Mammal Study 40: 29–33 (2015)

Short communication

Mitochondrial DNA evidence suggests challenge to the conspecific status of the hairy-footed flying squirrel *Belomys pearsonii* from Taiwan and Vietnam

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At present, the genus *Belomys* has only one species: the hairy-footed flying squirrel (*B. pearsonii* (Gray, 1842)), which is widely distributed in eastern Nepal, Bhutan, India (Sikkim to Assam), southern China (Guizhou, Yunnan, Guangxi, Guangdong, Hainan, and possibly Sichuan and Hunan), Taiwan, and the Indochina Peninsula (Myanmar, Thailand, Laos, and Vietnam) (Thorington et al. 2012) (Fig. 1). Previously, this species was classified into the genus *Trogopterus* and named *T. pearsonii* (Corbet and Hill 1992). Wilson and Reeder (2005), however, put it into the genus *Belomys*, regarding this genus as monotypic. Presently, this taxonomic status is generally accepted (e.g., Thorington et al. 2012).

This species has four subspecies: *pearsonii*, *blandus*, *kaleensis*, and *trichotis* (Thorington et al. 2012). Of them, *B. pearsonii kaleensis* is geographically isolated from other subspecies and is an endemic subspecies to Taiwan Island (e.g., Lin and Lin 1983). In Taiwan, there are two additional species of flying squirrels: the Indian giant flying squirrel (*Petaurista philippensis grandis*) and the red and white giant flying squirrel (*Petaurista alborufus lena*) (Ci 1998). Two *Petaurista* species were regarded as endemic subspecies in Taiwan (e.g., Lin and Lin 1983), showing differences from continental forms. *Petaurista alborufus lena*, however, is now thought to be a distinct endemic species (*P. lena*) based on morphological characteristics (Corbet and Hill 1992), molecular data (Oshida et al. 2000a), and cytogenetic data (Oshida et al. 2000c). Also, *P. philippensis grandis* is treated as a distinct endemic species (*P. grandis*) based on molecular data (Oshida et al. 2005; Yu et al. 2006). Moreover, Taiwan has other endemic species of mammals, such as *Microtus kikuchi*, *Macaca cyclopis*, and *Capricornis swinhoei* (Ci 1998). This unique zoogeographical situation is thought to have occurred with the geographical isolation of Taiwan Island. Like two *Petaurista* species, *Belomys* occurring in Taiwan may be distinct from continental species. To test this hypothesis, we preliminary compared *Belomys* from Vietnam with that from Taiwan, by using mitochondrial (mt) cytochrome *b* gene sequences. Here, we discuss the phylogenetic relationship between continental and Taiwanese *Belomys*.

Materials and methods

Specimens

We collected four *Belomys pearsonii kaleensis* specimens from the central part of Taiwan and three *B. pearsonii* from northeastern Vietnam and one from northwestern Vietnam (Fig. 1 and Table 1). Since it was difficult to determine the exact subspecies name for Vietnamese specimens, we treated them as *B. pearsonii* ssp. Specimens from Taiwan are in our laboratory. Specimens from Vietnam are deposited in the Institute of Ecology and Biological Resources, Vietnam Academy of Sciences and Technology, Hanoi, Vietnam.

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Table 1. Flying squirrel specimens examined in this study and retained in our private collection preserved in the Laboratory of Wildlife Biology, Obihiro University of Agriculture and Veterinary Medicine. Species name and code numbers correspond to those used in Table 2 and Fig. 2.

