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Short communication

Phylogenetics of *Petaurista* in light of specimens collected from northern Vietnam

Tatsuo Oshida[1,*], Can Ngoc Dang[2], Son Truong Nguyen[2], Nghia Xuan Nguyen[2], Hideki Endo[3], Junpei Kimura[4], Motoki Sasaki[5], Akiko Hayashida[3], Ai Takano[6] and Yoshihiro Hayashi[7]

[1] *Laboratory of Wildlife Ecology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan*
[2] *Institute of Ecology and Biological Resources, Hanoi, Vietnam*
[3] *The University Museum, The University of Tokyo, Tokyo 113-0033, Japan*
[4] *College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea*
[5] *Laboratory of Veterinary Anatomy, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan*
[6] *United Graduate School of Agricultural Science and Veterinary Science, Gifu University, Gifu 501-1193, Japan; Department of Bacteriology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan*
[7] *Faculty of Agriculture, The University of Tokyo, Tokyo 113-8657, Japan*

The southern part of China and northern part of Indochina Peninsula is one of the hot-spots of biodiversity (e.g., Cincotta et al. 2000; Cox and Moore 2005). In this area, there are many endemic mammalian species, such as giant panda *Ailuropoda melanoleuca*, saola *Pseudoryx nghetinhensis*, Tonkin snub-nosed monkey *Rhinopithecus avunculus*, Inornate squirrel *Callosciurus inornatus*, black-eared red-backed vole *Eothenomys olitor*, and Yunnan hare *Lepus comus* (e.g., Wilson and Reeder 2005). Meijaard and Groves (2006) also found high mammalian diversity in the east side of the Mekong River including this area. Therefore, this area would be very important for the speciation and diversity of mammals in Asia.

Giant flying squirrels (genus *Petaurista*) are widely distributed throughout South and Southeast Asia and in southern China, Taiwan, and Japan (Corbet and Hill 1992; Wilson and Reeder 2005). At present, they are split into eight species: *P. alborufus*, *P. elegans*, *P. leucogenys*, *P. magnificus*, *P. nobilis*, *P. petaurista*, *P. philippensis*, and *P. xanthotis* (Wilson and Reeder 2005). In each species, many variations and synonyms are reported (e.g., Ellerman and Morrison-Scott 1951; Corbet and Hill 1992; Wilson and Reeder 2005). In the southern China and northern Indochina Peninsula, three giant flying squirrel species (*P. alborufus*, *P. petaurista*, and *P. philippensis*) are commonly found (Lekagul and McNeely 1988; Corbet and Hill 1992). Based on genetic variations and morphological characteristics, in southern China, Yu et al. (2006) recognized as distinct two additional species (*P. hainana* from Hainan Island and *P. yunanensis* from Yunnan), although these species had been classified as *P. philippensis* by Wilson and Reeder (2005). Thus, this area may also be a biodiversity hotspot for *Petaurista* species. Based on molecular data, Oshida et al. (2004a) proposed that *Petaurista* might have some geographical evolutionary units or groups. Southern China and the northern Indochina Peninsula may be the source of one geographical evolutionary unit of *Petaurista*. To explore this conjecture further, we examined the phylogenetic position of two *Petaurista* species collected from northern Vietnam, the Indian giant flying squirrel (*P. philippensis*) and the spotted giant flying squirrel (*P. elegans*). These two forms were phylogenetically compared with those from southern China and the island of Sumatra. Here, we discuss the phylogenetic relationships of *P. philippensis* and *P. elegans*.

Materials and methods

Specimens

We collected four *P. philippensis* and one *P. elegans* from northern Vietnam (Table 1, Fig. 1). These specimens are stocked in the Institute of Ecology and Biological Resources, Hanoi, Vietnam. Total genomic DNA was extracted from muscle tissues using the QuiaQuick Kit (QUIAGEN K.K., Tokyo).

