

PHYLOGENETIC RELATIONSHIPS
AMONG CALLOSCIURUS SQUIRRELS
IN THE INDOCHINA PENINSULA: PHYLOGENETIC POSITION
OF *C. PYGERYTHRUS* FROM MYANMAR

TATSUO OSHIDA¹, WYNN THAN², THIDA OO², KHIN YU YU SWE³
HIROAKI SAITO⁴, MASAHARU MOTOKAWA⁵, JUNPEI KIMURA⁶, SON TRUONG NGUYEN⁷
HAI TUAN BUI⁸ and PHUONG HUY DANG⁹

- ¹Laboratory of Wildlife Ecology, Obihiro University of Agriculture and Veterinary Medicine
Obihiro 080-8555, Japan; E-mail: oshidata@obihiro.ac.jp; <https://orcid.org/0000-0003-0863-9530>
- ²Zoology Department, West Yangon University, Htantabin Township, Yangon 11401, Myanmar;
E-mails: demniswynn@gmail.com, <https://orcid.org/0000-0002-2236-8899>
prof.thidaoo@gmail.com, <https://orcid.org/0000-0002-7941-610X>
- ³Zoology Department, Mandalay University, Amarapura Township, Mandalay 0561, Myanmar;
E-mail: yuyuswe.dr@gmail.com, <https://orcid.org/0000-0001-7417-5952>
- ⁴Graduate School of Science, Kyoto University, Kyoto 606-8501, Japan
E-mail: keiryu.fishing@gmail.com, <https://orcid.org/0000-0002-6488-9887>
- ⁵The Kyoto University Museum, Kyoto University, Kyoto 606-8501, Japan
E-mail: motokawa@e23.jp, <https://orcid.org/0000-0002-5359-0070>
- ⁶Seoul National University, Seoul 151-742, Korea
E-mail: jay.kimura@me.com, <https://orcid.org/0000-0003-3130-9716>
- ⁷Department of Vertebrate Zoology, Institute of Ecology and Biological Resources
Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Street, Hanoi, Vietnam
E-mail: truongsoneibr@gmail.com, <https://orcid.org/0000-0003-3214-4407>
- ⁸Vietnam National Museum of Nature, Vietnam Academy of Science and Technology
18 Hoang Quoc Street, Hanoi, Vietnam
E-mail: tuanhai@eulipotlyphla.com, <https://orcid.org/0000-0003-4065-7229>
- ⁹Me Linh Station for Biodiversity, Institute of Ecology and Biological Resources
Vietnam Academy of Science and Technology, 18 Hoang Quoc Street, Hanoi, Vietnam
E-mail: phuongiebr@gmail.com, <https://orcid.org/0000-0002-2323-9511>

The phylogenetic relationships among seven *Callosciurus* species from the Indochina Peninsula, including the *C. honkhoaiensis* which is endemic to Hon Khoai Island, were studied using complete mitochondrial cytochrome *b* gene sequences (1140 bases). We primarily focused on the phylogenetic position of *C. pygerythrus*, which is distributed in the western part of the peninsula. We identified two main lineages: 1) *C. caniceps*, *C. honkhoaiensis*, *C. inornatus*, *C. phayrei* and *C. pygerythrus*, and 2) *C. erythraeus* and *C. finlaysonii*. Estimated divergence time between the two lineages was at the junction of the Zanclean and Piacenzian in the Pliocene. Within the first lineage, the divergence time of sub-lineages corresponded to the Pliocene-Pleistocene boundary, although phylogenetic relationships were unclear. These two divergence times estimated in the present study correspond to episodes of global cooling, suggesting that climate may have contributed to the divergence of these *Callosciurus* squirrels.

Keywords: cytochrome *b*, global cooling, Irrawaddy squirrel, Phayre's squirrel.

