pi = 0.0014$). Past, comprehensive studies tended to exclude genetic diversity analysis, instead, they focused mainly on the phylogeny of studied species (Datzmann et al. 2012; Kruskop et al. 2012, 2020; Simmons 2005). Nesi et al. (2011) and Park et al. (2019) had been mentioned some genetic diversity parameters. In particular, Park et al. (2019) investigated the population genetic structure of *M. ikonnikovi* in Korea R.P. They found 16 haplotypes (mean $Hd = 0.74$) from 42 sequences, with a mean nucleotide diversity of 0.004 (Park et al. 2019). Unfortunately, we had a limited number of samples of *M. ikonnikovi* ($n = 1$) to compare with this previous finding.

Unlike genetic diversity analyses, phylogenetic relationships of vesper bats were examined in several studies (Datzmann et al. 2012; Kruskop et al. 2012, 2020; Simmons 2005). Of these, Datzmann et al. (2012) included Mongolian samples in their comprehensive study. Our phylogenetic tree showed four distinct clades that were genera specific, excluding clade II (I–IV). Detailed relationship patterns of each genus follow.

1. Genus *Myotis*

Genus *Myotis* inhabits most parts of the World, with approximately 100 species (Koopman 1994; Stadelmann et al. 2004). Thus, the phylogeny of this genus is relatively well known. Yet, it is extremely difficult to include all of these species in a single comprehensive study. However, numerous studies attempted to clarify the phylogenetic status of different species in the genus *Myotis* (Datzmann et al. 2012; Kawai et al. 2003; Kruskop et al. 2012; Simmons 2005; Stadelmann et al. 2004). In our study, we included five species with 39 individuals in our phylogenetic analysis. In contrast to our tree, *M. bombinus* and *M. petax* did not cluster together in previous studies (Datzmann et al. 2012; Kruskop et al. 2012), which might be the result of different mtDNA genes.

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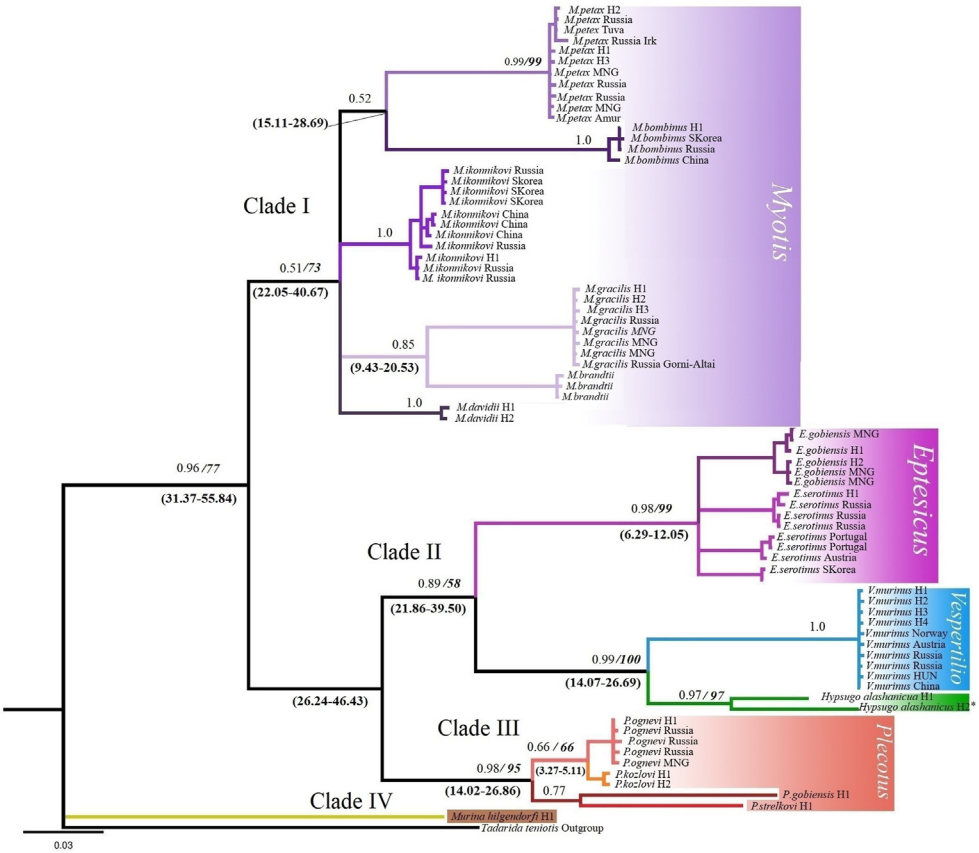

Figure 2. The 50% majority-rule consensus tree based on the 598 bp *COI* gene. The numbers adjacent to nodes are Bayesian inference posterior probabilities and Bootstrap values (in italic and bold). *Tadarida teniotis* was used as outgroup to the tree. Estimated values of divergence times of nodes of interest were provided below nodes (95% HPDs).