*To whom correspondence should be addressed. E-mail: oshidata@obihiro.ac.jp

Table 1. Giant flying squirrel specimens examined in this study

Species name	Identity number or Code	Species name according to Wilson and Reeder (2005)	Collecting locality	Accession No.
P. albiventer	SP800	*P. petaurista*	Ayubia National Park, Pakistan	AB092612
P. alborufus	ALC1	*P. alborufus*	southern China*	AB092613
	ALC2		southern China*	AB092614
P. elegans	No. 5	*P. elegans*	Jambi, Sumatra Island, Indonesia	AB092610
	181		Son La, Vietnam	AB510036
P. grandis	PHG	*P. philippensis*	Nantou, Taiwan	AB092611
P. hainana	PHA**	*P. philippensis*	Hainan Island, China	DQ072108
P. lena	ALL1	*P. alborufus*	Nantou, Taiwan	AB092615
P. leucogenys	LEL1	*P. leucogenys*	Fukuoka, Japan	AB092616
	LEL2		Ehime, Japan	AB092617
	LEN1		Wakayama, Japan	AB092618
	LEN2		Nagano, Japan	AB092619
P. petaurista	PEM1	*P. petaurista*	Laos?*	AB092608
	PEM2		southern China*	AB092609
P. philippensis	PPH**	*P. philippensis*	Yunnan, China	DQ072107
	150		Sa Pa, Vietnam	AB510040
	151		Sa Pa, Vietnam	AB510039
	OS634		Ninh Binh, Vietnam	AB510038
	180		Son La, Vietnam	AB510037
P. xanthotis	PXA**	*P. xanthotis*	Gansu, China	DQ072111
P. yunanensis	PYU**	*P. philippensis*	Yunnan, China	DQ072110

Note: *Unknown exact collecting locality. **Codes correspond to those reported by Yu et al. (2006), all other identity numbers are for our private specimens.

PCR and DNA sequencing

The entire mitochondrial cytochrome *b* gene (1,140 bases) was amplified with polymerase chain reaction (PCR), using a primer set (L14724 5'-GATATGAAAA ACCATCGTTG-3' and H15910 5'-GATTTTTGGTTT ACAAGACCGAG-3') reported by Oshida et al. (2000a). Primer names are concordant with the light (L) or heavy (H) strand and the 3' end-position of primers in human mitochondrial DNA sequences (Anderson et al. 1981). The 50 µl of reaction mixture contained approximately 100 ng of genomic DNA, 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 (ABgene, Epsom, UK). 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, ABI PRISM 3100 Genetic Analyzer, Applied Biosystem, CA, USA) and 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. Purification of PCR products and sequencing were carried out with Mission Biotech Co. Ltd. (Taipei, Taiwan).

Sequence analysis

Sequences of *P. alborufus*, *P. petaurista*, *P. grandis*, *P. lena*, *P. hainana*, *P. yunanensis*, *P. elegans*, and *P. philippensis* reported previously were obtained from the DNA Data Bank of Japan (DDBJ) (Table 1). Although the species names, *hainana*, *grandis*, and *yunanensis* are not commonly used, we followed Allen (1940), Zhang et al. (1997), and Yu et al. (2006) in using these names in the present study. According to Thomas (1907), the species name *lena* is a name for a Taiwan subspecies of *P. alborufus*. The taxonomic status of *P. lena* as a species is supported by Corbet and Hill (1992) and Oshida et al. (2000a, 2000b, 2004a). To root phylogenetic trees, sequences of the hairy-footed flying squirrel *Belomys pearsonii* (accession number: AB126285) and the Siberian flying squirrel *Pteromys volans* (accession

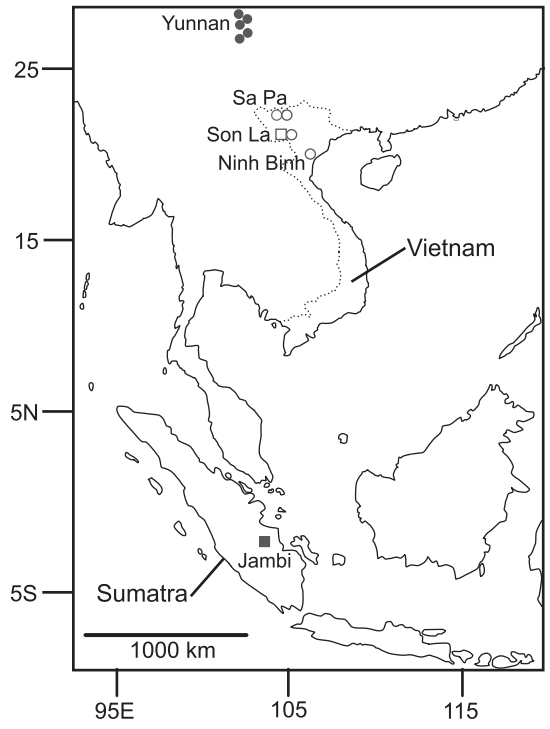

Fig. 1. Collecting localities of *Petaurista philippensis* and *P. elegans* examined in the present study. Each symbol indicates one individual. Solid circles are for five *P. philippensis* reported previously by Yu et al. (2006), which have the same haplotype. Open circles are for *P. philippensis* collected in the present study. The solid square is for *P. elegans* reported previously by Oshida et al. (2004a). The open square is for *P. elegans* collected in the present study. Dotted line shows the border between Vietnam and its neighbors.

