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Authors: Oshida, Tatsuo, Lin, Liang-Kong, Masuda, Ryuichi, and Yoshida, Michihiro C.

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Phylogenetic Relationships among Asian species of *Petaurista* (Rodentia, Sciuridae), Inferred from Mitochondrial Cytochrome *b* Gene Sequences

Tatsuo Oshida^{1*}, Liang-Kong Lin², Ryuichi Masuda^{1,3} and Michihiro C. Yoshida^{1,3}

¹Chromosome Research Unit, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan,

²Laboratory of Wildlife Ecology, Department of Biology, Tunghai University, Taichung Taiwan 407, R. O. C., and

³Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan

ABSTRACT—To elucidate the phylogenetic relationships among four species belonging to the genus *Petaurista* (*P. alborufus castaneus*, *P. alborufus lena*, *P. leucogenys leucogenys*, *P. leucogenys nikkonis*, *P. petaurista melanotus*, and *P. philippensis grandis*), we investigated the partial sequences (1,068 bp) of the mitochondrial cytochrome *b* gene for these giant flying squirrels. Phylogenetic trees (NJ, MP, and ML trees) constructed from cytochrome *b* sequences indicated that *P. leucogenys* was grouped independently with other species, and that *P. philippensis* was most closely related to *P. petaurista* with 99–100% bootstrap values. In addition, two subspecies of *P. alborufus* did not form a single clade: *P. alborufus castaneus* from China was most distantly related to the other species, whereas *P. alborufus lena* from Taiwan was closely related to *P. petaurista* and *P. philippensis* with 82–90% bootstrap values. This result suggests that it is reasonable to regard *P. alborufus lena* as a distinct species from *P. alborufus castaneus*.

INTRODUCTION

Flying squirrels belonging to the genus *Petaurista* had been classified traditionally into five species: *P. alborufus*, *P. elegans*, *P. leucogenys*, *P. magnificus*, and *P. petaurista* (Corbet and Hill, 1980), each of which was intricately divided into various subspecies (Lekagul and McNeely, 1988). Recently, Corbet and Hill (1991, 1992) renewed the classification and recognized five additional species: *P. caniceps*, *P. nobilis*, *P. philippensis*, *P. sybilla*, and *P. xanthotis*, from five species classified previously (Corbet and Hill, 1980). Such classification disrupts phylogenetic study of the giant flying squirrels. Oshida *et al.* (1992) investigated the karyotaxonomy of *Petaurista* and concluded that *P. petaurista melanotus* was more closely related to *P. alborufus lena* than to *P. petaurista grandis* (*P. philippensis grandis*). In addition, Oshida *et al.* (1996) examined the mitochondrial 12S ribosomal RNA sequences on *P. leucogenys* from Japan, *P. petaurista* from Laos (*P. petaurista melanotus*), and *P. petaurista* from Taiwan (*P. philippensis grandis*), and reported that two subspecies of *P. petaurista* were closely related to each other and that *P. leucogenys* could have early diverged from *P. petaurista*.

Mitochondrial DNA (mtDNA) is a valuable molecule in investigating the phylogenetic relationships among populations, subspecies, and species. Features of mtDNA such as the maternal inheritance and rapid evolutionary rate advance the rapid geographic sorting of haplotypes in the absence of gene flow (Avise *et al.*, 1984). Accordingly, using the information of mtDNA, we are able to infer the interspecific relationships, the intraspecific situations of population subdivision, and the genetic differentiation beyond the resolving ability of non-molecular approaches. In the present study, we determined partial sequences (1,068 base pairs: bp) of the mitochondrial cytochrome *b* gene for four species: *P. alborufus*, *P. leucogenys*, *P. petaurista*, and *P. philippensis*, and discuss the phylogenetic relationships among them as well as the taxonomic status of *P. alborufus* from continental China and Taiwan.

