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## Phylogenetic position of *Callosciurus erythraeus griseimanus* from Vietnam in the genus *Callosciurus*

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*Callosciurus* squirrels are widely distributed in Southeast Asia, eastern parts of South Asia, and southern parts of East Asia (Wilson and Reeder 2005). In this genus, there are 15 species; each species has many geographical forms (Corbet and Hill 1992; Wilson and Reeder 2005). Especially, at present, the Pallas's squirrel (*Callosciurus erythraeus*) has 25 subspecies (*atrodorsalis*, *bartoni*, *bhutanensis*, *bonhotei*, *castaneiventris*, *erythrogaster*, *flavimanus*, *gloveri*, *gordoni*, *griseimanus*, *harringtonii*, *hendeei*, *hyperythrus*, *intermedius*, *michianus*, *ningpoensis*, *pranis*, *rubeculus*, *shanicus*, *siamensis*, *sladeni*, *styani*, *thai*, *thaiwanensis*, and *zimmeensis*) (Wilson and Reeder 2005). These many geographical forms clearly make sub-specific classification of *C. erythraeus* complicated (e.g., Lekagul and McNeely 1988).

Of these subspecies, 18 (*atrodorsalis*, *bonhotei*, *castaneiventris*, *flavimanus*, *gloveri*, *gordoni*, *griseimanus*, *hendeei*, *michianus*, *ningpoensis*, *pranis*, *rubeculus*, *shanicus*, *siamensis*, *styani*, *thai*, *thaiwanensis*, and *zimmeensis*) were previously assigned to a distinct species, the belly-banded squirrel *C. flavimanus* (Ellerman and Morrison-Scott 1951; Moore and Tate 1965). Corbet and Hill (1992), however, regarded *C. flavimanus* as a subspecies of *C. erythraeus* (i.e., *C. erythraeus flavimanus*) and assigned all *C. flavimanus* subspecies to *C. erythraeus*. At present, this taxonomic status is generally accepted (Wilson and Reeder 2005). Debates on subspecific classification of *C. erythraeus* have been based on morphological characteristics, such as skull shapes and pelage patterns, but not on any other systematic characteristics, such as molecular and cytogenetic data. To provide

additional evidence for resolving the taxonomic and systematic status of *C. erythraeus* subspecies, we examined the phylogenetic position of *C. erythraeus griseimanus* among *Callosciurus* squirrel species by using mitochondrial (mt) cytochrome *b* gene sequences. *Callosciurus erythraeus griseimanus* is found in only the southeastern parts of the Indochina Peninsula. Judging from its restricted distribution pattern, this subspecies may be genetically distinct from other *C. erythraeus* subspecies and other *Callosciurus* species. We discuss here where we place this squirrel in the phylogeny of *Callosciurus*.

### Materials and methods

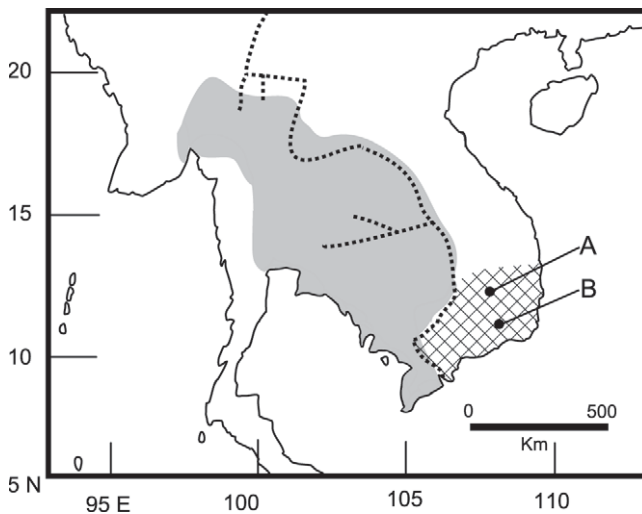
#### Specimens

We collected five *C. erythraeus griseimanus* specimens from southern Vietnam (Fig. 1 and Table 1), as the type specimen of this form is from near Ho Chi Minh (Milne-Edwards 1867). These squirrels have grayish backs and light buff ventral areas. According to description of Moore and Tate (1965), we identified these specimens as *C. erythraeus griseimanus*. These specimens are deposited 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).

