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A preliminary study on origin of *Callosciurus* squirrels introduced into Japan

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Abstract. To examine the origin of *Callosciurus erythraeus* introduced to Japan, we compared mitochondrial DNA control region sequences of eight haplotypes from Japan with those of 42 haplotypes from Taiwan. There were two distinct phylogroups in Japan. Six haplotypes from Japan were included in the Taiwan population, suggesting that they could have been introduced from Taiwan. Two haplotypes, however, were distantly related to the cluster consisting of all Taiwanese haplotypes and six Japanese haplotypes. The uncorrected genetic distances (5.2–5.7%) between these two haplotypes and the out-group (*Callosciurus finlaysonii* from Laos) were less than those (8.3–9.8%) between these two haplotypes and the cluster consisting of the other six Japanese haplotypes and all Taiwanese haplotypes. Both *C. erythraeus* and *C. finlaysonii* have variable external characteristics, such as pelage color, and include many variable subspecific forms, so that it is difficult to evidently identify both species on the basis of their external characteristics. *Callosciurus finlaysonii* may have been also introduced in Japan with both species now coexisting in Japan.

Key words: alien squirrel, *Callosciurus erythraeus*, *Callosciurus finlaysonii*, mitochondrial DNA control region, Taiwan.

Biological invasion is one of most important causes of bio-diversity loss (e.g. Diamond 1989; Alonso et al. 2001). Many invasions have led to essential changes in species composition, habitat structure, and ecosystem processes (e.g. Elton 1958; Simberloff 1991; Williamson 1998). Alien arboreal squirrels may cause severe damage to forests and commercial tree plantations (Rowe and Gill 1985; Dagnall et al. 1998) and the progressive disappearance of native squirrel species (Reynolds 1985; Gurnell and Pepper 1993; Bertolino and Genovesi 2003). To prevent such drastic changes by invasive squirrel species, it is first important to ascertain how the species was introduced from place of its origin. If we could identify the route of introduction, we could manage further invasions, even if the species had already been introduced for a long time.

The Pallas's squirrel (*Callosciurus erythraeus*) was originally distributed in eastern India, Bhutan, southeast China, Taiwan, Indochina, and Malaya (Corbet and Hill 1992; Wilson and Reeder 2005). This squirrel is arboreal and inhabits montane forests, cultivated areas, and even in gardens (Nowak 1991). This squirrel was exported to other countries as an exotic pet. As a result of this commercial activity, this species is now found in Argentina (e.g. Aprile and Chicco 1999), France (Jouanin 1986; Mitchel-Jonés et al. 1999), and Japan (Abe et al. 2005). It is considered serious threat to forests and tree plantations (Jouanin 1986; Setoguchi 1990; Aprile and Chicco 1999).

Callosciurus erythraeus has recently increased in

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numbers in Japan (Ozaki 1986; Torii 1989, 1993; Tamura and Ohara 2005). This squirrel is thought to have been introduced into central to southwestern parts of Japan from Taiwan (Tamura 2002; Abe et al. 2005). However, it is doubtful that the origin of this Japanese population was only Taiwan. Since this species is a common pet in the Southeast Asian countries such as Vietnam and Thailand, we may find several pathways of introduction into Japan.

To test whether *C. erythraeus* of Japan was originated from only Taiwan population, we compared the mitochondrial (mt) DNA control region sequences of *C. erythraeus* of Japan with those of Taiwan. This molecular marker is useful to for resolving phylogeographical characteristics of squirrels (Barratt et al. 1999; Oshida et al. 2001a). Also, the phylogeographical structure of *C. erythraeus* population in Taiwan was identified by using mtDNA control region sequences (Oshida et al. 2006). If all individuals of Japan are included in phylogroups of the Taiwan *C. erythraeus*, we can consider that the Japanese population was derived from the Taiwanese population. If, however, we find clear genetic differences between the Japanese and Taiwanese populations, the Japanese population could not have been introduced from Taiwan and its place of origin remains unknown. We discuss whether the Japanese population was derived from Taiwan.

Materials and methods

Specimens

Seventeen *C. erythraeus* specimens from 5 localities (Izu-oshima Island, Hamamatsu, the Izu Peninsula, Miyazaki, and Fukue Island) of Japan were examined in this study (Table 1 and Fig. 1).