Species name and code number	Collecting locality (locality number in Fig. 1)	Identity number	Haplotype	Accession number	Resource/Supplier
Belomys pearsonii kaleensis 1	Cueifong, Nantou,Taiwan (1)	TW06	–	AB126245	Oshida et al. (2004b)
Belomys pearsonii kaleensis 2	Taroko National Park, Hualien, Taiwan (1)	T-1716	BPT1	LC006019	National Museum of Natural Science, Taiwan
Belomys pearsonii kaleensis 3	Puli, Nantou, Taiwan (1)	TW15	BPT2	LC006020	Collected in the present study by us.
Belomys pearsonii kaleensis 4	Tonfushan, Nantou, Taiwan (1)	S2159	BPT3	LC006021	Taiwan Endemic Species Research Institute, Taiwan
Belomys pearsonii ssp. 1	Hoang Lien National Park, Lao Cai province, Vietnam (2)	IEBR-M-1047	BPV1	LC006022	Institute of Ecology and Biological Resources, Vietnam
Belomys pearsonii ssp. 2	Pia Oac National Park, Cao Bang, Vietnam (3)	IEBR-M-1423	BPV2	LC006023	Institute of Ecology and Biological Resources, Vietnam
Belomys pearsonii ssp. 3	Pia Oac National Park, Cao Bang, Vietnam (3)	IEBR-M-1435	BPV3	LC006024	Institute of Ecology and Biological Resources, Vietnam
Belomys pearsonii ssp. 4	Phong Nha-Ke Bang National Park, Quang Binh, Vietnam (4)	BN-KB-13	BPV4	LC006025	Institute of Ecology and Biological Resources, Vietnam

Fig. 1. Distribution of *Belomys pearsonii* (in light gray; Francis 2008; Thorington et al. 2012); this distribution map seems to be based on the collecting localities of specimens and probably it would be wider, as this species occurs widely in forest areas. Closed circles indicate collecting localities of *B. pearsonii* examined in the present study.

PCR and DNA sequencing

Total genomic DNA was extracted from muscle tissues using the DNeasy Blood & Tissue Kit (QIAGEN K.K., Tokyo). The complete mt cytochrome *b* gene sequence (1,140 bases) was amplified with polymerase chain reaction (PCR) using a primer set: L14724 5'-GATATGA AAAACCATCGTTG-3' and H15910 5'-GATTTTTGGT TTACAAGACCGAG-3'. Former and latter primers are reported by Kocher et al. (1989) and Oshida et al. (2000b), respectively. The 50 μl reaction mixture contained approximately 100 ng of genomic DNA, 0.25 pM of each primer, 200 μM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 units of r*Taq* DNA polymerase (Takara, Tokyo). Amplification was carried out for 35 cycles. The program was 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. A final extension reaction was at 72°C for 10 min. The PCR products purified with the PCR Clean Up-M (Viogen, Taipei, Taiwan) were directly sequenced using an automated DNA sequencer (ABI PRISM 377-96 Sequencer, the ABI PRISM 3100 Genetic Analyzer, Applied Biosystem, CA, USA) and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1 (Applied Biosystems, CA, USA). For sequencing, we used the same primers used for PCR and two additional primers newly designed: Cb1(1) 5'-CAGAATGATACTTCCTATTTGC-3' and Cb2(1) 5'-GCCTATGAATGCTGTGGCTAT-3'. Purification of PCR products and sequencing were carried out by Mission Biotech Co. Ltd. (Taipei, Taiwan).

Sequence analysis

To root phylogenetic trees, sequences of two *Pteromys* species (*P. volans* [accession number AB097683; Oshida et al. 2004b] and *P. momonga* [accession number AB164675; Oshida et al. 2005]) and three *Petaurista* species (*P. albiventer* [accession number AB092612], *P. elegans* [accession number AB510036], and *P. grandis* [accession number AB092611]) (Oshida et al. 2010) were used as out-groups. Since Mercer and Roth (2003) reported that *Belomys* was relatively closer to *Petaurista* and *Pteromys* than to other flying squirrel genera, they were thought to be suitable out-groups for resolving phylogenetic relationships among *Belomys* subspecies. Although genera *Aeretus* and *Trogopterus* are most closely related to *Belomys*, unfortunately their cytochrome *b* sequence data were not available in any databases.