INTRODUCTION

The Indochina Peninsula of Southeast Asia is a biodiversity hotspot (Cox & Moore 2005, Mittermeier *et al.* 2011), harbouring many endemic mammals, such as saola (*Pseudoryx nghetinhensis*), Tonkin snub-nosed monkey (*Rhinopithecus avunculus*), Delacour's langur (*Trachypithecus delacouri*) and southern white-cheeked gibbon (*Nomascus siki*) (Groves 2005, Grubb 2005). Recently, new bat species (*Myotis indochinensis* and *Murina kontumensis*) have been reported from this region (Nguyen *et al.* 2013, 2015), further highlighting the importance of this region in understanding the evolutionary forces driving mammalian speciation.

Arboreal squirrels, such as *Callosciurus* Gray, 1867, are good models to better understanding the patterns of speciation in the Indochina Peninsula because of their high diversity (Oshida *et al.* 2011). Out of 15 *Callosciurus* species in Southeast Asia (Koprowski *et al.* 2016, Nguyen *et al.* 2018), seven occur in the Indochina Peninsula and adjacent islands: grey-bellied squirrel (*C. caniceps* (Gray, 1842)), Pallas's squirrel (*C. erythraeus* (Pallas, 1779)), Finlayson's squirrel (*C. finlaysonii* (Horsfield, 1823)), Hon Khoai squirrel (*C. honkhoaensis* Nguyen, Oshida, Dang, Bui et Motokawa, 2018), Inornate squirrel (*C. inornatus* (Gray, 1867)), Phayre's squirrel (*C. phayrei* (Blyth, 1856)) and Irrawaddy squirrel (*C. pygerythrus* (I. Geoffroy Saint Hilaire, 1833)) (Corbet & Hill 1992, Thorington & Hoffmann 2005, Nguyen *et al.* 2018). Boonkhaw *et al.* (2017) reported that species from the Indochina Peninsula form monophyletic group consisting of two major lineages: *erythraeus-finlaysonii* lineage and *caniceps-inornatus-phayrei* lineage. Furthermore, Nguyen *et al.* (2018) reported that *C. honkhoaensis*, which is endemic to Hon Khoai Island located off the Indochina Peninsula, was closely related to *C. caniceps*.

In the Indochina Peninsula, high divergences of *Callosciurus* squirrels are recognized. Based on mitochondrial DNA sequences, Oshida *et al.* (2011) suggested the role of the Mekong River in the divergence of *C. caniceps* and *C. inornatus*, thus supporting the riverine barrier hypothesis (e.g. Salo *et al.* 1986, MacKinnon *et al.* 1996, Meijaard & Groves 2006, Oshida *et al.* 2001, 2016), which also explains intra-specific diversity in *C. erythraeus* (Oshida *et al.* 2013) and *C. finlaysonii* (Boonkhaw *et al.* 2017). Boonkhaw *et al.* (2017) showed that *C. erythraeus* and *C. finlaysonii* did not form each monophyletic group and that the *C. erythraeus/C. finlaysonii* complex in Thailand consisted of seven groups. Recently, based on morphological and genetic data, Balakirev and Rozhnov (2019) explained the inconsistency of the present subspecific classification of the *C. erythraeus/finlaysonii* complex. These phylogenetic studies on *Callosciurus* squirrels in the Indochina Peninsula have mainly focused on the species distributed in central and eastern regions. To better understand the evolutionary history of *Callosciurus*, the species distributed in the western

part of the peninsula (*C. phayrei* and *C. pygerythrus*; KOPROWSKI *et al.* 2016; Fig. 1) need to be included in the phylogenetic analyses. Although BOONKHAW *et al.* (2017) reported the close relationship of *C. phayrei* to *C. caniceps* within *caniceps–inornatus–phayrei* lineage, the phylogenetic position of *C. pygerythrus* is still unknown.

To examine the phylogenetic position of *C. pygerythrus*, we investigated the phylogenetic relationships among the seven peninsular *Callosciurus* species. *Callosciurus pygerythrus* covers the westernmost area of the distribution of the genus *Callosciurus*, occurring from central Nepal to Myanmar (CORBET & HILL 1992). Therefore, this species may be distantly related to others owing to its different evolutionary history. As *C. pygerythrus* resembles *C. caniceps* in external morphology (ELLERMAN & MORRISON-SCOTT 1966), it may be included in the *caniceps–inornatus–phayrei* lineage proposed by BOONKHAW *et al.* (2017). Here, we test these phylogenetic hypotheses using mitochondrial DNA (cytochrome *b*) sequences and discuss the evolutionary history of *Callosciurus*, including *C. pygerythrus* in the Indochina Peninsula.