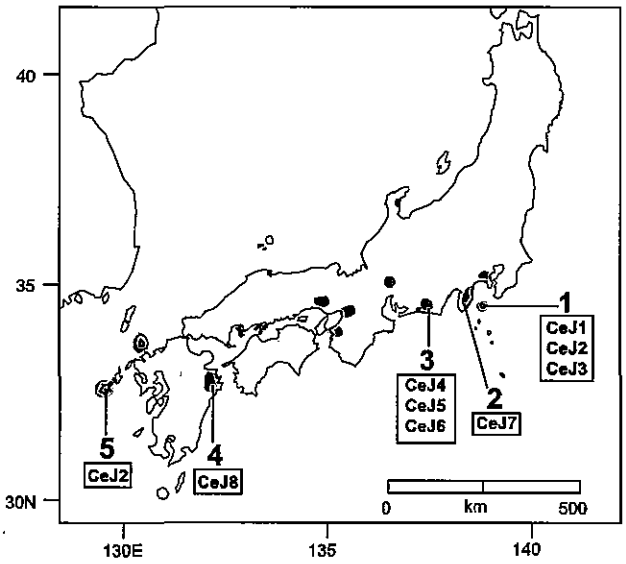

Fig. 1. Sampling localities of the Pallas's squirrel (*Callosciurus erythraeus*) in Japan. Shaded areas show the distribution of *C. erythraeus* (Abe et al. 2005). Locality numbers 1 (Izu-oshima Island), 2 (the Izu Peninsular), 3 (Hamamatsu), 4 (Miyazaki), and 5 (Fukue Island) correspond to those in Table 1. Haplotypes identified were shown in boxes.

Table 1. Specimens of *Callosciurus erythraeus*, *C. finlaysonii*, and *C. prevostii* examined in this study.

Species	Haplotype (linage)	Identity number	Number of individual	Locality number (name)	Accession number
C. erythraeus	CeJ1 (B)	182	1	1 (Izu-ohshima Island)	AB259592
	CeJ2 (B)	183, 185, 186	3	1 (Izu-ohshima Island)	AB259593
		273, 274	2	5 (Fukue Island)	
	CeJ3 (B)	184	1	1 (Izu-ohshima Island)	AB259594
	CeJ5 (B)	190	1	3 (Hamamatsu)	AB259596
	CeJ7 (B)	192	1	2 (Izu Peninsular)	AB259598
	CeJ8 (B)	275	1	4 (Miyazaki)	AB259599
C. finlaysonii	CeJ4 (A)	187, 188, 189, 191, 507, 508	6	3 (Hamamatsu)	AB259595
	CeJ6 (A)	506	1	3 (Hamamatsu)	AB259597
	Cfi1	M31312*	1	nearby Vientianne, Laos	AB259600
	Cfi2	M31313*	1	nearby Vientianne, Laos	AB259601
C. prevostii	Cpr1	311	1	Sumatra Island, Indonesia	AB259602
	Cpr2	312	1	Sumatra Island, Indonesia	AB259603

Haplotypes and accession numbers are for the mtDNA control region sequences. Locality numbers 1–5 match those in Fig. 1. Specimens of Taiwan are not included. * Specimens number of the National Science Museum, Tokyo, Japan.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from 99% ethanol-preserved muscle tissue with the phenol-chloroform method and suspended in Tris-EDTA (TE) buffer (Sambrook et al. 1989). The mtDNA control region sequence was amplified using polymerase chain reaction (PCR) with primers reported by Oshida et al. (2001b): L15933 5'-CTCTGGTCTTGTAAACCAAAAATG-3' and H637 5'-AGGACCAAACCTTTGTGTTTATG-3'. Primer names correspond to the light (L) or heavy (H) strand and the 3'end-position of the primers in the human mtDNA sequence (Anderson et al. 1981). 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 $MgCl_2$, and 2.5 units of r*Taq* DNA polymerase (Takara). Amplification was carried out for 35 cycles using the following cycle program: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The extension reaction was completed by incubation at 72°C for 10 min. The PCR products were purified with PCR Clean Up-M (Viogen) and directly sequenced using an automated DNA sequencer (PRISM 377-96 Sequencer and PRISM 3100 Genetic Analyzer Applied Biosystem, ABI). The PCR primers and one internal primer (L-cer 5'-CGGCACATACCCCATTCAGTC-3') reported by Oshida et al. (2006) were used for sequencing. Purification of PCR products and sequencing were done by Mission Biotech Co. Ltd. (Taipei). To root in the phylogenetic tree, we also determined control region sequences of two *Callosciurus finlaysonii* specimens from Laos (Table 1). In phylogenetic analysis based on the cytochrome *b* gene sequence, this species is most closely related to *C. erythraeus* (Oshida et al. 2001b), making it a suitable out-group. Also, to carefully ascertain phylogenetic relationships between *C. erythraeus* and *C. finlaysonii* and between phylogroups of *C. erythraeus*, we used the out-group (*Callosciurus prevostii*) distantly related to both *C. erythraeus* and *C. finlaysonii* (Oshida et al. 2001b).

