

Preliminary study on phylogeography of *Callosciurus prevostii* in Southeast Asia: mitochondrial DNA evidence supports riverine barrier hypothesis

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Abstract. Evolutionary history of *Callosciurus* squirrel species seems affected by geographical isolation by huge rivers in Southeast Asia. Geographical isolation by rivers may have influenced phylogeographical structure between and within *Callosciurus* species. In Sundaland, Southeast Asia, however, phylogeographical structure within *Callosciurus* species may be explained by geographical isolation among islands. In a test of the effects of rivers and islands, we examined phylogeographical characteristics of *C. prevostii* based on mitochondrial cytochrome *b* gene sequences. This squirrel is distributed from peninsular Thailand to Sumatra and Borneo and adjacent small islands. We collected specimens from four geographically different populations (the Malay Peninsula, central Sumatra, eastern Sumatra, and Borneo) and conducted phylogeographical analyses. Interestingly, the two Sumatra populations, geographically separated by the Batang Hari River, the longest river in Sumatra, did not show monophyly: the central Sumatra population was closely related to the Malay Peninsula population. The specimen from Borneo had clearly diverged from the others. Present geographical isolation by the ocean does not completely explain the phylogeographical structure of this species. The riverine barrier hypothesis helped explain their evolutionary history. Future studies should examine more *C. prevostii* specimens from more different places in Sundaland.

Key words: cytochrome *b*, geographical isolation, Prevost's squirrel, Sumatra Island.

Huge rivers sometimes act as important geographical barriers to separate mammalian populations (riverine barrier hypothesis) (e.g., Salo et al. 1986; MacKinnon et al. 1996; Meijaard and Groves 2006; Arora et al. 2010). Phylogeography of arboreal mammals is especially affected by rivers, as it is difficult for these animals to cross huge rivers. In fact, clear genetic divergences because of huge rivers were found in arboreal primates (Eriksson et al. 2004; Jalil et al. 2008; Arora et al. 2010) and tree squirrels (Moncrief 1993; Oshida et al. 2001, 2011, 2013).

Oshida et al. (2001) suggested huge rivers as a possible geographical barrier for speciation within the *Callosciurus* in Southeast Asia. Moreover, Oshida et al. (2011) suggested that *C. caniceps* and *C. inornatus* were divided by the Mekong River in the northern Indo-

china Peninsula. Complicated divergence patterns between *C. erythraeus* and *C. finlaysonii* with the Mekong River were reported in the southern Indochina Peninsula (Oshida et al. 2013). As all *Callosciurus* species have similar ecological habits (e.g., Francis 2008), there may be evidence in the Sundaland that reinforces the riverine barrier hypothesis (Fig. 1).

Sumatra Island, Indonesia, could be suitable to examine the riverine barrier hypothesis. When Sumatra Island and the other parts of the Sundaland were connected during the Pleistocene, much larger river systems flowed through eastern Sumatra toward the east with present huge rivers, former tributaries of that system (Tjia 1980; Whitten et al. 1997, Fig. 1). Therefore, those rivers on Sumatra Island probably acted as strong geographical barriers for many terrestrial mammal species since the

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Pleistocene glaciation.

In the present study, we examined the Prevost's squirrel (*Callosciurus prevostii*). This squirrel is widely distributed from peninsular Thailand to Sumatra Island, Borneo Island, and adjacent small islands (Corbet and Hill 1992; Francis 2008). It is morphologically so variable (e.g., Heaney 1978), having 47 named subspecies: *humai*, *prevostii*, and *wrayi* from the Malay Peninsula; *melanops*, *piceus*, and *rafflesii* from Sumatra Island; *atricapillus*, *atrox*, *borneoensis*, *caedis*, *coomansi*, *palustri*, *pluto*, *rufonigra*, *sanggaus*, *sarawakensis*, and *waringensis* from Borneo Island; and 30 others from 15 smaller islands (Thorington et al. 2012). This squirrel uses the middle and upper parts of the canopy as a niche. At the Krau Wildlife Reserve of Malaysia, this squirrel spends most of its time at heights from 6 m to more than 37 m above the forest floor (Thorington et al. 2012). Therefore, it is easily geographically isolated in forest patches, producing genetically different populations. On Borneo Island, distribution of two subspecies, *C. p. palustris* and *C. p. sanggaus*, are divided by the Kapuas River (Thorington et al. 2012).