MATERIALS

Flying squirrels examined in the present study are shown in Table 1. Classification of species and subspecies followed the description of Corbet and Hill (1991, 1992), Imaizumi (1960), and Lekagul and McNeely (1988). Two samples of *P. alborufus lena* and one individual of *P. philippensis grandis* were captured in central Taiwan. Three individuals of *P. alborufus castaneus* imported from Hong-Kong to Japan in 1996 were commercially obtained. Muscle tissues of two individuals of *P. leucogenys leucogenys* were provided from Mr. Koichi Ikeda of the Fukuoka Prefecture Forest Research and Extension Center, Fukuoka, Japan, and Mr. Takehito Okayama of the Omogo

* Corresponding author: Tel. +81-11-706-3541;
FAX. +81-11-736-6304.
E-mail: oshidata@sci.hokudai.ac.jp

Table 1. Species of the genera *Petaurista* and *Pteromys* examined in this study

Species name	Code	Common name	Collecting locality	Accession No. of sequence*
<i>Petaurista alborufus castaneus</i>	ALC1	Red and white giant flying squirrel	southern China	AB023898
	ALC2			AB023899
	ALC3			AB023900
<i>Petaurista alborufus lena</i>	ALL1	Japanese giant flying squirrel	Nantou, Taiwan	AB023901
	ALL2		Hualien, Taiwan	AB023902
<i>Petaurista leucogenys leucogenys</i>	LEL1		Ehime Pref., Japan	AB023903
	LEL2		Fukuoka Pref., Japan	AB023904
<i>Petaurista leucogenys nikkonis</i>	LEN1	Red giant flying squirrel	Wakayama Pref., Japan	AB023905
	LEN2		Aomori Pref., Japan	AB023906
<i>Petaurista petaurista melanotus</i>	PEM1		Laos	AB023908
	PEM2		southern China	AB023909
<i>Petaurista philippensis grandis</i>	PHG	Indian giant flying squirrel	Nantou, Taiwan	AB023907
<i>Pteromys volans orii</i> (out-group)	PVO	Russian (Siberian) flying squirrel	Hokkaido, Japan	AB023910

*Sequence data will appear in the DDBJ nucleotide sequence databases with accession numbers.

Mountain Museum, Ehime, Japan. The other *Petaurista* specimens were commercially obtained. The outgroup taxon, *Pteromys volans* was provided from the Noboribetsu Bear Park, Hokkaido, Japan.

METHODS

From homogenated muscle tissues, genomic DNAs were extracted according to the phenol/proteinase K/sodium dodecyl sulfate method of Sambrook *et al.* (1989). The whole region of the mtDNA cytochrome *b* gene was amplified with polymerase chain reaction (PCR) using the following two primers: L14724 5'-GATATGAAAAA-CCATCGTTG-3' and H15910 5'-GATTTTGGTTTACAAGACCGAG-3'. Primer names correspond to the light (L) or heavy (H) strand and the 3'-end-position of the primers in the human mtDNA sequence (Anderson *et al.*, 1981). The former was made from the sequence described by Kocher *et al.* (1989) and the latter was newly designed from the conservative sequences between human (Anderson *et al.*, 1981) and *Rattus norvegicus* (Gadaleta *et al.*, 1989). The 50 µl of reaction mixture contained 100 ng of genomic DNA, 25 picomoles of each primer, 200 µM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 2.5 units of *rTaq* DNA polymerase (Takara). Amplification was carried out for 35 cycles using the following cycle program: 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The extension reaction was completed by incubation at 72°C for 10 min. PCR products were purified with the Qia-quick PCR purification kit (QIAGEN) and directly sequenced using an automated DNA

sequencer (SQ5500, Hitachi).

Sequence alignment was carried out using GeneWorks (Intelligenetics). The phylogenetic trees were constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987) in Clustal W (Thompson *et al.*, 1994), using the maximum parsimony (MP) method with the branch and bound search algorithm (Hendy and Penny, 1982) in PAUP (Swofford, 1993), and using the maximum likelihood (ML) method with DNAML in PHYLIP package program (Felsenstein, 1993). In NJ and ML methods, numbers of nucleotide substitutions per site were estimated for multiple substitutions using the Kimura's two-parameter method (Kimura, 1980). To assess the confidence of branching, the bootstrap analyses (Felsenstein, 1985) were performed with 1,000 replications in NJ and MP methods and at 100 replications in ML method.

Based on 0.5%/million years (Myr) reported as the transversion divergence rate at the third codon positions of mammalian cytochrome *b* gene (Irwin *et al.*, 1991), divergence time was estimated.