MATERIAL AND METHODS

Material

We examined seven *Callosciurus* species from the Indochina Peninsula region (*C. caniceps*, *C. erythraeus*, *C. finlaysonii*, *C. honkhaiensis*, *C. inornatus*, *C. phayrei* and *C. pygerythrus*), and three from the Sunda region: *C. nigrovittatus* (Horsfield, 1823), *C. notatus* (Boddaert, 1785) and *C. prevostii* (Desmarest, 1822). We collected two *C. phayrei* and seven *C. pygerythrus* from Myanmar (Table 1 & Fig. 1). The genus *Lariscus* is closely related to *Callosciurus* (MERCER & ROTH 2003), and hence the three-striped ground squirrel (*Lariscus insignis*) was used as an outgroup in the phylogenetic analyses. DNA sequence data of *L. insignis* and the *Callosciurus* species except for *C. phayrei* and *C. pygerythrus* were obtained from the DNA Data Bank of Japan (DDBJ) (Table 1).

Extraction, amplification and sequencing of DNA

Total genomic DNA of *C. phayrei* and *C. pygerythrus* was extracted with the Qui-aQuick Kit (QUIAGEN K. K., Tokyo, Japan) from muscle tissue preserved in 99% ethanol. Complete cytochrome *b* gene sequences (1140 bases) were amplified with polymerase chain reaction (PCR) using primer set: L14724 5'-GATATGAAAAACCATCGTTG-3' (KOCHER *et al.* 1989) and H15910 5'-GATTTTTGGTTTACAAGACCGAG-3' (OSHIDA *et al.* 2000). The 50 µl reaction mixture contained 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 MgCl₂, and 2.5 units of rTaq DNA polymerase (Takara, Otsu, Japan). 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 (OSHIDA *et al.* 2011). The extension reaction was completed by incubation at 72 °C for 10 min. The PCR products were purified with PCR Clean Up-M (Viogen, Taiwan) and directly sequenced using an automated DNA sequencer (ABI PRISM 377-96 Sequencer and ABI PRISM 3100

Table 1. Squirrels examined in this study and retained in our private collections in the Laboratory of Wildlife Biology, Obihiro University of Agriculture and Veterinary Medicine (indicated by identity numbers beginning with OS). Numbers with species name correspond to those in Fig. 2. †Unknown exact collecting locality. *Samples collected in this study.

Species and haplotype	Collecting locality	Identity no.	Accession No.
<i>C. caniceps</i> 1	Pasoh Forest Res., Negeri Sembilan, Malaysia	Y3	AB499918
<i>C. caniceps</i> 2	Pasoh Forest Res., Negeri Sembilan, Malaysia	Y25	AB499919
<i>C. erythraeus</i> 1	Tam Dao, Vietnam	33	AB499908
<i>C. erythraeus</i> 2	Tam Dao, Vietnam	34	AB499909
<i>C. finlaysonii</i> 1	Thailand†	OS254	AB499910
<i>C. finlaysonii</i> 2	Thailand†	OS255	AB499911
<i>C. honkhvaiensis</i> 1	Hon Khoai Island, Vietnam	HK-2017.31, HK-2017.44	LC306835
<i>C. honkhvaiensis</i> 2	Hon Khoai Island, Vietnam	HK-2017.48	LC306836
<i>C. inornatus</i> 1	Co Ma, Thuan, Chau, Son La, Vietnam	183	AB499905
<i>C. inornatus</i> 2	Hon, Phu Yen, Son La, Vietnam	191	AB499906
<i>C. inornatus</i> 3	Hon, Phu Yen, Son La, Vietnam	199	AB499907
<i>C. nigrovittatus</i> 1	Pasoh Forest Res., Negeri Sembilan, Malaysia	Y26	AB499916
<i>C. nigrovittatus</i> 2	Pasoh Forest Res., Negeri Sembilan, Malaysia	Y28	AB499917
<i>C. notatus</i> 1	Pasoh Forest Res., Negeri Sembilan, Malaysia	Y11	AB499912
<i>C. notatus</i> 2	Pasoh Forest Res., Negeri Sembilan, Malaysia	Y12	AB499913
<i>C. phayrei</i>	Taungoo, Myanmar	OS822, OS823	LC552998
<i>C. prevostii</i> 1	Palembang, Indonesia	OS311	AB499914
<i>C. prevostii</i> 2	Palembang, Indonesia	OS312	AB499915
<i>C. pygerythrus</i> 1	Yangon, Myanmar	OS786, OS787, OS788, OS789	LC552995
<i>C. pygerythrus</i> 2	Mandalay, Myanmar	OS806	LC552996
<i>C. pygerythrus</i> 3	Mandalay, Myanmar	OS807, OS808	LC552997
<i>Lariscus insignis</i>	Pasoh Forest Res., Negeri Sembilan, Malaysia	Y8	AB499904
<i>Menetes berdmorei</i>	Cat Tien National Park, Vietnam	OS345(66)	LC552999

