GEOGRAPHIC ISOLATION CREATED DISTINCT PELAGE CHARACTERS IN FINLAYSON’S SQUIRREL ON ISOLATED ISLAND OFFSHORE OF THE INDOCHINA PENINSULA IN CENTRAL VIETNAM

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We found distinct pelage characters in Finlayson’s squirrel (Callosciurus finlaysonii) population, which is endemic to Lao Island of the Cham Islands, located off the coast of central Vietnam. Among squirrels of the ‘C. erythraeus-finlaysonii complex’, which consists of all forms of C. erythraeus and C. finlaysonii, mitochondrial cytochrome-b sequences show that the Lao Island squirrel forms a cluster with C. finlaysonii with external characters of C. erythraeus flavimanus, defined previously as ‘C. finlaysonii morpha flavimanus’. Both squirrel forms, however, differed in pelage colour. This different colouration may have arisen from the effect of geographic isolation.

Key words: Finlayson’s squirrel, Callosciurus erythraeus-finlaysonii complex, cytochrome-b sequence, Vietnam, Lao Island, pelage colour.
INTRODUCTION

Sundaland is a region of Southeast Asia that encompasses the Sunda shelf, the part of the Asian Continent that was previously submerged until the last glacial period, 110,000 to 12,000 years ago. Irwanto (2019) reported that the sea level has risen by approximately 130±10 m since 21,000 years ago, reaching its present level about 4,500 years ago. The Sunda shelf includes the Malay Peninsula, Sumatra, Borneo, Java, Madura, Bali, and surrounding small islands (Ben-Avraham 1973). After the sea level rose, the high peaks located near the coastlines of Sundaland became small islands near the Indochina and Malay peninsulas and large islands such as Sumatra and Borneo (Hall & Morley 2004, Nguyen et al. 2010). Thus, the rise in sea level after the last glacial period may be the main force reasonable for the isolation of several terrestrial mammals on these newly formed islands.

Mammalian populations isolated from the mainland often evolve under different environments and may become endemic to their localities, such as the Cozumel harvest mouse (*Reithrodontomys spectabilis*) (Pardiñas et al. 2017) and the Amami rabbit (*Pentalagus furnessi*) (Schai-Braun & Hackländer 2016). Recently, endemic mammalian species have also been found in small isolated islands off the Indochina Peninsula in southern Vietnam, including the Phu Quoc shrew (*Crocidura phuquocensis*) and Hon Khoai squirrel (*Callosciurus honkhoaensis*), which were found in the Phu Quoc Island (Abramov et al. 2007) and Hon Khoai Island (Nguyen et al. 2018), respectively. Therefore, it is possible that there are several yet to be identified mammalian forms in other coastal islands of Vietnam. Lao Island is located 15 km from the east coast of the Indochina Peninsula in central Vietnam, and is a part of the Cham Islands (“Cù Lao Chàm” in Vietnamese), which consist of Lao, Dai, Mo, Ong, Tai, La, Kho Me, and Kho Islands) (Fig. 1). The Cham Islands are included in the Triton Horst region of Sundaland, where the sea depth is less than 200 m (Dang & Sladen 1997). During the last glacial period, this area is thought to be a part of the mainland, because of the existence of some non-volant mammals, such as rhesus monkey (*Macaca mulatta*).

In the present study, we compared the biological characters of the Lao Island population and mainland population. To examine the effect of geographic isolation in a short period (approximately 10,000–20,000 years), we chose Finlayson’s squirrel *Callosciurus finlaysonii* (Horsfield, 1823). *Callosciurus finlaysonii*, common in Lao Island, is widely distributed in Thailand, southern Laos, southwestern Vietnam, southwestern Cambodia and southcentral Myanmar and the 16 subspecies are recognized (Koprowski et al. 2016). This species is closely related to Pallas’s squirrel *C. erythraeus* (Pallas, 1779) (Oshida et al. 2001), which also has many various forms (e.g. Koprowski et al. 2016). Recently, Boonkaw et al. (2017) reported that *C. erythraeus* and *C. finlaysonii* did not form each mono-
phyletic group as respective distinct species. In addition, Balakirev & Rozh
nov (2019) described that both species are taxonomically similar to each other, forming the ‘C. erythraeus-finlaysonii complex’. In the present study, based on external characters and mitochondrial DNA sequences, we discussed the phylogeographic status of the Lao Island squirrel in C. erytheaus-finlaysonii complex, putting the effect of geographic isolation into consideration.