RESULTS AND DISCUSSION

Phylogenetic relationships within the genus *Petaurista*

In this study, the partial sequences (1,068 bp) of cytochrome *b* gene were determined from 12 individuals of the genus *Petaurista* and one individual of the genus *Pteromys* as an out-group. Table 2 shows percentage sequence differ-

Table 2. Pairwise comparisons of cytochrome *b* nucleotide sequences (1,068 bp) between 13 flying squirrel specimens

	ALC1	ALC2	ALC3	ALL1	ALL2	LEL1	LEL2	LEN1	LEN2	PEM1	PEM2	PHG	PVO
ALC1		0.3	0.1	16.8	16.7	13.9	14.3	14.3	14.1	13.7	13.9	13.9	21.5
ALC2	3 / 0		0.4	16.9	16.8	14.3	14.6	14.6	14.5	13.9	14.1	14.2	21.5
ALC3	1 / 0	4 / 0		16.9	16.8	14.0	14.4	14.4	14.3	13.4	14.0	14.0	21.6
ALL1	135 / 20	136 / 20	136 / 20		1.2	14.7	15.3	15.0	15.2	12.1	12.9	13.2	23.8
ALL2	133 / 21	134 / 21	134 / 21	10 / 3		14.0	14.6	14.6	15.0	12.4	12.2	13.3	23.8
LEL1	111 / 21	114 / 21	112 / 21	119 / 19	113 / 20		1.0	1.4	1.5	14.5	14.5	14.6	20.5
LEL2	114 / 21	117 / 21	115 / 21	122 / 21	116 / 22			1.3	1.4	14.5	14.5	14.9	21.6
LEN1	113 / 22	116 / 22	114 / 22	121 / 20	117 / 21	15 / 1	13 / 1		0.7	14.4	14.4	14.5	20.6
LEN2	112 / 22	115 / 22	113 / 22	122 / 20	120 / 21	14 / 1	14 / 1	7 / 1		14.8	14.8	14.9	20.9
PEM1	108 / 22	110 / 22	109 / 22	104 / 12	106 / 13	116 / 21	114 / 23	114 / 22	117 / 22		0.5	3.8	20.5
PEM2	110 / 22	112 / 22	111 / 22	102 / 12	104 / 13	116 / 21	114 / 23	114 / 22	117 / 22	5 / 0		3.9	20.5
PHG	112 / 20	114 / 20	113 / 20	113 / 12	113 / 13	117 / 21	119 / 21	115 / 22	118 / 22	35 / 4	36 / 4		21.2
PVO	129 / 67	129 / 67	130 / 67	151 / 61	150 / 62	126 / 62	134 / 62	128 / 61	130 / 61	123 / 65	123 / 65	130 / 63	

Data above the diagonal represent percentage differences between specimens. Data below the diagonal are the numbers of nucleotide substitutions (transitions/transversions).

Table 3. Pairwise comparisons of the transversional substitution at the third codon positions of cytochrome *b* gene between 12 specimens of *Petaurista*

	ALC1	ALC2	ALC3	ALL1	ALL2	LEL1	LEL2	LEN1	LEN2	PEM1	PEM2	PHG
ALC1		0	0	4.2	4.5	4.5	4.5	4.8	4.8	5.6	5.6	5.1
ALC2	0		0	4.2	4.5	4.5	4.5	4.8	4.8	5.6	5.6	5.1
ALC3	0	0		4.2	4.5	4.5	4.5	4.8	4.8	5.6	5.6	5.1
ALL1	15	15	15		0.3	4.5	4.8	4.5	4.5	3.1	3.1	3.7
ALL2	16	16	16	1		4.5	5.1	4.8	4.8	3.4	3.4	3.9
LEL1	16	16	16	15	16		0.6	0.3	0.3	5.1	5.1	4.2
LEL2	16	16	16	17	18	2		0.3	0.3	5.3	5.3	4.2
LEN1	17	17	17	16	17	1	1		0	5.3	5.3	4.5
LEN2	17	17	17	16	17	1	1	0		5.3	5.3	4.2
PEM1	20	20	20	11	12	18	19	19	19		0	0.6
PEM2	20	20	20	11	12	18	19	19	19	0		0.6
PHG	18	18	18	13	14	15	15	16	16	2	2	

Data above the diagonal represent transversional percentage differences between specimens. Data below the diagonal are the numbers of transversions.