number: AB097683) reported by Oshida et al. (2004b) were used as out-groups because Mercer and Roth (2003) reported that *Belomys* and *Pteromys* were phylogenetically grouped together with *Petaurista* in the subfamily Pteromynae on the basis of nucleotide sequence analyses of the interphotoreceptor retinoid-binding protein and 12S and 16S ribosomal RNA genes. 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 test selected the general time reversible (GTR) model of substitution (Rodríguez et al. 1990; Yang et al. 1994), took into account the proportion of invariable sites (0.5781), and followed a gamma distribution for variable sites (1.9107) (GTR + I + G). Base frequencies were estimated as A = 0.3037, C = 0.3227, G = 0.0952, and T = 0.2784. The rate matrix was estimated as A-C = 2.1613, A-G = 44.2228, A-T = 3.7487, C-G = 1.7558, C-T = 44.2228, and G-T of 1.0000. Using the genetic distances correlated by this model, we also conducted neighbor-joining (NJ) analysis (Saitou and Nei 1987). In addition, we made un-weighted maximum parsimony (MP) analysis. All phylogenetic analyses were performed by PAUP* 4.0b10 (Swofford 2001). The ML and MP trees were constructed with the heuristic search option with tree-bisection-reconnection (TBR). To assess the nodal supports, the bootstrapping (Felsenstein 1985) was carried out with 100 replications in ML analysis and 10,000 replications in MP and NJ analyses.

Results

Complete sequences (1,140 bases) of the cytochrome *b* gene of all specimens were determined. Uncorrected percentage sequence divergence (*p*-distances) and nucleotide substitutions among sequences are shown in Table 2. The *p*-distances among *Petaurista* species examined ranged from 3.86 to 15.53%. The *p*-distances between *P. philippensis* from Yunnan of China and those from northern Vietnam were from 1.23 to 1.75%. The *p*-distance between *P. elegans* from Jambi of Sumatra Island and that from northern Vietnam was 10.18%.

An ML search, assuming the GTR + I + G model of evolution, produced a single tree (Fig. 2). The branching patterns of NJ and MP trees were essentially similar to those of ML tree (data not shown). In these trees, there were five major lineages: 1) *P. alborufus*, *P. alvibenter*, *P. yunaensis*, *P. hainana*, and *P. philippensis*, 2) *P. elegans*, 3) *P. lena*, *P. grandis*, and *P. petaurista*, 4) *P. xanthotis*, and 5) *P. leucogenys*. In the first lineage, *P. albiventer* was closely related to *P. yunanensis* with high support values (98% in ML tree, 99% in MP tree, and 100% in NJ tree). The sub-linage consisting of *P. alborufus*, *P. hainana*, and *P. philippensis* also had with high bootstrap values (89% in ML tree, 91% in MP tree, and 100% in NJ tree). In the third lineage, *P. grandis* was most closely related to *P. petaurista* with high bootstrap values (96% in ML tree and 100% in MP and NJ trees). *Petaurista philippensis* from Vietnam were most closely related to that from southern China and had high bootstrap values (95% in ML tree and 100% in MP and NJ trees). *Petaurista elegans* from northern Vietnam was most closely related to that from Sumatra Island with high bootstrap values (100% in ML, NJ, and MP trees).