Fig. 1. Location of Lao Island, Vietnam. Dark gray areas indicate Vietnam. Dotted circles mean geographic distribution of phylogenetic groups G9 and G10 described previously by Balakirev and Rozhnov (2019)
Table 1. Squirrels of *Callosciurus erythraeus–finlaysonii* complex used in the phylogenetic analyses in the present study.
*No information on exact collecting localities, but in Thailand. **Non-native individuals (introduced into Japan).

| Species             | Subspecies | Identity | Accession number | Locality                                           | Reference               |
|---------------------|------------|----------|------------------|---------------------------------------------------|-------------------------|
| *C. finlaysonii*     | albivexilli| C45      | LC192452         | Koh Kut Island, Thailand                          | Boonkha et al. (2017)  |
|                     | annellatus | C21      | LC101292         | Huai Thap Than–Huai Samran Wildlife Sanctuary, Thailand | Boonkha et al. (2017)  |
| bocourtii           |            | C22      | LC101293         | Sublungka Wildlife Sanctuary, Thailand             | Boonkha et al. (2017)  |
| bocourtii           |            | C23      | LC101294         | Sublungka Wildlife Sanctuary, Thailand             | Boonkha et al. (2017)  |
| bocourtii           |            | C24      | LC101295         | Sublungka Wildlife Sanctuary, Thailand             | Boonkha et al. (2017)  |
| bocourtii           |            | C25      | LC101296         | Sublungka Wildlife Sanctuary, Thailand             | Boonkha et al. (2017)  |
| bocourtii           |            | C26      | LC101297         | Sublungka Wildlife Sanctuary, Thailand             | Boonkha et al. (2017)  |
| boonsongi           |            | C27      | LC101298         | Phu Phan National Park, Thailand                   | Boonkha et al. (2017)  |
| cinnamomeus/nox     |            | C12      | LC101283         | Khao Kheow Wildlife Conservation Station, Thailand | Boonkha et al. (2017)  |
| cinnamomeus/nox     |            | C17      | LC101288         | Khao Kheow Wildlife Conservation Station, Thailand | Boonkha et al. (2017)  |
| finlaysonii         |            | C18      | LC101289         | Koh Sichang Island, Thailand                       | Boonkha et al. (2017)  |
| floweri             |            | C42      | LC192449         | Bangkok, Thailand                                  | Boonkha et al. (2017)  |
| folletti            |            | C39      | LC192446         | Koh Phai Island, Thailand                          | Boonkha et al. (2017)  |
| frandseni           |            | C43      | LC192450         | Koh Chang Island, Thailand                         | Boonkha et al. (2017)  |
| menamicus           |            | C8       | LC101279         | Doi Phu Kha National Park, Thailand                | Boonkha et al. (2017)  |
| menamicus           |            | C9       | LC101280         | Tham Chao Ram Wildlife Sanctuary, Thailand         | Boonkha et al. (2017)  |
| menamicus           |            | C10      | LC101281         | Tham Chao Ram Wildlife Sanctuary, Thailand         | Boonkha et al. (2017)  |
| trotteri            |            | C38      | LC192445         | Koh Lan Island, Thailand                           | Boonkha et al. (2017)  |
| unidentified        |            | OS254    | AB499910         | Thailand*                                          | Oshida et al. (2013)   |
| unidentified        |            | OS255    | AB499911         | Thailand*                                          | Oshida et al. (2013)   |
| Species        | Subspecies | Identity | Accession number | Locality                                      | Reference                          |
|----------------|------------|----------|------------------|-----------------------------------------------|------------------------------------|
| unidentified   | CMR-29     | MK256803 | Chu Mom Ray Nature Reserve, Vietnam | Balakirev & Rozhnov (2019)                |
| unidentified   | Sq-1       | MK256804 | Nam Cat Tien Nature Reserve, Vietnam | Balakirev & Rozhnov (2019)                |
| unidentified   | Sq-4       | MK256806 | Nam Cat Tien Nature Reserve, Vietnam | Balakirev & Rozhnov (2019)                |
| unidentified   | 132-09     | MK256807 | Bu Gia Map Nature Reserve, Vietnam | Balakirev & Rozhnov (2019)                |
| unidentified   | NTr-1      | MK256810 | Tre Island       | Balakirev & Rozhnov (2019)                  |
| unidentified   | QB-4       | MK256812 | Sa Khia, Vietnam | Balakirev & Rozhnov (2019)                  |
| unidentified   | CLC.2019.05.38 | LC589711 | Lao Island, Vietnam | This study                                    |
| unidentified   | CLC.2019.05.40 | LC589712 | Lao Island, Vietnam | This study                                    |
| unidentified   | CLC.2019.05.42 | LC589713 | Lao Island, Vietnam | This study                                    |
| C. erythraeus   | C1         | LC101272 | Japan**          | Boonkhaw et al. (2017)                       |
| unidentified   | C2         | LC101273 | Japan**          | Boonkhaw et al. (2017)                       |
| unidentified   | C3         | LC101274 | Japan**          | Boonkhaw et al. (2017)                       |
| unidentified   | C4         | LC101275 | Doi Suthep-Pui National Park, Thailand | Boonkhaw et al. (2017)                  |
| unidentified   | C5         | LC101276 | Doi Suthep-Pui National Park, Thailand | Boonkhaw et al. (2017)                  |
| unidentified   | C6         | LC101277 | Khao Sanam Preang Wildlife Sanctuary, Thailand | Boonkhaw et al. (2017)                  |
| unidentified   | C7         | LC101278 | Japan**          | Boonkhaw et al. (2017)                       |
| unidentified   | OS341      | AB716958 | Cat Tien National Park, Vietnam | Oshida et al. (2013)                       |
| unidentified   | 33         | AB499908 | Tam Dao, Vietnam | Oshida et al. (2011)                        |
| unidentified   | 34         | AB499909 | Tam Dao, Vietnam | Oshida et al. (2011)                        |
| griseimanus     | 63         | AB716959 | Cat Tien National Park, Vietnam | Oshida et al. (2013)                       |
| griseimanus     | 64         | AB716960 | Cat Tien National Park, Vietnam | Oshida et al. (2013)                       |
| griseimanus     | 186        | AB716961 | Vinh Cuu Nature Reserve, Vietnam | Oshida et al. (2013)                       |
MATERIALS AND METHODS