Sequence and phylogeographic analyses

Sequence alignment was carried out using the software program DNASIS (Hitachi, Tokyo, Japan). In this alignment, the 43 haplotype sequences of *C. erythraeus* of Taiwan reported by Oshida et al. (2006) were included (Table 2). We recognized 14 gap-sites to generate most consensus sequence alignments, but in all analyses, gap-sites were excluded. Modeltest version 3.06 (Posada and Crandall 1998) was used to find the nucleotide substitution model that best fit the data. The hierarchical likelihood ratio tests (hLRT) without out-group, implemented in Modeltest, selected the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985). This model included the proportion of invariable sites and the gamma shape parameter. Neighbor-joining (NJ) analysis (Saitou and Nei 1987) was conducted with the Hasegawa-Kishino-Yano model taking into account the proportion of invariable sites and following a gamma distribution for variable sites in PAUP* 4.0b10 (Swofford 2001). To assess the nodal supports, bootstrapping (Felsenstein 1985) was performed with 5,000 replicates.

Results

Complete sequences (1,076–1,081 bp) of the mtDNA control region were successfully determined from 17 individuals of *C. erythraeus* from Japan, resulted in eight unique haplotypes. All sequences were deposited in the DNA Data Bank of Japan (DDBJ) (Table 1). Of all haplotypes including the closer out-group (*C. finlaysonii*), there were 890 constant sites, 38 sites were parsimony-uninformative, and 155 were parsimony-informative.

The rooted neighbor-joining tree with Hasegawa-Kishino-Yano model, a gamma distribution, and proportion of invariable sites, including the closer out-group (*C. finlaysonii*), showed two distinct mtDNA lineages: lineage A consisting of haplotypes CeJ4 and CeJ6 and lineage B consisting of the other all haplotypes: CeJ1, CeJ2, CeJ3, CeJ5, CeJ7, and CeJ8 (Fig. 2). Both lineages were supported with a 100% bootstrap value. The uncorrected genetic distance between lineages A and B ranged from 8.3 to 9.8%. In lineage B, there were four geographical phylogroups (northern, western, eastern, and southern phylogroups) as reported by Oshida et al. (2006). Three haplotypes, CeJ1, CeJ5, and CeJ8, were included in the eastern phylogroup, showing a single cluster (lineage B-I) with 99% nodal support (Fig. 2). The other three haplotypes (CeJ2, CeJ3, and CeJ7) were not included in any of the four phylogroups, forming a single cluster (lineage B-II) with 100% nodal support (Fig. 2). When all hypothesized gap sites were excluded, the average numbers of nucleotide differences between two haplotypes (*K*, Tajima 1983) in lineage A and in lineage B were 0 and 16.2, respectively. To confirm the phylogenetic relationship among lineage A, *C. finlaysonii*, and *C. erythraeus*, we further reconstructed the NJ tree under the Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) with gamma shape parameter and

Table 2. MtDNA control region sequences of *Callosciurus erythraeus* of Taiwan used in this study.