Table 2. Pairwise comparisons of cytochrome *b* nucleotide sequences (1,140 bp) among 21 *Petaurista* specimens

	albiventer (SP800)	*alborufus* (ALC1)	*alborufus* (ALC2)	*elegans* (No. 5)	*elegans* (181)	*grandis* (PHG)	*hainana* (PHA)	*lena* (ALL1)	*leucogenys* (LEL1)	*leucogenys* (LEL2)
albiventer (SP800)		10.35	10.35	14.74	13.25	13.16	9.74	14.12	13.86	13.60
alborufus (ALC1)	107/11		0.35	14.65	14.21	12.46	7.28	14.65	12.54	12.37
alborufus (ALC2)	108/10	3/1		14.83	14.39	12.63	7.19	14.74	12.72	12.54
elegans (No. 5)	145/23	143/24	146/23		10.18	13.16	14.39	14.30	15.00	14.91
elegans (181)	127/24	137/25	140/24	107/9		12.81	14.04	13.95	13.68	13.95
grandis (PHG)	123/27	116/26	119/25	120/30	115/31		12.19	11.58	12.90	13.07
hainana (PHA)	96/15	73/10	73/9	136/28	131/29	111/28		13.68	11.84	11.40
lena (ALL1)	138/23	145/22	147/21	135/28	128/31	116/16	130/26		13.25	12.90
leucogenys (LEL1)	133/25	117/26	120/25	139/32	127/29	123/24	105/30	129/22		1.40
leucogenys (LEL2)	129/26	116/25	119/24	139/31	131/28	126/23	101/29	126/21	13/3	
leucogenys (LEN1)	134/28	119/27	122/26	144/33	132/30	127/25	106/31	129/23	16/3	15/4
leucogenys (LEN2)	138/26	117/25	120/24	144/31	132/28	131/23	104/29	131/21	18/1	17/2
petaurista (PEM1)	126/26	117/27	120/26	128/29	124/32	36/9	108/27	106/15	123/25	124/24
petaurista (PEM2)	126/24	119/25	122/24	126/27	123/30	37/7	107/25	104/13	123/23	124/22
philippensis (PPH)	92/11	65/6	65/5	139/26	128/27	116/26	50/10	129/20	114/24	111/23
philippensis (150)	97/11	67/6	65/5	146/26	132/25	117/26	61/10	138/20	121/24	118/23
philippensis (151)	96/10	66/5	64/4	145/25	130/26	118/25	60/9	137/19	122/23	119/22
philippensis (OS634)	99/10	69/5	67/4	144/25	135/26	123/25	59/9	137/19	118/23	115/22
philippensis (180)	95/10	69/5	67/4	146/25	135/26	118/25	59/9	138/19	121/23	118/22
xanthotis (PXA)	125/19	130/22	134/21	124/28	122/29	104/20	111/26	122/18	102/22	99/22
yunanensis (PYU)	71/5	99/12	101/11	140/26	129/27	120/28	88/16	135/24	130/26	127/27

	leucogenys (LEN1)	*leucogenys* (LEN2)	*petaurista* (PEM1)	*petaurista* (PEM2)	*philippensis* (PPH)	*philippensis* (150)	*philippensis* (151)	*philippensis* (OS634)	*philippensis* (180)	*xanthotis* (PXA)	*yunanensis* (PYU)
albiventer (SP800)	14.21	14.39	13.33	13.16	9.04	9.47	9.30	9.56	9.21	12.63	6.67
alborufus (ALC1)	12.81	12.46	12.63	12.63	6.23	6.40	6.23	6.49	6.49	13.33	9.74
alborufus (ALC2)	12.98	12.63	12.81	12.81	6.14	6.14	5.97	6.23	6.23	13.60	9.83
elegans (No. 5)	15.53	15.35	13.77	13.42	14.47	15.09	14.91	14.83	15.00	13.33	14.56
elegans (181)	14.21	14.04	13.68	13.42	13.60	13.77	13.68	14.12	14.12	13.25	13.68
grandis (PHG)	13.33	13.51	3.95	3.86	12.02	12.54	12.54	12.98	12.54	10.88	12.98
hainana (PHA)	12.02	11.67	11.84	11.58	5.26	6.23	6.05	5.97	5.97	12.02	9.12
lena (ALL1)	13.33	13.33	10.61	10.26	13.07	13.86	13.68	13.68	13.77	12.28	13.95
leucogenys (LEL1)	1.67	1.67	12.98	12.81	12.11	12.72	12.72	12.37	12.63	10.88	13.68
leucogenys (LEL2)	1.67	1.67	12.98	12.81	11.75	12.37	12.37	12.02	12.28	10.53	13.51
leucogenys (LEN1)		0.88	13.07	12.90	12.37	12.98	12.98	12.81	12.90	11.32	13.95
leucogenys (LEN2)	8/2		13.25	13.07	12.02	12.63	12.63	12.46	12.54	10.97	13.86
petaurista (PEM1)	123/26	127/24		0.61	12.28	12.72	12.72	12.90	12.81	10.97	12.81
petaurista (PEM2)	123/24	127/22	5/2		12.11	12.54	12.54	12.72	12.63	10.61	12.81
philippensis (PPH)	116/25	114/23	115/25	115/23		1.75	1.58	1.67	1.23	12.98	8.16
philippensis (150)	123/25	121/23	120/25	120/23	18/2		0.18	1.49	0.61	13.68	9.12
philippensis (151)	124/24	122/22	121/24	121/22	17/1	1/1		1.32	0.44	13.51	8.95
philippensis (OS634)	122/24	120/22	123/24	123/22	18/1	16/1	15/0		1.23	13.16	8.68
philippensis (180)	123/24	121/22	122/24	122/22	13/1	6/1	5/0	14/0		13.60	8.95
xanthotis (PXA)	106/23	104/21	106/19	104/17	128/20	136/20	135/19	131/19	136/19		13.95
yunanensis (PYU)	130/29	131/27	119/27	121/25	81/12	92/11	91/11	88/11	91/11	137/22	