Study site – The study was conducted in Lao Island (15°52′–16°00′ N, 108°22′–108°44′ E), part of the Cham Islands. The Cham Islands consisting of eight small islands, have been recognized by UNESCO as a World Biosphere Reserve since 2009 (main island shown in Fig. 1) and Lao Island with a land area of 13.17 km² and its highest peak is 517 m above the sea level is the largest. Lao Island has only freshwater (Tran 2019). Lao Island is covered with a sub-tropical humid forest consisting of Brownlowia tabularis, Firmiana colorata, Aglaia macrocarpa, Dimocarpus longan ssp., Syzygium jambos, Millettia nigrescens, Heritiera angustata, Syzygium cumini, and Vitex tripinnata (Tran et al. 2018, Tran 2019). Average annual temperature, humidity, and rainfall are 25.6 °C, 82%, and 2000 mm, respectively (Lê 2011).

Collecting – In May 2019 and July 2020, we had 300 trap days for collecting squirrels. We did not set traps every day in the two months because of weather conditions. Instead, we used cage traps (15 cm × 15 cm × 25 cm), which were set on trees about 1–2 m from the ground to collect seven C. finlaysonii specimens from Lao Island: five females (field numbers CLC.2019.05.26, CLC.2019.05.39, CLC.2019.05.40, CLC.2019.05.41, and NTS.2020.212) and two males (CLC.2019.05.38 and CLC.2019.05.42), following methods approved in the Animal Care and Use Guidelines of the American Society of Mammalogists (Sikes et al. 2016). Specimens were deposited in the Department of Vertebrate Zoology, Institute of Ecology and Biological Resources (IEBR), Vietnam Academy of Science and Technology, Hanoi, Vietnam.

External characteristics – We recorded the pelage colour of this squirrel in detail. In addition, we measured head and body length, tail length, hind foot length without a nail, and ear length using tapeline and digital calliper (NTD12-15PMX, Mitsutoyo, Japan) in millimetres (mm) and body mass using in grams (g).