#### PCR and DNA sequencing

The entire mitochondrial cytochrome *b* gene sequence (1,140 bases) was amplified with polymerase chain reaction (PCR), using primer set: L14724 5'-GATAT

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**Fig. 1.** Distribution of *Callosciurus erythraeus griseimanus* (in cross-hatched area, Moore and Tate 1965) and *C. finlaysonii* (in light gray, Francis 2008). Solid circles indicate collecting localities of *C. e. griseimanus* examined in the present study: A) Cat Tien National Park and B) Vinh Cuu Nature Reserve. Dotted line shows the Mekong River.

**Table 1.** Squirrel specimens examined in this study and retained in our private collections preserved in Laboratory of Wildlife Biology, Obihiro University of Agriculture and Veterinary Medicine (indicated by identity numbers)

Species name with code	Common name	Collecting locality	Identity number	Accession No.
<i>Callosciurus caniceps</i> 1	gray-bellied squirrel	Pasoh Forest Reserve, Negeri Sembilan, Malaysia	Y3	AB499918
<i>Callosciurus caniceps</i> 2	gray-bellied squirrel	Pasoh Forest Reserve, Negeri Sembilan, Malaysia	Y25	AB499919
<i>Callosciurus erythraeus</i> cf. <i>hendeei</i> 1	Pallas's squirrel	Tam Dao, Vietnam	33	AB499908
<i>Callosciurus erythraeus</i> cf. <i>hendeei</i> 2	Pallas's squirrel	Tam Dao, Vietnam	34	AB499909
<i>Callosciurus erythraeus griseimanus</i> 1	belly-banded squirrel	Cat Tien National Park, Vietnam	OS341	AB716958
<i>Callosciurus erythraeus griseimanus</i> 2	belly-banded squirrel	Cat Tien National Park, Vietnam	63	AB716959
<i>Callosciurus erythraeus griseimanus</i> 3	belly-banded squirrel	Cat Tien National Park, Vietnam	64	AB716960
<i>Callosciurus erythraeus griseimanus</i> 4	belly-banded squirrel	Vinh Cuu Nature Reserve, Vinh Cuu, Vietnam	186	AB716961
<i>Callosciurus erythraeus griseimanus</i> 5	belly-banded squirrel	Vinh Cuu Nature Reserve, Vinh Cuu, Vietnam	187	AB716962
<i>Callosciurus finlaysonii</i> 1	Finlayson's squirrel	Thailand*	OS254	AB499910
<i>Callosciurus finlaysonii</i> 2	Finlayson's squirrel	Thailand*	OS255	AB499911
<i>Callosciurus inornatus</i> 1	Inornate squirrel	Co Ma, Thuan, Chau, Son La, Vietnam	183	AB499905
<i>Callosciurus inornatus</i> 2	Inornate squirrel	Hon, Phu Yen, Son La, Vietnam	191	AB499906
<i>Callosciurus inornatus</i> 3	Inornate squirrel	Hon, Phu Yen, Son La, Vietnam	199	AB499907
<i>Callosciurus nigrovittatus</i> 1	black-striped squirrel	Pasoh Forest Reserve, Negeri Sembilan, Malaysia	Y26	AB499916
<i>Callosciurus nigrovittatus</i> 2	black-striped squirrel	Pasoh Forest Reserve, Negeri Sembilan, Malaysia	Y28	AB499917
<i>Callosciurus notatus</i> 1	plantain squirrel	Pasoh Forest Reserve, Negeri Sembilan, Malaysia	Y11	AB499912
<i>Callosciurus notatus</i> 2	plantain squirrel	Pasoh Forest Reserve, Negeri Sembilan, Malaysia	Y12	AB499913
<i>Callosciurus prevostii</i> 1	Prevost's squirrel	Palembang, Indonesia	OS311	AB499914
<i>Callosciurus prevostii</i> 2	Prevost's squirrel	Palembang, Indonesia	OS312	AB499915
<i>Lariscus insignis</i>	three-striped ground squirrel	Pasoh Forest Reserve, Negeri Sembilan, Malaysia	Y8	AB499904

Numbers with species name correspond to those in Table 2 and Fig. 2. \*Unknown exact collecting locality.