Haplotype	Phylogroup	Identity number	Accession no.
Ce01	Northern	NMNS3748	AB181249
Ce02	Northern	NMNS6422	AB181250
Ce03	Northern	NMNS4244	AB181251
Ce04	Northern	NMNS5401	AB181252
Ce05	Northern	NMNS1191	AB181253
Ce06	Northern	NMNS5439	AB181254
Ce07	Northern	NMNS6406	AB181255
Ce08	Northern	NMNS842, NMNS1345	AB181256
Ce09	Northern	NMNS1184, NMNS4366, NMNS6408	AB181257
Ce10	Northern	NMNS1905, NMNS6609	AB181258
Ce11	Northern	NMNS5280, NMNS5383	AB181259
Ce12	Northern	NMNS1025, NMNS1178, NMNS6616	AB181260
Ce13	Western	NMNS5440	AB181261
Ce14	Western	NMNS6410, NMNS6413, NMNS6597	AB181262
Ce15	Western	NMNS6606	AB181263
Ce16	Western	NMNS278, NMNS6614	AB181264
Ce17	Western	NMNS5281, NMNS5402	AB181265
Ce18	–	NMNS5393	AB181266
Ce19	Western	NMNS5381	AB181267
Ce20	Eastern	NMNS5436	AB181268
Ce21	Eastern	NMNS5419	AB181269
Ce22	–	NMNS5420, NMNS5421	AB181270
Ce23	Eastern	NMNS5426	AB181271
Ce24	Western	NMNS5380	AB181272
Ce25	–	NMNS6415	AB181273
Ce26	Eastern	NMNS5414, NMNS6595	AB181274
Ce27	Eastern	NMNS5418, NMNS5425, NMNS5437, NMNS5438	AB181275
Ce28	Eastern	NMNS5424, NMNS5432, NMNS5434, NMNS5435	AB181276
Ce29	Eastern	NMNS6612	AB181277
Ce30	Eastern	NPUST15	AB181278
Ce31	Southern	NMNS5415, NMNS5416	AB181279
Ce32	Eastern	NMNS5423	AB181280
Ce33	Eastern	NMNS5422	AB181281
Ce34	–	NMNS5417, NPUST14	AB181282
Ce35	Eastern	NMNS5406, NMNS5287	AB181283
Ce36	Southern	NMNS5283, NMNS5396, NMNS6412	AB181284
Ce37	Southern	NMNS5403, NMNS5407, NMNS5399	AB181285
Ce38	Eastern	NMNS6611	AB181286
Ce39	Western	NMNS6610	AB181287
Ce40	Eastern	NMNS6615	AB181288
Ce41	Eastern	NMNS6603, NMNS6613	AB181289
Ce42	Western	NMNS6805	AB181290
Ce43	Western	NMNS6806, NMNS6834	AB181291

Identity numbers indicate the specimens from the National Museum of Natural Science, Taichung, Taiwan (NMNS) and the National Pingtung University of Science and Technology, Pingtung, Taiwan (NPUST). Phylogroups were definded by Oshida et al. (2006). Four haplotypes do not belong to any phylogroup (–).