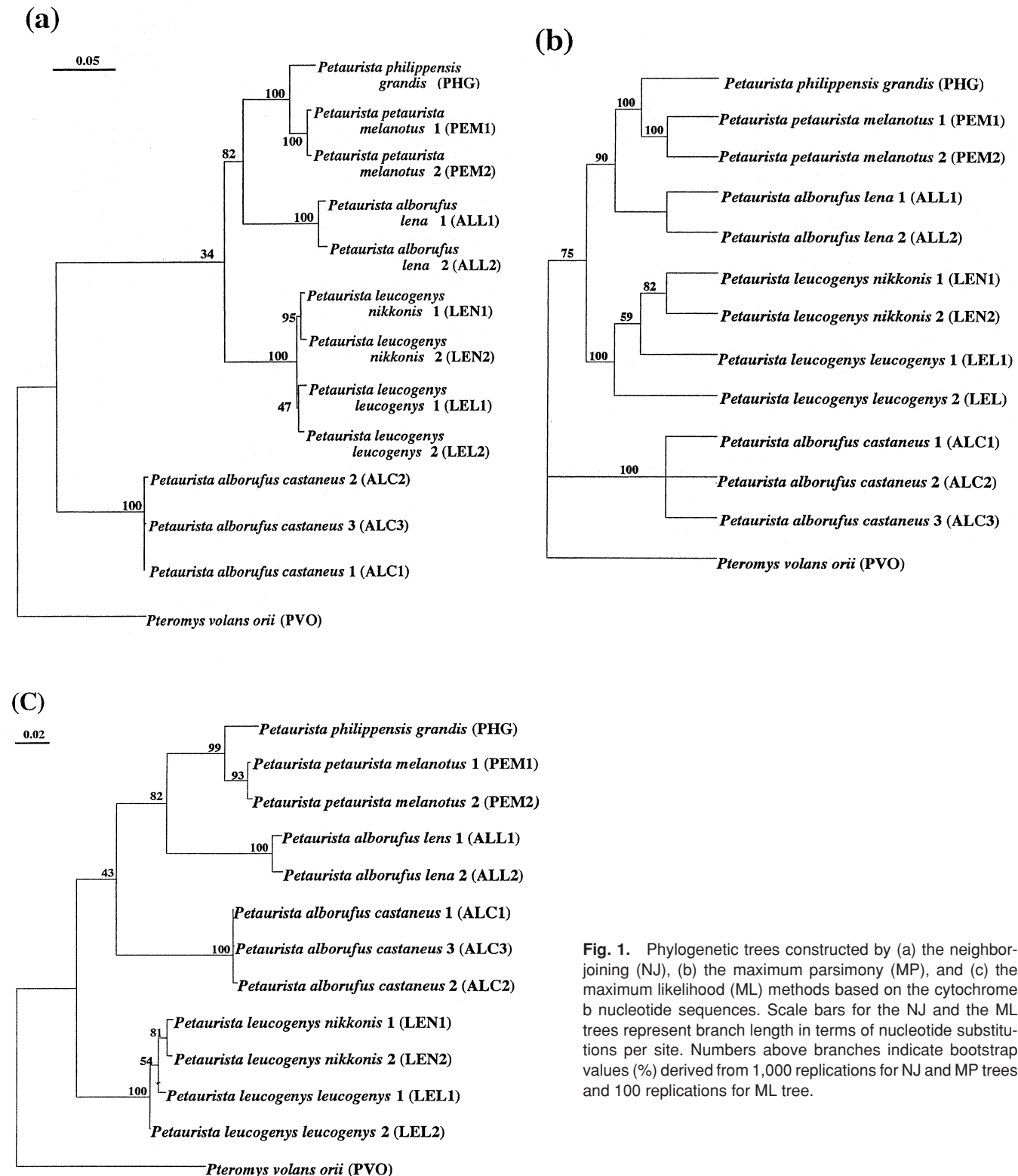
ences and numbers of transversions and transitions obtained from pairwise comparison. In addition, to estimate divergence between species, the transversional substitutions at the third codon positions were obtained by pairwise comparison (Table 3). Phylogenetic trees reconstructed using the NJ and the MP methods indicated essentially the same branching patterns (Figs. 1a and b): the first dichotomy isolated *P. alborufus castaneus* from the other flying squirrels and then *P. leucogenys* split from a clade formed by *P. alborufus lena*, *P. philippensis*, and *P. petaurista*. Consequently, the giant flying squirrels analyzed in the present study formed three groups: *P. alborufus castaneus*, *P. leucogenys*, and the other species or subspecies (82–90% bootstrap values). Although the branching order in the phylogenetic tree constructed with the ML method (Fig. 1c) was different from those of NJ and MP trees, three groups recognized in NJ and MP trees were obviously observed in ML tree (82–100% bootstrap values). However, the bootstrap values to support the branching orders of three groups were not high: 34% in NJ tree (Fig. 1a), 75% in MP tree (Fig. 1b), and 43% in ML tree (Fig. 1c). Owing to these low bootstrap values and the branching order differences between NJ and MP trees and ML tree, their phylogenetic relationships were not obvious in the present study.