To test the riverine barrier hypothesis, we investigated phylogenetic relationships among four *C. prevostii* populations from peninsular Malaysia, central and eastern Sumatra Island separated by the Batang Hari River, and Borneo Island (Fig. 1). Here, we discuss the phylogeographical relationships of *C. prevostii* populations with special mention to riverine barrier hypothesis in the Sundaland, especially the barrier produced by the Batang Hari that is the longest river in Sumatra Island.

Materials and methods

Specimens

We collected a total of 14 *Callosciurus prevostii* specimens from four different localities: seven specimens from the Malay Peninsula, two from central Sumatra Island, four from eastern Sumatra Island, and one from southern Borneo Island (Table 1 and Fig. 1). The Sumatra populations were collected from both sides of the Batang Hari River (Fig. 1). Unfortunately, it was difficult to identify the correct subspecies name for each population due to variable pelage colors. Skin or muscle tissue specimens of all individuals were fixed in 99% ethanol and deposited in the Laboratory of Wildlife Biology, Obihiro University of Agriculture and Veterinary Medicine, Japan.

PCR and DNA sequencing

Total genomic DNA was extracted from the tissues using the DNeasy Blood & Tissue Kit (QIAGEN K.K., Tokyo). The complete mitochondrial cytochrome *b* gene sequence (1,140 bases) was amplified with polymerase chain reaction (PCR) using primer set L14724 5'-GATATGAAAAACCATCGTTG-3' and H15910 5'-GATTTTGTGTTTACAAGACCGAG-3'. Former and latter primers were reported by Kocher et al. (1989) and Oshida et al. (2000), respectively. The 50 µl reaction mixture contained approximately 100 ng of genomic DNA, 0.25 µM 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, Tokyo). Amplification was carried out for 35 cycles. The PCR condition for a cycle was 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. A

Table 1. *Callosciurus prevostii* specimens examined in this study and preserved in the Laboratory of Wildlife Biology, Obihiro University of Agriculture and Veterinary Medicine

Locality number	Collecting locality	Haplotype	Identity number	Accession number	Supplier
1	Mayay Peninsula (Pasoh F. R., Negeri Sembilan, Malaysia)	Cpr1	Y01	LC138029	
		Cpr2	Y17, Y18, Y32, Y35	LC138030	
		Cpr3	Y33	LC138031	
		Cpr4	Y34	LC138032	
2	central Sumatra Island (Muarabungo, Jambi, Indonesia)	Cpr5	F02	LC138028	Yuki Fujita
		Cpr6	F89	LC138033	Yuki Fujita
3	eastern Sumatra Island (Palembang, Indonesia)	Cpr7	OS311*	AB499914	
		Cpr8	OS312*, OS313	AB499915	
		Cpr9	OS314, OS315	LC138034	
		Cpr10	OS316	LC138035	
4	Borneo Island (Bangkirai, Indonesia)	Cpr11	Y46	LC138036	

Locality numbers correspond to those in Fig. 1. Asterisks indicate specimens used in Oshida et al. (2011).