Extraction, amplification and sequencing of DNA – Total genomic DNA was extracted from 99% ethanol-preserved muscle tissue using the DNeasy Blood & Tissue Kit (QIAGEN K.K., Tokyo, Japan). The complete mitochondrial cytochrome-b (Cytb) gene sequence (1140 bases) was amplified using polymerase chain reaction (PCR) with the primer set L14724 5′–GATATGAAAAACCATCGTTG–3′ and H15910 5′–GATTTTTGGTTTAC AAGACCGAG–3′. The 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 performed 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 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 Biosystems, CA, USA). For sequencing, we used the same primers used for the PCR. Purification of PCR products and sequencing were carried out by Mission Biotech Co. Ltd. (Taipei, Taiwan).

Molecular phylogenetic analyses – Sequence alignment was carried out to detect unique haplotypes using the software program DNAsIS (Hitachi, Tokyo, Japan). As a result, we found three complete Cytb haplotypes of three Lao Island squirrels (CLC.2019.05.38, CLC.2019.05.40, and CLC.2019.05.42) and deposited them in the DNA Data Bank of Japan (DDBJ) (Fig. 1). Unfortunately, we did not obtain the complete Cytb sequences of the other specimens because of sample conditions.

Using 39 available partial Cytb sequences (571 bp) (Table 1), we analyzed the phylogenetic position of Lao Island squirrel among C. erythraeus-finlaysonii complex. To root
the phylogenetic trees, we used sequences of *C. caniceps* and *C. inornatus*. For ML and MP analyses, we used the program MEGA version X (Kumar et al. 2018, Stecher et al. 2020). The BIC test selected the Hasegawa-Kishino-Yano model of substitution (Hasegawa et al. 1985) with a gamma distribution (2.85) for variable sites (0.59) (HKY + Γ + I) for maximum likelihood (ML) analysis. Base frequencies were estimated as A = 0.256, C = 0.307, G = 0.140 and T = 0.297. The rate matrix was estimated as A-C and G-C = 0.010, A-G = 0.131, A-T and G-T = 0.009, T-A = 0.008, T-C = 0.288, C-G and T-G = 0.004 and G-A = 0.240. We also conducted unweighted maximum parsimony (MP) analysis. The MP tree was constructed using tree-bisection-regrafting (TBR). Additionally, we conducted neighbour-joining (NJ) using the Kimura 2-parameter method (Kimura 1980). Bootstrapping (Felsenstein 1985) was performed with 1000 replications in ML, MP, and NJ analyses for the assessment of nodal supports. Bayesian inference (BI) was performed using BEAST 1.10.4 (Suchard et al. 2018). Bayesian analysis also used the HYK + Γ + I substitution model and involved two runs for ten million steps, using four Markov chain Monte Carlo chains sampling every 10000 steps and a burn-in of 10%. Posterior probabilities assessed the nodal support of the BI tree.

RESULTS

**External characters**

Photographs of one of the Lao Island squirrel is shown in Fig. 2. All specimens were almost similar in pelage colour. From the muzzle to the ears and around the cheeks were honey-yellow (dark orange) on the rostrum. Ears were dark orange (or buff) on both sides. Dorsal pelage was agouti light grey with black colour at the base and two pale brown bands in the middle. Ventral is agouti bright orange (or buff) consisting of two bands, dark greyish base-band and light orange band at middle with darker fur tip. Feet were bright orange, similar to the ventrum. The tail is agouti light brown consisting of four light bands and three dark bands. The head and body length, tail length, hind foot length, and ear length were 200.0–205.0 mm, 197.5–207.5 mm, 38.8–42.5 mm, and 18.5–20.2 mm, respectively. Body weight was 195–201 g.

**Molecular phylogenetic analysis**

The ML, MP, NJ, and BI trees of the *C. erythraeus-finlaysonii* complex somehow differed in branching patterns but were essentially similar to each other (Fig. 3). Within the *C. erythraeus-finlaysonii* complex, Lao Island squirrel formed a cluster and was closely related to *C. finlaysonii* with external characters of *C. erythraeus flavimanus*; this form was tentatively defined as ‘*C. finlaysonii* morpha *flavimanus* (lineage G10)’ by Balakirev and Rozhnov (2019). Moreover, the cluster consisting of Lao Island squirrel and lineage 10 was closely related to a cluster (lineage G9) consisting of *C. erythraeus griseimanus* and *C. finlaysonii* with external characters of *C. erythraeus griseimanus* defined tentatively as ‘*C. finlaysonii* morpha *griseimanus*’ by Balakirev and Rozhnov.
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Fig. 2. A Callosciurus squirrel (field number NTS.2020.212) from Lao Island, Vietnam: A = dorsal pelage, B = lateral pelage, C = ventral pelage, D = agouti band of dorsal fur, E = agouti band of tail fur, F = agouti band of ventral fur, G = left forelimb fur and H = left hindlimb fur
These phylogenetic relationships were supported with high bootstrap values and posterior probabilities (Fig. 3).