Phylogeny of *Petaurista alborufus*

P. alborufus from southern China, Taiwan, Burma, and Thailand had been divided into seven subspecies: *barroni* (Kloss, 1916; Ellerman and Morrison-Scott, 1951), *castaneus* (Thomas, 1923), *candidula* (Wroughton, 1911; Ellerman and Morrison-Scott, 1951), *lena* (Thomas, 1907), *leucocephalus* (Hilzheimer, 1905), *ochraspis* (Thomas, 1923), and *taylori* (Thomas, 1914; Ellerman and Morrison-Scott, 1951). However, Corbet and Hill (1992) have recognized only four variations as subspecies of *P. alborufus*: *castaneus*, *lena*, *leucocephalus*, and *ochraspis*. In the present study, it is noteworthy that *P. alborufus castaneus* was distantly related to *P. alborufus lena* (Fig. 1). Based on pelage characteristics, *P. alborufus lena* was once treated as a distinct species *P. pectoralis* (Swinhoe, 1870). In addition, Corbet and Hill (1992)

also suggested that *P. alborufus lena* is distinct enough to merit specific rank. Phylogenetic trees (Fig. 1) obtained in the present study did not conflict with the phylogenetic position of the form *lena* proposed by Swinhoe (1870) and Corbet and Hill (1992). Assuming that *castaneus* distributed in the mainland is a representative subspecies of *P. alborufus*, *lena* living only in Taiwan may be regarded to be distinct from *P. alborufus*. Moreover, *P. alborufus lena* was closely related to the clade of *P. petaurista* and *P. philippensis* with high bootstrap values (82% in NJ tree of Fig. 1a, 90% in MP tree of Fig. 1b, and 82% in ML tree of Fig. 1c). Based on the morphological characteristics such as externals and dental forms, Corbet and Hill (1992) regarded three subspecies (*barroni*, *candidula*, and *taylori*) of *P. alborufus* as *P. petaurista*. *P. alborufus* has often been confused with *P. petaurista* owing to the complicated morphological variation of these species. Based on the chromosomal characteristics, Oshida *et al.* (1992) have reported that *P. alborufus lena* was more closely related to *P. petaurista melanotus* than to *P. petaurista grandis* (*P. philippensis grandis*). The present molecular findings support their view.

Hsu (1990) reported that the Taiwan island rose from the sea floor on the Eurasian Continent approximately 4.0 Myr ago. Moreover, based on the faunistic and geological analyses, Kano (1940) and Liu and Ding (1984) concluded that the Taiwan island had been connected with the Eurasian Continent at least twice due to the glacial eustasy, initially during the Pliocene and subsequently during the Pleistocene. It is likely that the multiple faunistic exchanges between the Taiwan island and the Eurasian Continent had occurred through these connections. Lin and Lin (1983) explained the complicated zoogeography of Taiwanese mammals as follows: from a paleoenvironmental point of view, the first mammal group which immigrated from the Eurasian Continent to the Taiwan island during the glacial period of the Pliocene had adapted themselves to the cold environment. However, after the glacial period, to avoid the environment being warm, they had to move to the high elevation areas of Taiwan. Subsequently, the mammal group which immigrated to the island during the



glacial periods of the Pleistocene had succeeded in expanding their ranges throughout the low elevation areas of Taiwan.