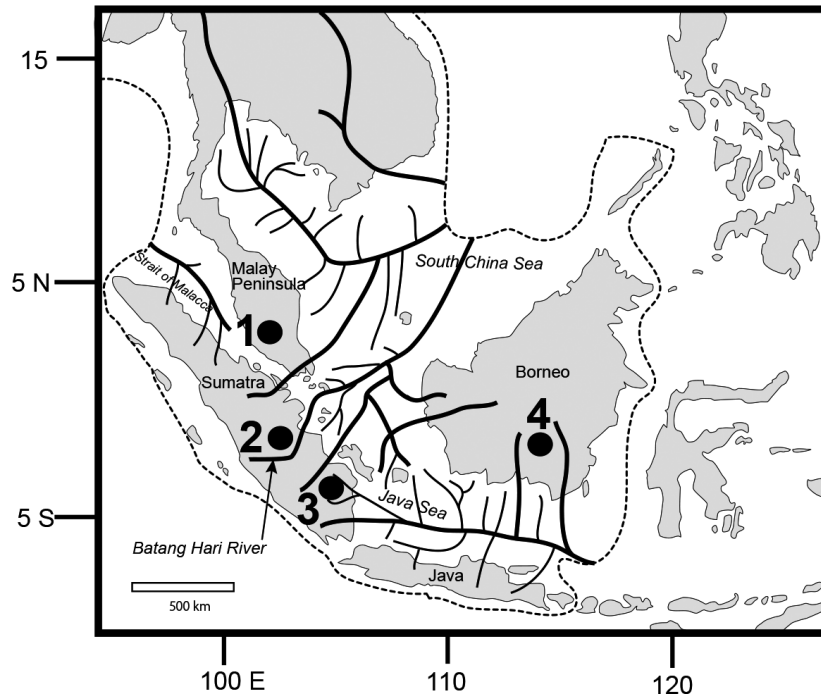


Fig. 1. Map of the Sundaland modified from MacKinnon et al. (1996). Broken line outlines the Sundaland during the Pleistocene glaciation. Solid lines indicate present and ancient rivers. Closed circles with numbers indicate collecting localities of *Callosciurus prevostii* examined in the present study.

final extension reaction after the 35 cycles was conducted at 72°C for 10 min. The PCR products were purified with the PCR Clean Up-M (Viogen, Taipei, Taiwan) and directly sequenced by the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1 (Applied Biosystems, CA, USA) and automated DNA sequencer (ABI PRISM 377-96 Sequencer, the ABI PRISM 3100 Genetic Analyzer, Applied Biosystem, 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 analyses

To root phylogenetic trees, we used sequences of two black-striped squirrels *Callosciurus nigrovittatus* (accession numbers AB499916 and AB499917, Oshida et al. 2011). Since *C. nigrovittatus* was closer to *C. prevostii* than to other *Callosciurus* species (Oshida et al. 2011), it was a suitable out-group for resolving phylogenetic relationships within *C. prevostii* species. We also included sequence data of two *C. prevostii* from eastern Sumatra Island (accession numbers AB499914 and AB499915, Oshida et al. 2011).

All sequences were aligned with DNASIS (Hitachi, Tokyo). We calculated pairwise uncorrected genetic

distances (p -distances) among *C. prevostii* haplotypes. For neighbor-joining (NJ) (Saitou and Nei 1987) and maximum-likelihood (ML) analyses and Bayesian inference (BI) reconstruction, the program MODELTEST 3.06 (Posada and Crandall 1998) was used to select the most appropriate substitution model of molecular evolution through the Akaike information criterion; the Tamura-Nei model (Tamura and Nei 1993) was selected with the proportion of invariable sites ($I = 0.6192$). Base frequencies were estimated as A = 0.2693, C = 0.2880, G = 0.1274, and T = 0.3152. Rate matrix was estimated as A-C = 1.0000, A-G = 27.6367, A-T = 1.0000, C-G = 1.0000, C-T = 30.2248, and G-T = 1.0000. Based on genetic distances calculated by this model, we conducted NJ analysis. In addition, we conducted an un-weighted maximum parsimony (MP) analysis with a branch-and-bound search option. The ML tree was constructed with the selected model by a heuristic search option having a tree-bisection-reconnection. To assess nodal supports, bootstrapping (Felsenstein 1985) was carried out with 10,000 replications in NJ and MP analyses and 1,000 replications in ML analysis. The NJ, MP, and ML analyses were performed by PAUP* 4.0b10 (Swofford 2001). The BI reconstruction was carried out with the selected model by MrBayes 3.0b4 (Huelsenbeck and Ronquist