Genetic Analyzer Applied Biosystem, CA, US). For sequencing, we used both forward and reverse primers used for PCR. Purification of the PCR products and sequencing were done by Mission Biotech Co. Ltd. (Taipei, Taiwan).

Sequence and phylogenetic analyses

Sequence alignment to detect unique haplotypes was with the software program dnasis (Hitachi, Tokyo, Japan), followed by phylogenetic analyses in MEGA 7 (KUMAR *et al.* 2016). The Hasegawa-Kishino-Yano (HKY) model (HASEGAWA *et al.* 1985) for nucleotide substitution with the proportion of invariable sites (0.54), and gamma distribution for vari-

able sites (1.85) (HKY + I + Γ) was selected based on the Bayesian Information Criterion values, and used for the maximum likelihood (ML) analysis. Additionally, the unweighted maximum-parsimony (MP) analysis with tree-bisection-reconnection and neighbor-joining (NJ) analysis (SAITOU & NEI 1987) using the genetic distances correlated by the Kimura 2-parameter method (KIMURA 1980) were performed. We assessed nodal supports by bootstrapping (FELSENSTEIN 1985) with 1000 replicates for ML and MP analyses, and 5000 replicates for NJ analysis. Bayesian inference (BI) was performed using BEAST 1.10.4 (SUCHARD *et al.* 2018) using the HKY + I + Γ substitution model, with two runs for ten million steps, using four Markov chain Monte Carlo chains sampling every 1000 steps, and a burn-in of 10%. Posterior probabilities were used to assess the nodal support of the BI tree. Divergence times among the *Callosciurus* species were estimated using the RelTime method (TAMURA *et al.* 2012) in MEGA7. The divergence time between *Callosciurus* and *Lariscus* (7.5 ± 1.2 million years ago (Ma); MERCER & ROTH 2003) was used as a calibration point, by including *Menetes berdmorei* (a sister group to the ancestor of *Callosciurus* and *Lariscus*; MERCER & ROTH 2003) as an outgroup (Table 1).