Fig. 3. Neighbour joining (NJ) phylogeny of squirrels of *Callosciurus erythraeus-finlaysonii* complex constructed using mitochondrial partial cytochrome-*b* sequences under the Kimura 2-parameter-model of evolution. From left, numbers above branches represent bootstrap values from: 1000 replicates of NJ, maximum likelihood (ML) and unweighted maximum parsimony (MP) analyses, and posterior probability supports in Bayesian (BI) analysis. G9 and G10 are phylogenetic group described previously by Balakirev and Rozhnov (2019).
DISCUSSION

Phylogenetic analyses showed that Lao Island squirrel and *C. finlaysonii* morpha *flavimanus* clustered together. Among *C. erythraeus-finlaysonii* complex, *Callosciurus finlaysonii* morpha *flavimanus*, distributed from central to southern Vietnam, is geographically most close to Lao Island squirrel. The pelage colour of Lao Island squirrel was different from those of *C. finlaysonii* morpha *flavimanus*. Moore & Tate (1965) described the pelage colour of *flavimanus* as follows. The rostrum and fore feet are orange-yellow, and hind feet are reddish-orange. Venter varies among individuals from mars orange to burnet sienna, which extends from wrist to ankle and covers the venter except for the agouti scrotum and chin. Compared to the *flavimanus*, Lao Island squirrel shows a uniquely dark orange cheek and forehead. The pelage colour of Lao Island squirrel was also different from those of *C. finlaysonii* morpha *griseimanus*. The feet of *griseimanus* are white cream buff or cartridge buff (Moore & Tate 1965), but those of Lao Island squirrel were bright orange.

After the last glacial maximum, Lao Island squirrel could be geographically isolated from the mainland population by the rise of sea level. Several researchers have reported that members of *C. erythraeus* and *C. finlaysonii* are easily confused taxonomically (Boonkhaw et al. 2017, Balakirev & Rozhnov 2019). This is because members of *C. erythraeus* and *C. finlaysonii* have many pelage colour variations (Lekagul & McNeeley 1988, Koprowski et al. 2016). It is difficult to explain how the pelage colour variations have been created during evolution. Lao Island squirrel was evidently different from *Callosciurus finlaysonii* morpha *flavimanus* in the colour of cheeks. The external character, such as coat colour may have evolved on the isolated island in a short time. In the Gulf of Thailand, some *C. finlaysonii* subspecies with each unique coat colour are confined to each isolated island: *albivexilli*, *finlaysonii*, *folletti*, *frandseni*, *germaini*, *harmandi*, and *trotteri* are distributed on Koh Kut, Koh Si-Chang, Koh Phai, Koh Chang, Con Son, Phu Quoc, and Koh Lan, respectively (Milne-Edwards 1877, Kloss 1915, 1916a, 1916b, 1916c, Koprowski et al. 2016). Therefore, it can be evidence that pelage colour variations of *C. erythraeus-finlaysonii* complex may be the result of geographic isolation. Although the isolated island populations (*C. f. folletti*, *C. f. trotteri*, and *C. f. finlaysonii*) are closely related to the continental populations occurring in the nearby continent (*C. f. cinnamomeus* and *C. f. nox*), both populations evidently differ in pelage colour; the island populations have white or beige coat colours, while *C. f. cinnamomeus* and *C. f. nox* are reddish and black, respectively (Boonkhaw et al. 2017). In addition, *C. f. albivexilli* and *C. f. frandseni* differ from the continental populations in pelage color, and also form a distinct genetic cluster (Boonkhaw et al. 2017, Balakirev & Rozhnov 2019).
In *C. finlaysonii*, however, the individual differences of pelage colour are often observed even within subspecies (e.g. Koprowski *et al.* 2016). In fact, in *Callosciurus* squirrels, the genetic mechanism to produce pelage colour variation has not yet been studied. Therefore, in further study, we also need to clarify how the genetic mechanism contributes to creating pelage colour variation of *C. finlaysonii*.

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