GAAAAACCATCGTTG-3' and H15910 5'-GATTTTTG GTTACAAGACCGAG-3'. The former and latter were reported by Kocher et al. (1989) and Oshida et al. (2000), respectively. The 50  $\mu$ l reaction mixture contained approximately 100 ng of genomic DNA, 25 pM of each primer, 200  $\mu$ M dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 2.5 units of *rTaq* 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 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 by Mission Biotech Co. Ltd. (Taipei, Taiwan).

#### Sequence analysis

Sequences of *C. caniceps*, *C. erythraeus* from northern Vietnam, *C. finlaysonii*, *C. inornatus*, *C. notatus*, and *C. prevostii* (reported previously by Oshida et al. (2011)) were obtained from the DNA Data Bank of Japan (DDBJ) (Table 1). Unlike *C. erythraeus griseimanus*, *C. erythraeus* from northern Vietnam had a reddish ventral area. Although we tentatively regarded this form as *C. erythraeus hendeei* according to Lunde and Nguyen (2001), it was difficult to determine its exact subspecific name. Therefore, in the present study, we treated this form as *C. erythraeus* cf. *hendeei*, not as to *C. erythraeus griseimanus*. To root phylogenetic trees, the sequence of the three-striped ground squirrel *Lariscus insignis*, reported by Oshida et al. (2011), was used as out-group. Since Mercer and Roth (2003) reported that *Lariscus* was most closely related to *Callosciurus*, it is a suitable out-group for resolving phylogenetic relationships among *Callosciurus* species. 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 (0.5587), and

followed a gamma distribution for variable sites (2.5339) (GTR + I + G). Base frequencies were estimated as A = 0.2755, C = 0.3280, G = 0.1184, and T = 0.2781. The rate matrix was estimated as A-C = 2.1941, A-G = 31.0463, A-T = 5.0941, C-G = 0.6392, C-T = 51.7451, 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. The ML and MP trees were constructed with a heuristic search option with tree-bisection-reconnection (TBR). To assess nodal supports, bootstrapping (Felsenstein 1985) was carried out with 200 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 (BS) reconstruction was carried out using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Bayesian analysis was conducted using the GTR substitution model selected by MODELTEST for our data set. The analysis involved two runs for one million iterations, using four Markov chain Monte Carlo (MCMC) chains sampling every 1,000 generations and a burn-in of 20%. A 50% majority rule consensus trees based on the remaining trees were generated. Posterior probabilities were used to assess nodal support of the BS tree. To estimate divergence time between *C. erythraeus griseimanus* and any closely related *Callosciurus* species, we used the transversal divergence rate (0.5%/million year) at the third codon position of mammalian cytochrome *b* gene (Irwin et al. 1991).

## Results

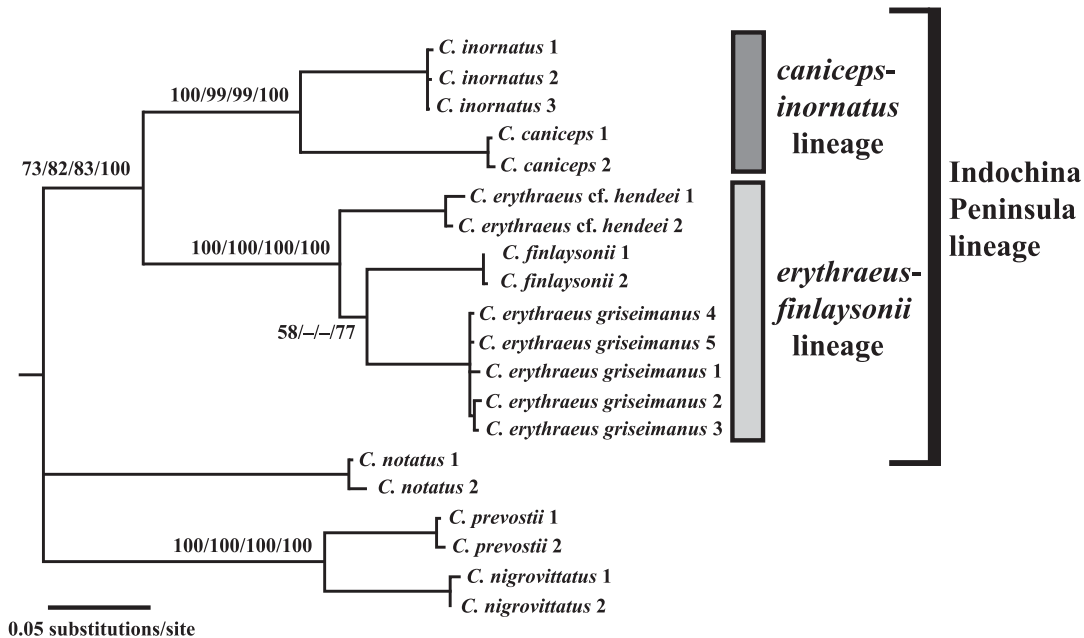
Complete sequences (1,140 bases) of the cytochrome *b* gene of all specimens were determined. Uncorrected percentage sequence divergence (*p*-distances) and nucleotide substitutions among sequences are shown in Table 2. The *p*-distances among *Callosciurus* species examined ranged from 10.53 to 17.46%. The *p*-distances between *C. e. griseimanus* and *C. finlaysonii* were 8.16 to 8.77%, showing that *C. finlaysonii* was most closely related to *C. e. griseimanus*. The *p*-distances between *C. e. griseimanus* and *C. erythraeus* cf. *hendeei*, were from 8.25 to 9.04%.