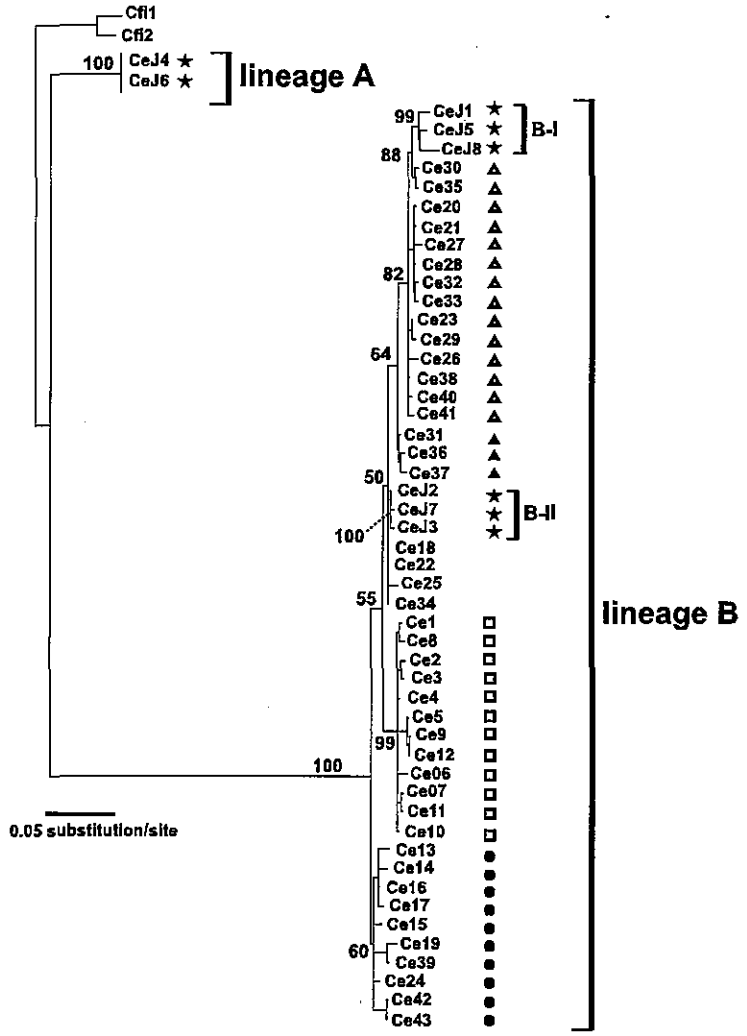

Fig. 2. Neighbor-joining tree showing phylogenetic relationships among the 51 mtDNA control region haplotypes of the Pallas's squirrels (*Callosciurus erythraeus*) from Taiwan and Japan, based on the Hasegawa-Kishino-Yano model with gamma shape parameter and proportion of invariable sites. Bootstrap supports are given on branches. Four mtDNA phylogroups from Taiwan are indicated: northern (open squares), western (solid circles), southern (solid triangles), and eastern (open triangles). The haplotypes from Japan are shown by solid stars.

proportion of invariable sites by PAUP* 4.0b10 (Fig. 3). Of all haplotypes including new out-group, there were 773 constant sites, 41 sites were parsimony-uninformative, and 252 were parsimony-informative. In this analysis, *Callosciurus prevostii* was used as a distantly related out-group (Table 1), and all gap-sites were excluded. In this NJ tree (Fig. 3), lineage A is closely related to *C. finlaysonii*, forming a single cluster with 98% nodal support.

Discussion

In Japan, there were at least two mtDNA lineages of *C. erythraeus* (Fig. 2). One lineage was closely related to Taiwanese population. Of this lineage, lineage B-I could have been introduced into Japan from the northeastern part of Taiwan, the location of the eastern phylogroup of Taiwanese population (Oshida et al. 2006). The origin of lineage B-II was unclear. Although both B-I and B-II lineages were included in Taiwan cluster, to exactly specify the origin of B-II, we would require analyzing more sequence data of *C. erythraeus* from different areas such as India, China, and Indochina Peninsular.

The uncorrected genetic distance between lineages A and B ranged from 8.3 to 9.8%. Interestingly, the uncorrected genetic distance between lineage A and the outgroup was 5.2–5.7%. Therefore, lineage A may be more closely related to *C. finlaysonii* rather than lineage B.