Table 2. Pairwise uncorrected percentage differences (*p*-distances) of cytochrome *b* nucleotide sequences (1,140 bp) between 11 *Callosciurus prevostii* haplotypes

Haplotype	Cpr2	Cpr3	Cpr4	Cpr5	Cpr6	Cpr7	Cpr8	Cpr9	Cpr10	Cpr11
Cpr1	0.18	0.35	0.70	0.79	0.61	1.75	2.11	1.84	2.02	5.26
Cpr2		0.18	0.53	0.61	0.44	1.78	1.93	1.68	1.84	5.09
Cpr3			0.53	0.79	0.61	1.76	2.11	1.84	2.02	5.26
Cpr4				1.14	0.97	2.11	2.46	2.19	2.37	5.44
Cpr5					0.53	1.67	2.02	1.75	1.93	5.18
Cpr6						1.32	1.84	1.58	1.75	5.00
Cpr7							0.53	0.26	0.44	5.09
Cpr8								0.26	0.09	5.44
Cpr9									0.18	5.18
Cpr10										5.35

2001). The analysis involved two runs for one million iterations, sampling four Markov chain Monte Carlo chains every 1,000 generations and using a burn-in of 20%. The 50% majority consensus trees were generated from sampled trees for each run. We assessed nodal supports of the BI tree with posterior probabilities.

Divergence times among lineages were estimated by BEAST version 1.8.3 (Drummond et al. 2016). Divergence time between two squirrel genera, *Callosciurus* and *Dremomys*, was estimated to be 14.0 million years ago (Mya) based on the fossil record (Qui 2002; Hawkins 2016). To set the calibration point, we therefore included two *Dremomys* squirrel's sequences: *D. pernyi* (accession number HQ698361, Chang et al. 2011) and *D. rufigenis* (accession number EF539341, Li et al. 2008) in this analysis.

Results

Complete sequences (1,140 bases) of the cytochrome *b* gene were determined for all *C. prevostii* specimens. All sequence data were deposited in the DNA Data Bank of Japan (Table 1). We found 11 *C. prevostii* cytochrome *b* haplotypes. Uncorrected *p*-distances among haplotypes are shown in Table 2. Among haplotypes, there were 85 variable sites. Of those, 24 were parsimony informative.

Branching patterns of NJ, MP, ML, and BI trees were similar to each other. The NJ tree is shown in Fig. 2. Trees showed three major geographical lineages: lineage I (the Malay Peninsula and central Sumatra Island), lineage II (eastern Sumatra Island), and lineage III (Borneo Island). Lineage I consisted of nine specimens from two different localities and had high support values (100% in NJ and BI trees, 94% in MP tree, and 82% in ML tree). In this line-