All sequences were aligned with DNASIS (Hitachi, Tokyo). For maximum-likelihood (ML) analysis, the program MODELTEST 3.06 (Posada and Crandall 1998) selected the most appropriate substitution model of molecular evolution through the Akaike information criterion (AIC). This program selected the general time reversible (GTR) model of substitution (Rodríguez et al. 1990; Yang et al. 1994), considered the proportion of invariable sites (I = 0.4895), and followed a gamma distribution for variable sites (G = 1.0128). Base frequencies were estimated as A = 0.2826, C = 0.3136, G = 0.1215, and T = 0.2789. The rate matrix was estimated as A-C = 10.5349, A-G = 86.6118, A-T = 22.1015, C-G = 3.1878, C-T = 242.5728, and G-T of 1.0000. The ML tree was constructed by a heuristic search option having a tree-bisection-reconnection (TBR). Using genetic distances correlated by this model, we also conducted neighbor-joining (NJ) analysis (Saitou and Nei 1987). In addition, we did an un-weighted maximum parsimony (MP) analysis. The MP tree was constructed with a branch-and-bound search option. To assess nodal supports, bootstrapping (Felsenstein 1985) was carried out with 500 replications in ML analysis and 10,000 replications in MP and NJ analyses. The ML, MP, and NJ analyses were performed by PAUP* 4.0b10 (Swofford 2001). Bayesian inference (BI) reconstruction was carried out using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Bayesian analysis was conducted using the GTR substitution model selected by MODELTEST. The analysis involved two runs for one million iterations, using four Markov chain Monte Carlo chains sampled every 1,000 generations and a burn-in of 20%. Based on the remaining trees, 50% majority rule consensus trees were generated. Posterior probabilities were used to assess nodal support of the BI tree.

Results

Complete sequences (1,140 bases) of the cytochrome *b* gene were determined for all specimens. We deposited all sequence data in the DNA Data Bank of Japan (Table 1). Uncorrected percentage sequence divergence (*p*-distances) and nucleotide substitutions among sequences are shown in Table 2. The *p*-distances among our *Belomys* specimens ranged from 0.09 to 13.33%.

An ML search, assuming the GTR + I + G model of evolution, produced a single tree (Fig. 2). Branching patterns of NJ, MP, and BI trees were essentially similar to those of the ML tree (data not shown). All trees showed three major geographical lineages: 1) Taiwan (Taiwan lineage), 2) northwestern Vietnam (Vietnam lineage I), and 3) northeastern and central Vietnam (Vietnam lineage II). The Taiwan lineage consisted of all *B. pearsonii kaleensis* specimens endemic to Taiwan (Fig. 2). This lineage was supported with highest support values (100% in all trees). The Vietnam lineage II, which was supported with 99% support values in ML and MP trees and 100% support value in the BI tree, included two specimens from northeastern Vietnam and one specimen from central Vietnam. The Vietnam lineage I had one specimen from northwestern Vietnam; BI analysis showed that this specimen was closely related to Taiwan's *B. pearsonii kaleensis* with 97% nodal support, but other trees showed

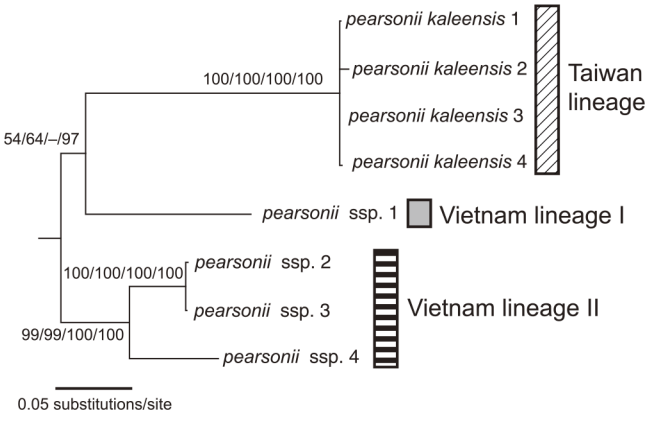

Fig. 2. Phylogeny of *Belomys pearsonii* constructed with the maximum-likelihood (ML) under GTR + I + G model for the mitochondrial cytochrome *b* sequences. From the left, numbers above branches represent: bootstrap values from 500 replicates of ML analysis and from 10,000 replicates of un-weighted maximum parsimony (MP) and neighbor-joining (NJ) analyses and posterior probability supports in Bayesian analysis. Specimens defined in Table 1.

Table 2. Pairwise comparisons of cytochrome *b* nucleotide sequences (1,140 bp) between eight *Belomys* specimens. Data above diagonal represent uncorrected percentage differences (*p*-distances). Data below diagonal are numbers of nucleotide substitutions (transitions/transversions).