Consistently with our results, the clustering of the subclade of *M. gracilis* and *M. brandtii* in our tree was previously shown in Kruskop et al. (2012; mtDNA *COI* gene) and Datzmann et al. (2012; mtDNA *ND1* gene). Historically, *M. gracilis* in Mongolia has been synonymized with *M. brandtii,* a species only distributed in the western Palearctic. The estimated time of divergence of these two species in our study (9-20 mya; 95% HPD) was higher than previous findings (2-11 mya; 95% HPD) (Datzmann et al. 2012). For *M. davidii*, we had two haplotypes in our phylogenetic tree (Figure 2A—B). Interestingly, samples belonging to haplotypes H4 of *M. davidii* were geographically distinct from H5 (Figure 1). More specifically, animals in H5 were captured from western Mongolia, including Bulgan village in Khovd province and Tugrug village in Gobi-Altai province, while animals in H4 came from Eastern and Central Mongolian. Further phylogenetic study is required to clarify the phylogenetic relationships and subspecies of this group. Park et al. (2019) investigated the phylogenetic status of *M. ikonnikovi* in P.R. Korea using *COI* and *Cytb* genes. They found closer phylogenetic relationships between *M. ikonnikovi* from Russia and P.R. Korea than between the Chinese and Korean populations (Park et al. 2019). Unfortunately, this previous study did not include samples from Mongolia. Generally, our phylogenetic tree samples clustered with those samples from Russia (especially, parts of the Eastern Palearctic: Chita, Tuva, Amur, etc. regions) and P.R. Korea.

2. Genera *Eptesicus, Vespertilio* and *Hypsugo*

As in previous studies (Datzmann et al. 2012; Kruskop et al. 2012), the genera *Eptesicus, Vespertilio and Hypsugo* clustered in the same clade (II) in this study. Consistent with a previous study (Datzmann et al. 2012), our findings showed that this clade diverged with its neighboring clade (genus *Plecotus*) around 26.24—46.43 mya (95% HPD).

Within the genus *Eptesicus*, we examined the species *E. gobiensis* and *E. serotinus*. We found two separate clusters for *E. gobiensis*, including our two haplotypes. Previous studies (Datzmann et al. 2012) demonstrated the sister clade status of *E. serotinus* and *E. nilssonii*, with a shallow genetic split between Eastern and Western Palearctic populations of *E. nilssoni*. Unfortunately, we had no samples from *E. nilssonii* for this study.

However, similar to a previous study (Datzmann et al. 2012) and unlike *E. nilssonii, E. serotinus* showed a relatively deep genetic split between Eastern and western Palearctic populations (Figure 2A). The intraspecies genetic variation of *E. serotinus*, especially populations in western Palearctic, was also disclosed by Coraman et al. (2013).

The next genus of clade II was *Vespertilio,* with a single species *V. murinus.* Since the species in this genus did not show significant genetic differentiation among western and Eastern Palearctic, *V.murinus* is one of few species with a wide Palearctic distribution range that shows genetic uniformity across its range (Kruskop et al. 2012). Our phylogenetic tree with four haplotypes from this study, and reference sequences from western and Eastern Palearctic regions, supported these previous findings. The relatively close phylogenetic relationship of *V. murinus* and its Asian congener *V. sinensis* was reported previously (Datzmann et al. 2012; Kruskop et al. 2012).