Data above the diagonal represent uncorrected percentage differences (*p*-distances). Data below the diagonal are the numbers of nucleotide substitutions (transitions/transversions). Identity number or code of each specimen are shown in parentheses.

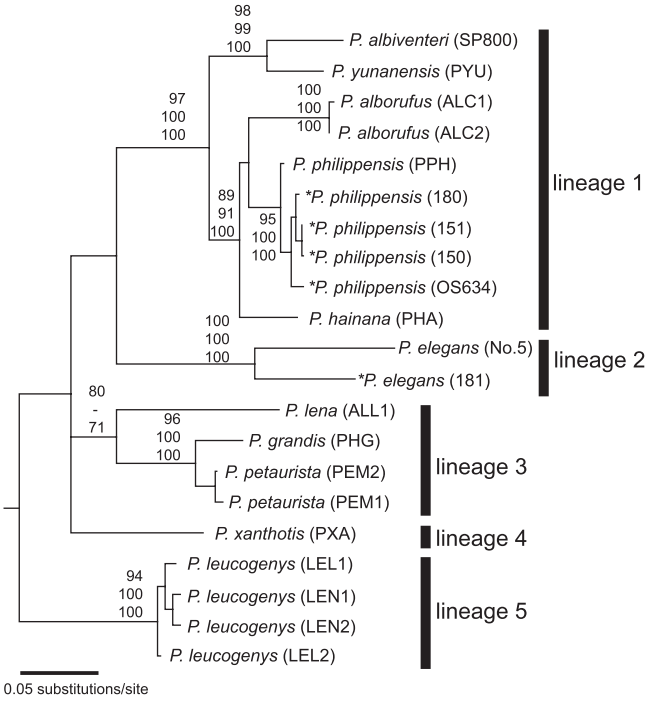

Fig. 2. Phylogeny of 11 *Petaurista* species constructed with the maximum-likelihood (ML) under GTR + I + G model for the cytochrome *b* sequences. Asterisks indicate specimens from northern Vietnam. Numbers above or under branches represent, from the top: bootstrap values from 100 replicates of ML analysis and from 10,000 replicates of un-weighted maximum parsimony (MP) and neighbor-joining (NJ) analyses. A hyphen means no data, because the clade was absent.

Discussion

Phylogenetic relationships among Petaurista species

Phylogenetic relationships among these 11 *Petaurista* species were essentially similar to those reported previously (Oshida et al. 2004a; Yu et al. 2006). We identified a lineage consisting of *P. alborufus*, *P. alvibenter*, *P. yunanensis*, *P. hainana*, and *P. philippensis* (Fig. 2). Oshida et al. (2001, 2004a) reported that *P. elegans* might be closely related to *P. alborufus*. We found in the present study that *P. elegans* was clustered with this lineage, but was not closely related to only *P. alborufus*. Therefore, we regarded *P. elegans* as a distinct linage. Also, *P. leucogenys* and *P. xanthosis* appeared to be each distinct linage. *Petaurista grandis*, *P. lena*, and *P. petaurista* were in one lineage (Fig. 2). The *p*-distances between *P. grandis* and *P. petaurista* were 3.86% and 3.95%, showing a very close relationship. *Petaurista grandis* is considered a subspecies of *P. philippensis* (Corbet and Hill 1992; Wilson and Reeder 2005). This form, however, could be regarded as a distinct species from *P. philippensis*, as Oshida et al. (2004a) and Yu et al. (2006) reported previously.