Specimens	*pearsonii kaleensis* 1	*pearsonii kaleensis* 2	*pearsonii kaleensis* 3	*pearsonii kaleensis* 4	*pearsonii* ssp. 1	*pearsonii* ssp. 2	*pearsonii* ssp. 3	*pearsonii* ssp. 4
pearsonii kaleensis 1		0.70	0.09	0.26	13.60	12.98	13.07	13.25
pearsonii kaleensis 2	8/0		0.61	0.61	13.60	13.16	13.25	13.25
pearsonii kaleensis 3	1/0	7/0		0.18	13.68	13.07	13.16	13.33
pearsonii kaleensis 4	3/0	7/0	2/0		13.68	13.07	13.16	13.33
pearsonii ssp. 1	130/25	130/25	131/25	131/25		11.84	11.75	12.63
pearsonii ssp. 2	120/28	122/28	121/28	121/28	120/15		0.26	7.37
pearsonii ssp. 3	121/28	123/28	122/28	122/28	119/15	3/0		7.28
pearsonii ssp. 4	124/27	124/27	125/27	125/27	130/14	79/5	78/5	

low or no support values. Therefore, we regarded this specimen as a unique lineage.

The *p*-distances between the Taiwan lineage and Vietnam linage I, those between the Taiwan linage and Vietnam lineage II, and those between Vietnam linages I and II were 12.98–13.33%, 13.68–13.68%, and 11.75–12.63%, respectively (Table 2).

Discussion

In the present study, we identified three lineages in *B. pearsonii*. Based on mammalian mt cytochrome *b* sequences, Bradley and Baker (2001) reported that distance values between 2 and 11% have a high probability of being indicative of conspecific populations or valid species. The genetic distances among the three *B. pearsonii* lineages are distinct enough to divide these lineages into separate species. In addition, the similar intrageneric genetic distances were reported in other flying squirrel genera: 10.26–15.00% among major lineages of *Petaurista* (Oshida et al. 2004a) and 14.60% between two *Pteromys* species (Oshida et al. 2000b). Therefore, *Belomys* could not be monotypic genus and may include at least three distinct species. The phylogenetic trees show that the Taiwan lineage probably separated first from the other two lineages. The two Vietnamese lineages could have diverged on the continent, although it is difficult to discuss their phylogenetic history because our study had too few sample numbers and collecting localities.

Taiwan Island is thought to have formed around 6.0 million years ago (Yang 2001), but was sometimes connected with Asian mainland during the Pleistocene (e.g., Huang 1984; Liu and Ding 1984). Yu (1995) suggests that several mammal species migrated from Asian mainland to Taiwan during glacial periods, due to lowered sea levels. One *Belomys* population could have stocked the island for making distinct species. Phylogenetic characteristics of Taiwan's *Belomys* population could be similar to these of two *Petaurista* species occurring in Taiwan (Oshida et al. 2000a, 2000b, 2005). The present study could not include many *Belomys* specimens from many different places of Taiwan, because of difficulty in collecting specimens. Our current sample, however, indicates that multiple migrations of this species to Taiwan might not have occurred during the time of peak glaciation and lowered sea level in the Pleistocene.

Pollen records show that a large part of the exposed Taiwan Strait was either grassland or barren during the last glacial maximum (Liew et al. 1998). The arboreal

Belomys could not have immigrated from Asian mainland to Taiwan across such a treeless exposure during the last Pleistocene glaciation. Further phylogeographical study of *Belomys* population in Taiwan is needed.

Acknowledgments: We are grateful to Dr. Cara Lin Bridgman for her critical reading of the manuscript. We also thank Ms. Yen-Jean Chen (National Museum of Natural Science, Taiwan) for supplying specimen. This study was partly supported by the Grant-in-Aid for Scientific Researches 22405002, 25304005, and 26304009 from the Ministry of Education, Science, Sports, and Culture, Japan and by JSPS core-to-core program HOPE.

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Received 21 October 2014. Accepted 1 December 2014.
Editor was Masaharu Motokawa.