In our phylogenetic tree, only one species, *Hypsugo alaschanicus,* showed a deep split as intraspecific genetic variation. So far, it is

reported that two species, *H. savii (caucasicus)* and *H. alaschanicus* occur in Mongolia (Dolch et al. 2021a). Yet, the occurrence of *H. savii (caucasicus)* remains questionable. We found two genetically distinct haplotypes from three samples in this study (Figure 2). More specifically, all samples in H16 and H17 were collected from western Mongolian localities, including Khovd and Gobi-Altai provinces (Table S1 and Figure 1). Unexpectedly, we no longer consider the animals in H17 as belonging to *H. alaschanicus*. Estimated divergent time between these haplotypes was 7.75–16.38 mya (95% HPD; BPP = 0.97; BS = 97). This genetic differentiation in this study was also supported by the estimated genetic distance value (*F*st = 1.0, as much as the other two individual species had). A recent study (Dolch et al. 2021a) proved the existence of a new and cryptic species, *Hypsugo stubbei* in Mongolia. This crucial discovery led us to conclude that animals in H17 represented the new and cryptic species *H. stubbei.* Similar to a previous study (Dolch et al. 2021a), we are reporting an overlapped distribution of *H. alaschanicus* and *H. stubbei* in western Mongolia. Two samples collected from Elgen-Us in Gobi-Altai province fell into both haplotypes. Previous comprehensive studies that examined western and Eastern Palearctic bat species did not report such intraspecific sequence divergence in *H. alaschanicus* (Datzmann et al. 2012; Kruskop et al. 2012).

3. Genus *Plecotus*

We included all four species of the genus *Plecotus* in our study, namely *P. ognevi, P. kozlovi, P. strelkovi,* and *P. gobiensis*. These species are all distributed in the Eastern Palearctic. Of these, *P.gobiensis* was previously known as *P. turkmenicus*, whose distribution is limited to northwest Turkmenistan, an area approximately 2,000 km away from Mongolia (Kruskop et al. 2020). Thus, Kruskop et al. (2020) strongly suggested reconsideration of *P. turkmenicus* in Mongolia. Dolch et al. (2021b) studied the genetic distinction between *P. turkmenicus* and other *Plecotus* species in Mongolia, and proposed a new species *P. gobiensis* based on morphological and molecular genetics characteristics.

The sister species status of *P. ognevi* and *P. kozlovi* found in our study (node supporting values, BPP = 0.66; BS = 66), was also reported in previous studies (Datzmann et al. 2012; Kruskop et al. 2012, 2020). Datzmann et al. (2012) suggested that these sister species diverged from each other around 3.27 − 5.11 mya (95% HPD). In addition, a subspecies of *P. ognevi* (*P. o. nomrogi*) was also strongly suggested in the previous study (Dolch et al. 2021b) based on the geographically disjunct distribution of this subspecies in the Eastern Mongolian steppes (Dolch et al. 2021b). However, in our study, we did not find any intraspecific divergence of *P. ognevi* ($n = 5$), even with a sample from the Eastern Mongolia steppe (Buir lake). However, we had a limited number of *P. ognevi* samples in our study. The new species *P. gobiensis* clustered together with *P. strelkovi* (BPP = 0.77), as in previous studies (Datzmann et al. 2012; Kruskop et al. 2012, 2020).

4. Genus *Murina*

M. hilgendorfi clustered as outgroups to other Vesper bats of Mongolia in our study (Figure 2A). This phylogenetic pattern was previously reported in Kruskop et al. (2012). *M. hilgendorfi* has been synonymized with the Asian tropical species *M. leucogaster,* species initially separated by Yoshiyuki (1989). Yoshiyuki (1989) applied the species name *hilgendorfi* based on Japanese specimens. Afterward, Simmons (2005) and Kruskop et al. (2012) found the existence of *M. hilgendorfi* in Eastern and central Asia. Nevertheless, *M. hilgendorfi* and *M. leucogaster* clustered as genetically close sister species (Kruskop et al. 2012).

In conclusion, an increased number of studies which used molecular genetics has resulted in substantial taxonomic changes within the numerous genera in the family Vespertilionidae. For example, over the last decade, the number of bat species registered in Mongolia increased dramatically from 14 to 20 species (with the inclusion of *H. stubbei*). In this study, the mtDNA barcoding gene revealed four distinct groups, which were genera specific (excluding clade II). We believe that future genetic analysis will detect new and cryptic vesper bat species in Mongolia, especially as the number of molecular genetic studies increases. This is true for genera that contain systematically problematic species, those that are difficult to distinguish based on morphological characteristics, such as *Myotis* and *Plecotus.*

Therefore, in the future, we plan to conduct research into the population genetic structure of specific bat species, such as *M. ikonnikovi, M. davidii, H. alaschanicus,* and *P. ognevi* using alternative molecular markers (mtDNA control region, short tandem repeat markers, etc.) with a larger number of samples from different Mongolian natural zones.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.japb.2022.04.006.

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