P. alborufus lina inhabits areas with elevation of 1,200 to 3,750 meters above sea level (Chang, 1985). In contrast, the distribution area of *P. philippensis grandis* living in Tai-

wan widely ranges from 700 to 2,600 meters above sea level (Chang, 1985). Accepting the hypothesis of Lin and Lin (1983), it is likely that the incursion of *P. alborufus lina* was earlier than that of *P. philippensis grandis*. In the present study, by using the available divergence rate estimated from the mammalian cytochrome *b* genes (Irwin *et al.*, 1991) and our data

(Table 3), the divergence between *P. alborufus lena* and *P. petaurista melanotus* and that between *P. alborufus lena* and *P. philippensis grandis* were estimated to have occurred approximately 6.2–6.8 Myr ago and 7.4–7.8 Myr ago, respectively. It seems reasonable to suppose that, after the deviation from the lineages of *P. petaurista* and *P. philippensis* in the Eurasian Continent during the late Miocene, *P. alborufus lena* immigrated to Taiwan island and adapted itself to the alpine region in Taiwan. *P. alborufus lena* might have evolved independently from other *Petaurista* species due to the geographic isolation. Thus, our results support that this giant flying squirrel should be treated as a distinct species, as described originally by Swinhoe (1870) and Thomas (1907).

Phylogeny of *Petaurista leucogenys*

P. leucogenys is indigenous to the Japanese main islands except for Hokkaido (Corbet and Hill, 1991). Although Corbet and Hill (1980) described that *P. leucogenys* is distributed on the Japanese islands and central China, recently they changed the classification and newly treated the *leucogenys* population of central China as a distinct species *P. xanthotis* (Corbet and Hill, 1991, 1992). Based on the poor information about the pelage, Imaizumi (1960) classified *P. leucogenys* into three subspecies: *leucogenys*, *nikkonis*, and *oreas*, although this classification of subspecies has not been generally accepted. In the present study, *P. leucogenys* clearly formed a single clade with high bootstrap values (100% in NJ, MP, and ML trees). Meanwhile, the divergence time estimated from our data (Table 3) was approximately 10.2–10.6 Myr ago between *P. leucogenys* and *P. petaurista*, 8.4–9.0 Myr ago between *P. leucogenys* and *P. philippensis*, 9.0–10.2 Myr ago between *P. leucogenys* and *P. alborufus lena*, and 9.0–9.6 Myr ago between *P. leucogenys* and *P. alborufus castaneus*. Although it is hard to determine which species is most closely related to *P. leucogenys*, this species could be an independent lineage in the genus *Petaurista* at least in the late Miocene. Based on the fossil records, Kawamura (1988, 1990) and Kawamura *et al.* (1989) showed that *P. leucogenys* immigrated from the Eurasian Continent to the Japanese islands through the land bridges around the early to the middle Pleistocene, and that this species had been isolated due to the separation of the Japanese islands from the Eurasian Continent in the Pleistocene. Accepting Kawamura's hypothesis, our results suggest that an ancestral stock of *P. leucogenys* had diverged from the other *Petaurista* species within the Eurasian Continent prior to its immigration to Japan.

Phylogeny of *Petaurista petaurista* and *P. philippensis*

P. petaurista melanotus and *P. philippensis grandis* formed a single clade with high bootstrap values (100% in NJ and MP trees and 99% in ML tree, Fig. 1). *P. petaurista*, which is one of the most dominant species in the genus *Petaurista*, is distributed throughout southern parts of the Eurasian Continent and Southeast Asia (Corbet and Hill, 1980; Lekagul and McNeely, 1988). On the other hand, *P. philippensis* had been treated as a subspecies of *P. petaurista* until Corbet and Hill

(1991, 1992) established it as a distinct species. A Taiwanese form (*P. philippensis grandis*) examined here was previously considered as *P. petaurista grandis* by Swinhoe (1870). Based on sequence data of the 12S rRNA gene, Oshida *et al.* (1996) reported that the genetic distance between *P. philippensis grandis* (*P. petaurista* from Taiwan) and *P. petaurista melanotus* (*P. petaurista* from Laos) was almost parallel to intraspecific differences within *P. leucogenys*. Cytochrome *b* data in the present study supported that, although the genetic distance between *P. philippensis grandis* and *P. petaurista melanotus* corresponded to approximately twice of intraspecific differences within *P. leucogenys* (Fig. 1a), *P. philippensis* is most closely related to *P. petaurista*.

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