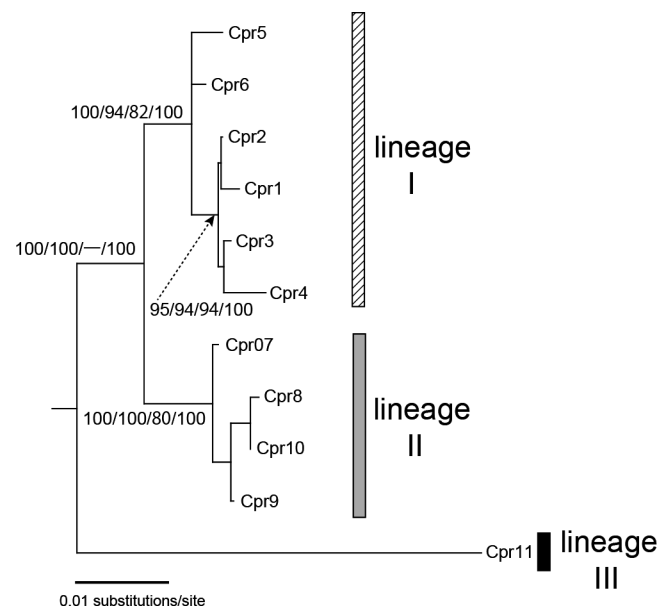


Fig. 2. Phylogeny of 11 *Callosciurus prevostii* haplotypes constructed with the neighbor-joining method under the Tamura-Nei + I model for mitochondrial cytochrome *b* sequences shows division into three lineages: 1) the Malay Peninsula and central Sumatra Island, 2) eastern Sumatra Island, and 3) Borneo Island. Haplotypes are defined in Table 1. From the left, numbers at branches represent: bootstrap values from 10,000 replicates of neighbor-joining and un-weighted maximum parsimony analyses and from 1,000 replicates of maximum likelihood analysis and posterior probability supports in Bayesian analysis. A hyphen means no data due to absence of node in the maximum likelihood tree.

age, the seven specimens from the Malay Peninsula were clustered together with high support values (95% in NJ, 94% in MP and ML trees, and 100% in BI tree), but the two specimens from central Sumatra Island did not form a single cluster. Lineage II consisted of all specimens from eastern Sumatra Island with high support values

(100% in NJ, MP, and BI trees and 80% in ML tree). The *p*-distances between two Sumatra populations ranged from 1.32% to 2.02% (Table 2). Lineage III included the one specimen from Borneo Island and was distantly related to other two lineages. The *p*-distances between lineage III and the other two lineages were from 5.00% to 5.44% (Table 2). In the chronological analyses, lineage III was estimated to have diverged from the other lineages approximately 1.7 Mya. The divergence time between lineages I and II were inferred to be 0.6 Mya.

Discussion

We identified three main lineages for the four *Callosciurus prevostii* populations. The two Sumatra populations, which are geographically closest to each other on the same island but are separated by the Batang Hari River did not show a monophyletic relationship. The central Sumatra population was more closely related to the Malay Peninsula population. This suggests that the Batang Hari River was one of effective geographical barriers for *C. prevostii*, supporting riverine barrier hypothesis. Divergence time (0.6 Mya) between lineages I and II also suggests that the two Sumatra populations were divided by this huge river during the middle Pleistocene glaciation. Populations from the Malay Peninsula and central Sumatra, however, may have been distributed on the Sundaland as a single population during the Pleistocene glaciation, although now they are geographically isolated by the Strait of Malacca.

The Borneo population was distantly related to the other populations in the present study. Between Sumatra and Borneo Islands, the Java Sea is currently a strong geographical barrier. There is clear genetic divergence between Sumatra and Borneo Islands for some forest mammal species: the common long-tailed macaque *Macaca fascicularis* (Liedigk et al. 2015), the orangutan *Pongo pygmaeus* (Muir et al. 2000), and the Sunda clouded leopard *Neofelis diarai* (Wilting et al. 2011). The shallow continental shelves of the South China Sea and Java Sea are incised by several ancient rivers channeling between Sumatra and Borneo Islands (e.g., MacKinnon et al. 1996). In addition, two large rivers run along the bed of the Java Sea between Java and Borneo Islands (Verstappen 1973). MacKinnon et al. (1996) suggested that these rivers probably served as barriers to floral and faunal distributions in the Sunda area. Judging from divergence time (1.7 Mya) between Borneo and the other populations during the Pleistocene glaciation,

Callosciurus prevostii may have also been geographically influenced by these ancient river systems.

The riverine barrier hypothesis was supported by the phylogeographical relationships among the four *C. prevostii* populations in the present study. We, however, did not examine individuals from every *C. prevostii* populations in the Sundaland. To improve understanding of their phylogeography, future studies should examine more specimens from more different places in Sundaland.

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