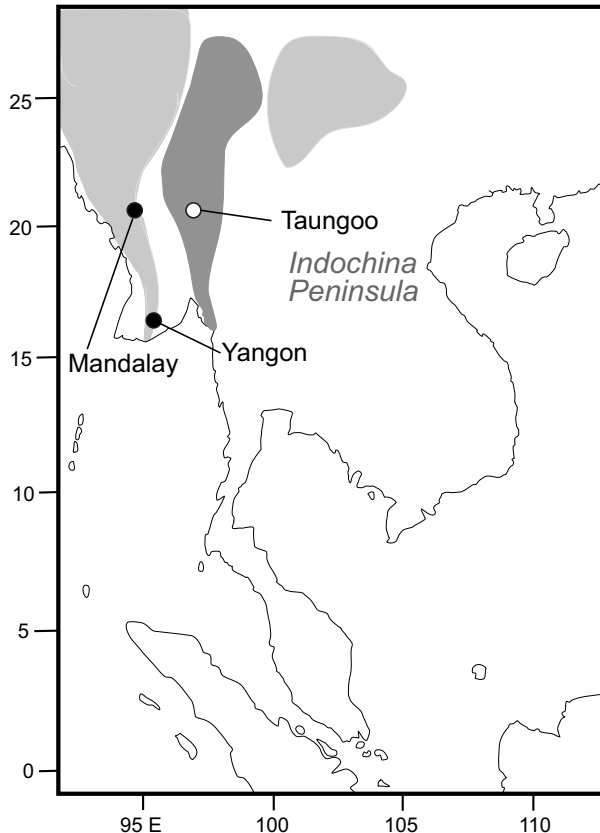


Fig. 1. Distribution of *Callosciurus phayrei* (dark gray area) and *C. pygerythus* (light gray area) in the Indochina Peninsula (KOPROWSKI *et al.* 2016) and collecting sites of squirrels examined in the present study. Open and closed circles indicate *C. phayrei* and *C. pygerythus*, respectively

RESULTS

Complete sequences (1140 bp) of cytochrome *b* gene were successfully obtained from two *C. phayrei* and seven *C. pygerythrus*. All sequences were deposited in the DNA Data Bank of Japan (DDBJ) (Table 1). The ML phylogenetic tree is shown in Fig. 2. The topology of NJ, MP and BI trees was essentially similar to that of ML tree. All trees showed two well-supported lineages, namely the Indochina lineage (*C. caniceps*, *C. erythraeus*, *C. finlaysonii*, *C. honkhoaiensis*, *C. inornatus*, *C. phayrei* and *C. pygerythrus*) and the Sunda lineage (*C. nigrovittatus* and *C. prevostii*). Within the Indochina lineage, two major lineages were observed, namely lineage A (*C. caniceps*, *C. honkhoaiensis*, *C. inornatus*, *C. phayrei* and *C. pygerythrus*) and lineage B (*C. erythraeus* and *C. finlaysonii*). Within the lineage A, we found two sub-lineages: one consisting of *C. inornatus* and *C. pygerythrus* and the other consisting of *C. caniceps*, *C.*