An ML search, assuming the GTR + I + G model of evolution, produced a single tree (Fig. 2). Branching patterns of NJ, MP, and BS trees were essentially similar to those of the ML tree (data not shown). In these trees, there were three major lineages: 1) *C. caniceps*, *C.*

**Table 2.** Pairwise comparisons of cytochrome *b* nucleotide sequences (1,140 bp) between 20 *Callosciurus* squirrel specimens

Species code	<i>caneiceps</i> 1	<i>caneiceps</i> 2	<i>erythraeus</i> cf. <i>hondaei</i> 1	<i>erythraeus</i> cf. <i>hondaei</i> 2	<i>erythraeus</i> <i>griseimanus</i> 1	<i>erythraeus</i> <i>griseimanus</i> 2	<i>erythraeus</i> <i>griseimanus</i> 3	<i>erythraeus</i> <i>griseimanus</i> 4	<i>erythraeus</i> <i>griseimanus</i> 5	<i>filipsonii</i> 1	<i>filipsonii</i> 2	<i>inornatus</i> 1	<i>inornatus</i> 2	<i>inornatus</i> 3	<i>nigrovittatus</i> 1	<i>nigrovittatus</i> 2	<i>notatus</i> 1	<i>notatus</i> 2	<i>prevostii</i> 1	<i>prevostii</i> 2
<i>caneiceps</i> 1		0.53	15.79	15.61	15.53	15.53	15.53	15.26	14.74	14.83	10.61	10.61	10.61	10.53	16.93	16.84	16.40	16.67	17.28	17.46
<i>caneiceps</i> 2	6/0		15.61	15.44	15.35	15.18	15.35	15.09	14.74	14.83	10.79	10.79	10.79	10.70	16.67	16.58	16.14	16.58	17.02	17.19
<i>erythraeus</i> cf. <i>hondaei</i> 1	148/32	146/32		9.04	8.95	8.77	8.51	8.51	8.95	9.04	13.51	13.60	13.60	13.60	16.14	16.14	15.44	15.18	15.97	16.32
<i>erythraeus</i> cf. <i>hondaei</i> 2	146/32	144/32	1.14		8.68	8.51	8.25	8.25	8.51	8.68	13.77	13.68	13.68	13.68	16.14	16.14	15.44	15.35	16.14	16.32
<i>erythraeus</i> <i>griseimanus</i> 1	150/28	148/28	91/12	88/12		0.97	0.79	0.61	0.53	8.33	8.51	13.33	13.42	13.42	16.05	16.05	15.97	15.70	15.79	16.14
<i>erythraeus</i> <i>griseimanus</i> 2	150/27	148/27	91/11	88/11	10/1		0.35	0.70	0.61	8.60	8.77	13.42	13.25	13.33	16.23	16.23	15.70	15.44	15.61	15.97
<i>erythraeus</i> <i>griseimanus</i> 3	150/27	146/27	91/11	88/11	8/1	4/0		0.44	0.44	8.42	8.60	13.25	13.07	13.16	16.05	16.05	15.53	15.26	15.44	15.79
<i>erythraeus</i> <i>griseimanus</i> 4	150/27	148/27	89/11	86/11	6/1	8/0	6/0		0.26	8.25	8.42	13.25	13.07	13.16	16.23	16.23	15.88	15.61	15.61	15.97
<i>erythraeus</i> <i>griseimanus</i> 5	147/27	145/27	86/11	83/11	5/1	7/0	5/0	3/0		8.16	8.33	12.98	12.81	12.90	16.00	15.97	15.61	15.35	15.53	15.88