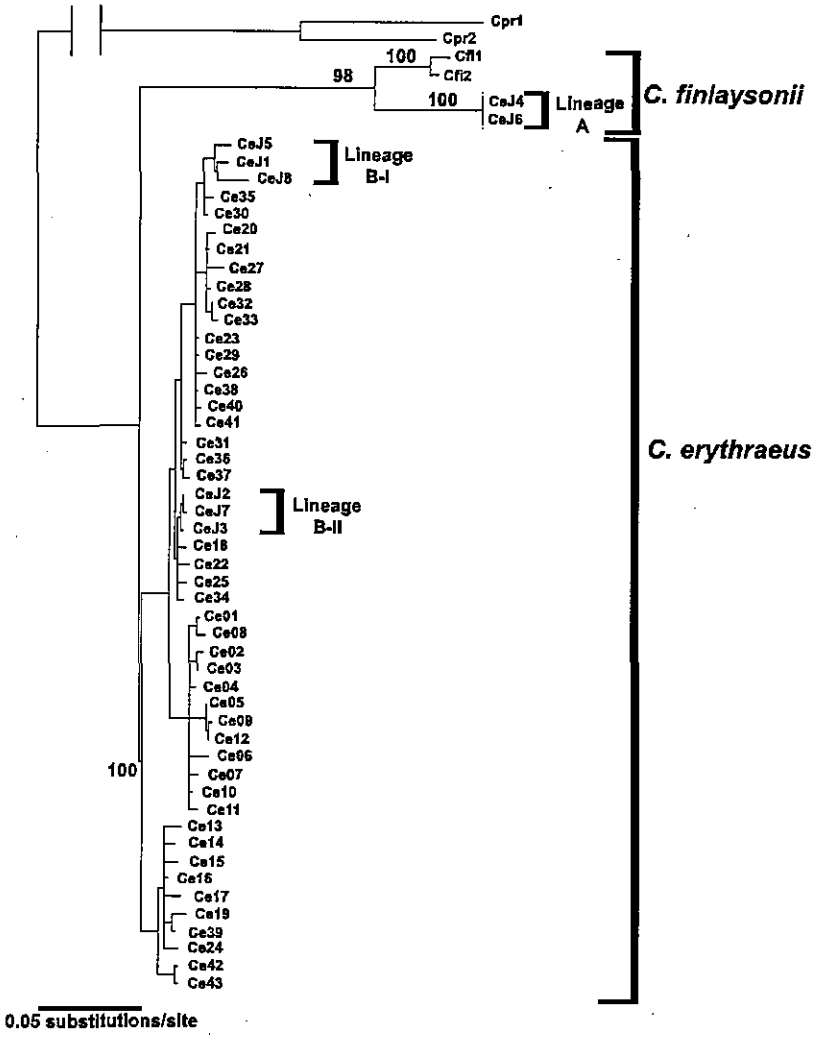

Fig. 3. Neighbor-joining tree showing phylogenetic relationships among the 51 mtDNA control region haplotypes of *Callosciurus* squirrels from Taiwan and Japan, based on the Hasegawa-Kishino-Yano model with gamma shape parameter and proportion of invariable sites. Bootstrap supports are given on branches.

Callosciurus squirrels are classified into 15 species based on morphological features such as baculum which has a sharp-edged accessory blade attached to its dorsal surface (Corbet and Hill 1992). Each species is intricately subdivided into several subspecies on the basis of pelage variations (Moore and Tate 1965; Lekagul and McNeely 1988; Corbet and Hill 1992; Wilson and Reeder 2005).

Actually, the many external variations of each species make difficult to clearly identify *Callosciurus* species and subspecies. Furthermore, hybridization or gene introgression between subspecies, and even between species, may occasionally take place. Therefore, specimens of lineage A may be regarded as *C. finlaysonii* or as hybrids between *C. finlaysonii* and *C. erythraeus*, although their external characteristics were more similar to those of *C. erythraeus*. In the NJ tree (Fig. 3) including the out-group (*C. prevostii*) distantly related to *C. erythraeus* and *C. finlaysonii*, lineage A is closely related to *C. finlaysonii*, forming a single cluster with 98% nodal support. This suggests that lineage A could be identified as a *C. finlaysonii*. There is no evidence that any *C. finlaysonii* introduced to Japan were released into the field. This species was certainly sold as a pet in Japan (e.g. Yanagawa 2000). If these specimens are derived from *C. finlaysonii* species that become wild, there could be a serious environmental problem. In Italy, the population of introduced *C. finlaysonii* is increasing, causing damage to natural conifer and broadleaf forests (Bertolino et al. 1999; Mitchell-Jones et al. 1999). In

addition, its bark-stripping behavior and fruit consumption could lead to significant commercial damage in the plantation forests (Bertolino et al. 2004). In Japan, this species may also increase and seriously damage forest resources. *Callosciurus finlaysonii* is distributed in Thailand, Cambodia, and Laos (Corbet and Hill 1992), but not in Taiwan. To avoid further imports this species into Japan, first we should consider the route from the Indochina Peninsula to Japan. Second, we should precisely identify its distribution range in Japan. Third, we need to find out whether it has caused forest damage or ecological disturbance in the natural environments of Japan. These studies should carefully distinguish the effects of *C. finlaysonii* from those of *C. erythraeus*. To identify the species, molecular markers such as mitochondrial DNA sequence are useful. Further studies using sequence data of *C. finlaysonii* in the Indochina Peninsula may be able to specify the origin of the Japanese population.

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