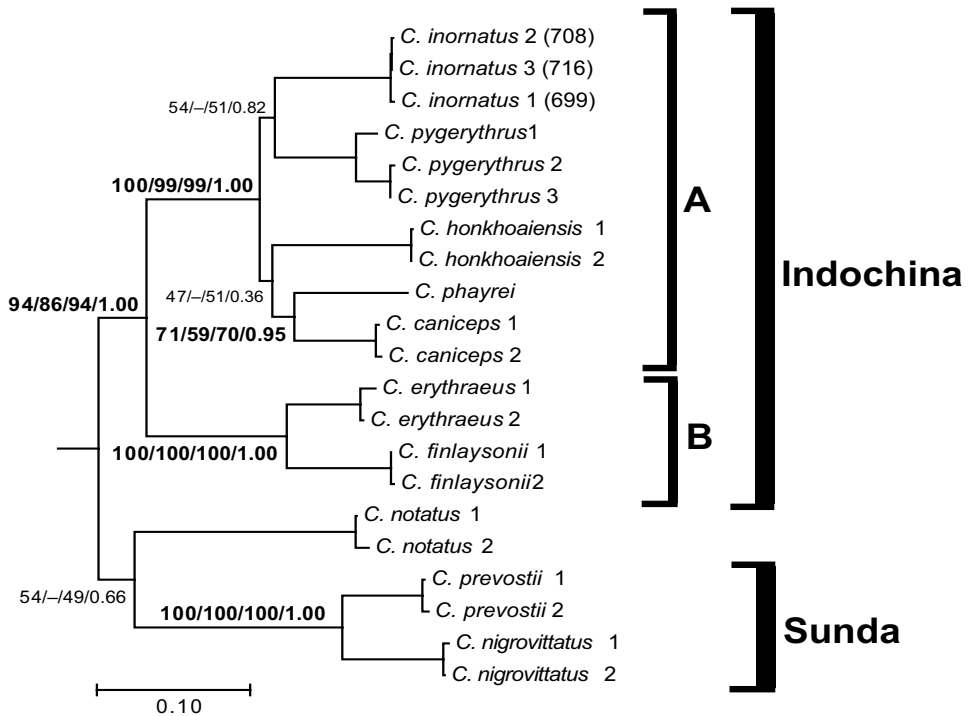


Fig. 2. Phylogeny of *Callosciurus* constructed with the maximum-likelihood (ML) under HKY + I + Γ model for cytochrome *b* sequences. From left, numbers above branches represent: bootstrap values from 1000 replicates of ML and un-weighted maximum parsimony (MP) analyses, bootstrap values from 5000 replicates of neighbor-joining (NJ) analysis and posterior probability supports in Bayesian analysis. Hyphens mean no data, because the clade was absent

honkhoaiensis and *C. phayrei*, but these were not reliably supported. *Callosciurus phayrei* was closely related to *C. caniceps*, although support values were not high (71%, 59%, 70% and 0.95 in the ML, MP, NJ and BI trees, respectively).

The divergence time estimates are shown in Figure 3. In the Indochina lineage, the divergence time between lineages A and B was estimated to be 3.73 Ma; while within lineage A, the divergence between the two sub-lineages was estimated to be 2.19 Ma. The estimated divergence time between *C. honkhoaiensis* and the ancestor of *C. caniceps* and *C. phayrei* was 1.96 Ma, and that between *C. caniceps* and *C. phayrei* was 1.76 Ma. Besides, the divergence time between *C. inornatus* and *C. pygerythus* was estimated to be 1.95 Ma.

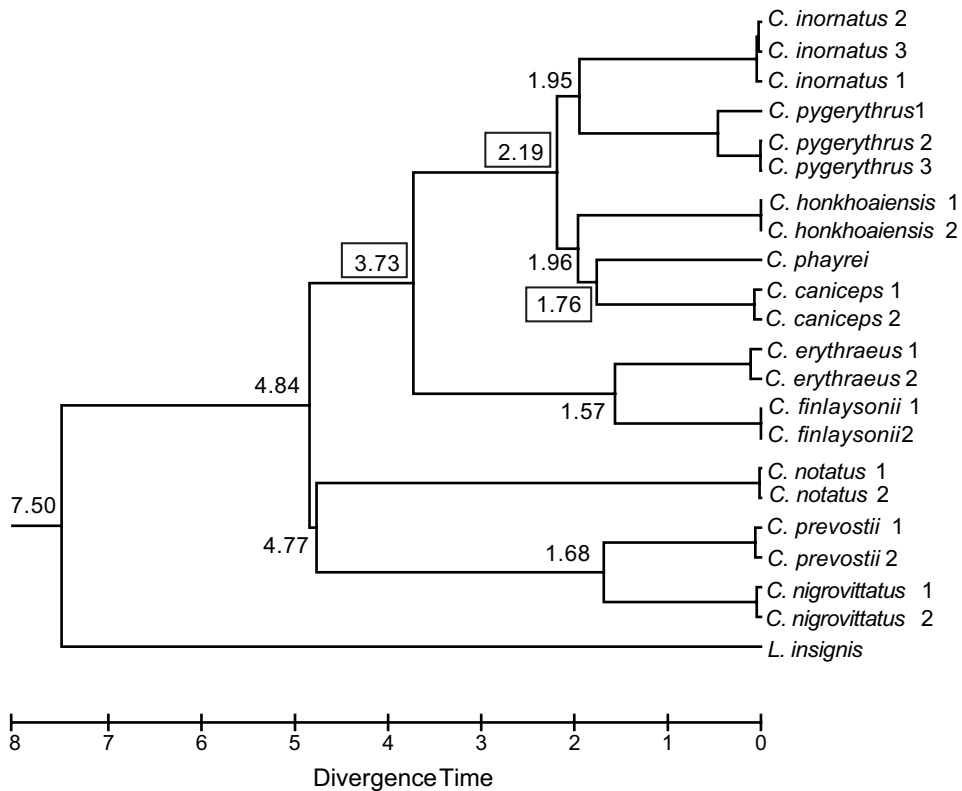


Fig. 3. Divergence time tree estimated by the RelTime method, based on the maximum-likelihood (ML) analysis under HKY + I + Γ model for the cytochrome *b* sequences. Divergence times among *Callosciurus* squirrels in millions of years before present are on branches. Divergence times with high support values are inside squares