<i>filipsonii</i> 1	139/29	139/29	91/11	86/11	90/5	92/6	90/6	87/6	87/6		0.18	14.83	14.65	14.74	16.93	16.93	16.58	16.32	16.58	16.93
<i>filipsonii</i> 2	139/29	139/29	90/13	86/13	90/7	92/8	90/8	87/8	87/8	0/2		14.74	14.56	14.65	16.93	16.93	16.58	16.32	16.58	16.93
<i>inornatus</i> 1	111/10	113/10	116/38	119/38	120/34	120/33	118/33	115/33	134/35	133/35	133/35		0.35	0.26	16.14	16.14	15.97	16.23	16.84	17.19
<i>inornatus</i> 2	112/9	114/9	118/37	119/37	119/33	119/32	117/32	114/32	133/34	132/34	3/1			0.09	16.14	16.14	15.79	16.05	16.84	17.02
<i>inornatus</i> 3	111/9	113/9	118/37	119/37	120/33	120/32	118/32	115/32	134/34	133/34	2/1	1/0			16.05	16.05	15.88	16.14	16.93	17.11
<i>nigrovittatus</i> 1	157/36	154/36	138/46	138/46	139/44	140/45	138/45	137/45	150/43	150/43	140/44	141/43	140/43		0.44	0.44	15.61	15.35	8.77	8.95
<i>nigrovittatus</i> 2	156/36	153/36	138/46	138/46	139/44	140/45	138/45	137/45	150/43	150/43	140/44	141/43	140/43	5/0			15.61	15.35	8.68	8.86
<i>notatus</i> 1	155/32	155/32	136/40	136/40	146/36	144/35	142/35	143/35	154/35	154/35	144/38	143/37	144/37	142/36	142/36		0.88	0.88	15.00	15.35
<i>notatus</i> 2	158/32	157/32	133/40	135/40	143/36	141/35	139/35	140/35	151/35	151/35	147/38	146/37	147/37	139/36	139/36	10/0			15.44	15.79
<i>prevostii</i> 1	163/34	160/34	136/46	138/46	136/44	133/45	131/45	132/45	146/43	146/43	150/42	151/41	152/41	93/6	93/6	139/32	144/32			0.53
<i>prevostii</i> 2	165/34	162/34	140/46	140/46	140/44	137/45	135/45	136/45	150/43	150/43	154/42	153/41	154/41	96/6	95/6	143/32	148/32	6/0		

Data above the diagonal represent uncorrected percentage differences (*p*-distances). Data below diagonal are numbers of nucleotide substitutions (transitions/transversions).



**Fig. 2.** Phylogeny of *Callosciurus* species constructed with the maximum-likelihood (ML) under GTR + I + G model for the cytochrome *b* sequences. From the left, numbers above branches represent: bootstrap values from 200 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. Hyphens mean no data because clades were absent.

*erythraeus* cf. *hendeei*, *C. e. griseimanus*, *C. finlaysonii*, and *C. inornatus*; 2) *C. notatus*; and 3) *C. nigrovittatus* and *C. prevostii*. This phylogenetic relationship was essentially similar to that without *C. e. griseimanus* presented by Oshida et al. (2011).

