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

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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.

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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. honkhoaiensis* 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). Boonkhw 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. honkhoaiensis*, 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* (Boonkhw et al. 2017). Boonkhw 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. erythroaeus, C. finlaysonii, C. honkhoaensis, 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 QuickStart 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...
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).

### 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. |
|-----------------------|---------------------|--------------|---------------|
| C. caniceps 1         | Pasoh Forest Res., Negeri Sembilan, Malaysia | Y3           | AB499918      |
| C. caniceps 2         | Pasoh Forest Res., Negeri Sembilan, Malaysia | Y25          | AB499919      |
| C. erythraeus 1       | Tam Dao, Vietnam    | 33           | AB499908      |
| C. erythraeus 2       | Tam Dao, Vietnam    | 34           | AB499909      |
| C. finlaysonii 1      | Thailand†           | OS254        | AB499910      |
| C. finlaysonii 2      | Thailand†           | OS255        | AB499911      |
| C. honkhoaiensis 1    | Hon Khoai Island, Vietnam | HK-2017.31, LC306835 |
| C. honkhoaiensis 2    | Hon Khoai Island, Vietnam | HK-2017.48  | LC306836      |
| C. inornatus 1        | Co Ma, Thuan, Chau, Son La, Vietnam | 183          | AB499905      |
| C. inornatus 2        | Hon, Phu Yen, Son La, Vietnam | 191          | AB499906      |
| C. inornatus 3        | Hon, Phu Yen, Son La, Vietnam | 199          | AB499907      |
| C. nigrovittatus 1    | Pasoh Forest Res., Negeri Sembilan, Malaysia | Y26          | AB499916      |
| C. nigrovittatus 2    | Pasoh Forest Res., Negeri Sembilan, Malaysia | Y28          | AB499917      |
| C. notatus 1          | Pasoh Forest Res., Negeri Sembilan, Malaysia | Y11          | AB499912      |
| C. notatus 2          | Pasoh Forest Res., Negeri Sembilan, Malaysia | Y12          | AB499913      |
| C. phayrei            | Taungoo, Myanmar    | OS822, OS823 | LC552998      |
| C. prevostii 1        | Palembang, Indonesia | OS311        | AB499914      |
| C. prevostii 2        | Palembang, Indonesia | OS312        | AB499915      |
| C. pygerythrus1       | Yangon, Myanmar     | OS786, OS787, OS788, OS789 | LC552995 |
| C. pygerythrus2       | Mandalay, Myanmar   | OS806        | LC552996      |
| C. pygerythrus3       | Mandalay, Myanmar   | OS807, OS808 | LC552997      |
| Lariscus insignis     | Pasoh Forest Res., Negeri Sembilan, Malaysia | Y8           | AB499904      |
| Menetes berdmorei     | Cat Tien National Park, Vietnam | OS345(66)   | LC552999      |

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).

![Map of Indochina Peninsula showing the distribution of Callosciurus phayrei and C. pygerythus](image)

**Fig. 1.** Distribution of Callosciurus phayrei (dark gray area) and C. pygerythus (light gray area) in the Indochina Peninsula (Korrowski 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.
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. pygerythrus was estimated to be 1.95 Ma.

![Divergence time tree](image)

**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, Boonkhw et al. (2017) described two Callosciurus linages 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 linages: linage A comprising C. caniceps, C. inornatus, C. phayrei, C. pygerythrus and C. honkhoaensis and B comprising C. erythraeus and C. finlaysonii, thus adding C. pygerythrus and C. honkhoaensis to the first linage described by Boonkhw et al. (2017). The estimated divergence time (about 3.73 Ma) between linages 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 linages A and B.

Phylogenetic relationships within linage 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 stenonis 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–honkhoaensis–inornatus–phayrei linage reported previously (Boonkhw et al. 2017, Nguyen et al. 2018), but it was difficult to explain the evolutionary history within this linage. Each Callosciurus species of this linage 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 linage, 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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