DISCUSSION

Recently, BOONKHAW *et al.* (2017) described two *Callosciurus* lineages from the Indochina Peninsula: one containing *C. caniceps*, *C. inornatus* and *C. phayrei* and the other containing *C. erythraeus* and *C. finlaysonii*. We observed two major lineages: lineages A comprising *C. caniceps*, *C. inornatus*, *C. phayrei*, *C. pygerythrus* and *C. honkhoaiensis* and B comprising *C. erythraeus* and *C. finlaysonii*, thus adding *C. pygerythrus* and *C. honkhoaiensis* to the first lineage described by BOONKHAW *et al.* (2017). The estimated divergence time (about 3.73 Ma) between lineages A and B was at the junction of the Zanclean and Piacenzian in the Pliocene. Drastic divergence of shrews (DUBEY *et al.* 2008, ESSELSTYN *et al.* 2009, YUAN *et al.* 2013) and murine rodents (RUEDAS & KIRSCH 1997, GOROG *et al.* 2004) are also found in the Indochina Peninsula during the early Pliocene. Sediment core data suggest that global climatic conditions were altered in the early and middle Pliocene (KASHIWAYA *et al.* 2001), with global cooling in the late Pliocene (RAVELO *et al.* 2004, HAYWOOD *et al.* 2009). This suggests a potential role of climate conditions in the divergence of two lineages A and B.

Phylogenetic relationships within lineage A were unclear because of lower support values (Fig. 2). The estimated divergence times among these five species (about 2.19 Ma) correspond to the Pliocene–Pleistocene boundary, which experienced a cooler global climate (RAVELO *et al.* 2004, HAYWOOD *et al.* 2009). Climatic change is known to affect the distribution of forests in the Northern Hemisphere (e.g. HEUERTZ *et al.* 2010, STEFENON *et al.* 2019), and these changes in forest environments could thereby affect arboreal *Callosciurus* squirrels.

The estimated divergence time between *C. caniceps* and *C. phayrei* was about 1.76 Ma, which lies at the junction of the Calabrian and Gelasian. In the 1.8–1.2 Ma interval, a decrease in global temperatures increased the specialization of mammal species and the fauna adapted to cooler environments (KAHLKE *et al.* 2011). For instance, in the Gelasian/Calabrian transition in Italy, fossils of extinct ungulates show an abundance of mixed feeders (such as *Leptobos etruscus*, *Pseudodama nestii* and *Stephanorhinus etruscus*) and grazers (such as *Equus stenorhinus* and *Gazella meneghini*) and a lack of browsers, indicating the development of open grasslands and open woodlands (STRANI *et al.* 2018). The ancestral form of these two *Callosciurus* species may have been affected by similar climatological alteration in the peninsula.

We found that *C. pygerythrus* was included in *caniceps–honkhoaiensis–inornatus–phayrei* lineage reported previously (BOONKHAW *et al.* 2017, NGUYEN *et al.* 2018), but it was difficult to explain the evolutionary history within this lineage. Each *Callosciurus* species of this lineage also exhibits wide regional variation (CORBET & HILL 1992, THORINGTON *et al.* 2012, KOPROWSKI *et al.* 2016). Therefore, to better understand the evolutionary history of this lineage, future

studies need to analyze the relationships among regional forms (subspecies) of each species, similar to the works of BOONKHAW *et al.* (2017) and BALAKIREV and ROZHNOV (2019) on the *C. erythraeus/finlaysonii* complex.

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