The first lineage included *Callosciurus* species occurring only in Indochina Peninsula (Indochina Peninsula lineage) (Fig. 2). This lineage was supported with high support values (73% in ML tree, 82% in MP tree, 83% in NJ tree and 100% in BS tree). In this lineage, a sub-lineage consisting of *C. erythraeus* cf. *hendeei*, *C. e. griseimanus*, and *C. finlaysonii* was significantly supported with 100% support values in all trees (Fig. 2). Divergence time between *C. erythraeus* cf. *hendeei* and *C. e. griseimanus* and that between *C. erythraeus* cf. *hendeei* and *C. finlaysonii* was 1.6–1.8 and 1.9–2.1 million years ago (Ma), respectively. Divergence time between *C. e. griseimanus* and *C. finlaysonii* was 0.7–1.2 Ma.

## Discussion

Phylogenetic relationships among *Callosciurus* species were essentially similar to those reported previously (Oshida et al. 2011). Therefore, we do not discuss the phylogenetic relationships among all *Callosciurus* species

used in the present study. We focus on our new finding: the phylogenetic relationships among *C. erythraeus* cf. *hendeei*, *C. e. griseimanus*, and *C. finlaysonii*. We found that the genetic distance between *C. erythraeus* cf. *hendeei* and *C. e. griseimanus* was similar to the genetic distances among different *Callosciurus* species. Based on mammalian cytochrome *b* sequences, Bradley and Baker (2001) reported that the distance values between 2 and 11% had a high probability of being indicative of conspecific populations or valid species. Therefore, the *p*-distance between *C. erythraeus* cf. *hendeei* and *C. e. griseimanus* is thought to be distinct enough to identify separate species. The *griseimanus* form could be considered a distinct species.

In ML and BS trees, *C. e. griseimanus* seemed more closely related to *C. finlaysonii*, although the support value in ML tree was not high (58%) (Fig. 2). In MP and NJ trees, however, this relationship did not appear. Therefore, we are not able to resolve this relationships among *C. erythraeus* cf. *hendeei*, *C. finlaysonii*, and *C. e. griseimanus*. Genetic distances among three forms are very similar, showing a polytomic phylogenetic relationship. Based on the transversal divergence rate at the third codon position of the cytochrome *b* gene sequence, however, *C. e. griseimanus* appeared most closely related to *C. finlaysonii*. Divergence time between *C. erythraeus*

cf. *hendeei* and *C. e. griseimanus* was 1.6–1.8 Ma. This was similar to divergence time between *C. erythraeus* cf. *hendeei* and *C. finlaysonii*: 1.9–2.1 Ma. Divergence time between *C. e. griseimanus* and *C. finlaysonii* was 0.7–1.2 Ma, suggesting a more recent divergence event. Therefore, first *C. erythraeus* cf. *hendeei* may have separated from the other two forms. Then *C. e. griseimanus* and *C. finlaysonii* may have separated.

At present, *C. e. griseimanus* is distributed in the southeastern parts of the Indochina Peninsula (Moore and Tate 1965; Timmins and Duckworth 2008) and *C. finlaysonii* occurs in the south central part of this peninsula (Francis 2008), showing their parapatric distribution (Fig. 1). The contact zone between both forms, however, is still unclear (Fig. 1), because the distribution of *C. e. griseimanus* is not yet studied in detail in Cambodia and Laos. This undefined contact zone makes it difficult to identify the trigger separating these two forms. Oshida et al. (2001, 2011) suggested the Mekong River as a possible geographical barrier for speciation within the *Callosciurus*. Oshida et al. (2011) specifically suggested that *C. caniceps* and *C. inornatus* were divided by this river in the northern Indochina Peninsula. After 1.5 Ma, the course of the Mekong River shifted eastwards to its present course towards Vientiane, Laos (Meijaard and Groves 2006). This huge river should be enough of a geographical barrier to cause speciation. Many forest mammal species are restricted to the east side of the Mekong River, such as Owston's palm civet (*Chrotogale owstoni*), pygmy slow loris (*Nycticebus pygmaeus*), redthroated squirrel (*Dremomys gularis*), and Tonkin snub-nosed monkey (*Rhinopithecus avunculus*) (Meijaard and Groves 2006). Since the divergence time between *C. finlaysonii* and *C. e. griseimanus* is estimated to be 0.7–1.2 Ma, speciation of these two forms may have been caused by the present course of the Mekong River. The riverine barrier hypothesis may explain the evolutionary history of these two *Callosciurus* forms in the southern Indochina Peninsula.

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