Phylogenetic relationship of P. philippensis

Petaurista philippensis is widely distributed in Sri Lanka, India, southern China, the Indochina Peninsula, and Taiwan (Wilson and Reeder 2005). The Taiwan form, *P. grandis*, however, is currently regarded as a distinct species (Oshida et al. 2004a; Yu et al. 2006) from *P. philippensis*. In Vietnam, this species occurs widely from north to south, but not in south of Long An (Lunde and Nguyen 2001; Dang et al. 2008). Currently, there are six subspecies (*annamensis*, *cineraceus, grandis*, *lylei*, *mergulus*, and *yunanensis*) in this species (Wilson and Reeder 2005). In our study, however, we recognize *grandis* and *yunanensis* as distinct species. It is difficult to precisely identify subspecies of *P. philippensis* because there are so many geographical variations. Based on the pelage color described by Corbet and Hill (1992), we tentatively identified Vietnam's specimens as *P. philippensis annamensis*. The *p*-distances between the southern China specimen and northern Vietnam specimens were 1.23 to 1.75%, showing moderate intraspecific genetic variation and suggesting that southern China and northern Vietnam populations are not geographically separated. To clarify the phylogenetic and taxonomic status of *P. philippensis*, we need to examine the remaining three subspecies: *cineraceus*, *lylei*, and *mergulus*.

Phylogenetic relationship of P. elegans

Petaurista elegans occurs in Nepal, India, southern China, northern and western Myanmar, Laos, northern Vietnam, Malay Peninsula, Sumatra Island, Java Island, and Borneo Island (Wilson and Reeder 2005). This species is confined to the northern part of Vietnam (Lunde and Nguyen 2001; Dang et al. 2008). Although we identified *P. elegans* from Vietnam as very closely related to that from Sumatra Island (Fig. 2), this *p*-distance between two forms was 10.18%. In the light of molecular systematics of mammals (Irwin et al. 1991), this *p*-distance is thought to be distinct enough to identify separate species. Geographical isolation between northern Vietnam population and Sumatra Island population may have resulted in speciation.

Petaurista elegans inhabits mountainous forests. In the Malay Peninsula, this species is found from 215 to 1,220 m elevation (Medway 1978). On Borneo Island, *P. elegans banksi* is known only in highlands: from the

Grand Kinabalu (1,070–1,080 m elevation) and the Crocker Range (1,140 m elevation) (Payne and Francis 1994). Our specimen from Vietnam was captured in mountainous forest at about 1,230 m elevation. Based on mtDNA cytochrome *b* gene sequences, Oshida et al. (2000a, 2004a) suggested that *P. lena*, which is confined to highlands (1,200–3,750 m elevation) in Taiwan (Chang 1985), might have independently evolved from other *Petaurista* species. Oshida et al. (2004a) suggested that the *P. albiventer* endemic to mountainous areas (1,353–3,050 m elevation) of northern Pakistan (Robert 1997) could have been isolated from other *Petaurista* species for a long time. Genetic differences among mammalian populations isolated in mountain highlands are often observed (Wettstein et al. 1995; Yu 1995; Osborne et al. 2000). Therefore, adaptation to mountainous forests may have promoted speciation in the *P. elegans* lineage, as well as in *P. lena* and *P. albiventer*.

Wilson and Reeder (2005) currently recognized six *elegans* subspecies (*banksi*, *caniceps*, *marica*, *punctatus*, *sumatrana*, and *sybilla*). Unfortunately, we could not precisely identify subspecific name of our specimen from northern Vietnam. According to its pelage coloration (Corbet and Hill 1992), it was thought to be *marica* or *sybilla*. Based on its place of origin, the specimen from Sumatra Island was considered *sumatrana*, but this identification was uncertain. Corbet and Hill (1992) regarded *caniceps* and *sybilla* as distinct species. Wilson and Reeder (2005), however, treated both forms as subspecies of *P. elegans*. Further taxonomic and phylogenetic studies may identify distinct species in the *P. elegans* complex.

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