

Mitochondria DNA sequences of Finlayson's squirrel found in Hamamatsu, Shizuoka Prefecture, Japan

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Alien *Callosciurus* squirrels cause severe damage to trees in forests, gardens, and commercial plantations (Jouanin 1986; Setoguchi 1990; Torii 1993; Aprile and Chicco 1999; Gurnell and Wauters 1999). Pallas's squirrel (*Callosciurus erythraeus*) originally occurs in eastern India, Bhutan, southeast China, Taiwan, Indochina, and Malaya (Corbet and Hill 1992; Wilson and Reeder 2005). This squirrel was introduced into other countries as an exotic pet. At present, *C. erythraeus* populations are found in Argentina (e.g., Aprile and Chicco 1999), France (Jouanin 1986; Gurnell and Wauters 1999), and Japan (Ishii 2005; Tamura 2002, 2009; Ikeda et al. 2011). Under law number 78 of the Invasive Alien Species Act (Ministry of the Environment, Government of Japan, 2004: <http://www.env.go.jp/nature/intro/index.html>), import of *C. erythraeus* to Japan is strictly prohibited. This species is treated as 'invasive alien species' in Japan.

Finlayson's squirrel (*C. finlaysonii*), which originally occurs in the Indochina Peninsula (Corbet and Hill 1992; Wilson and Reeder 2005), was also introduced to Italy (Bertolino et al. 1999; Currado et al. 1999). Similar to *C. erythraeus*, *C. finlaysonii* is thought to cause damage to environments (Bertolino et al. 2004). It is difficult to distinguish this species from *C. erythraeus* based on external characteristics such as pelage patterns (e.g., Lekagul and McNeely 1988). Based on mitochondrial (mt) DNA control region sequences, Oshida et al. (2007) reported that *C. finlaysonii* was probably introduced to Hamamatsu, Shizuoka Prefecture, Japan (Fig. 1). They captured eight *Callosciurus* individuals in Hamamatsu. Of them, seven individuals had haplotypes closely related to that of *C. finlaysonii* from Laos (two *C.*

finlaysonii haplotypes were detected), and one individual had a *C. erythraeus* haplotype. Therefore, we expect these two *Callosciurus* species exist sympatrically in Hamamatsu. *Callosciurus finlaysonii*, however, is not treated as an invasive alien species in Japan (law number 78 of the Invasive Alien Species Act, Ministry of the Environment, Government of Japan, 2004). Currently this species is treated as unidentified alien species. If the population of *C. finlaysonii* increases, this species should be added to the list of invasive alien species.

To estimate whether *C. finlaysonii* is increasing, we investigated ratios of *C. finlaysonii* haplotype and *C. erythraeus* haplotype in the Hamamatsu population by using mtDNA control region sequences. We discuss the present situation of introduced *C. finlaysonii* in Hamamatsu. In addition, we compare control region sequences of specimens from Hamamatsu, Laos and Thailand, to identify the origin of introduced *C. finlaysonii*. The exact origin would be difficult to determine because we do not have sequence data from all *C. finlaysonii* populations occurring in the Indochina Peninsula. We, however, obtained successfully *C. finlaysonii* sequences from Thailand and Laos. Identifying whether the Hamamatsu population is more closely related to specimens from Thailand or Laos may help to prevent subsequent introductions of this squirrel into Japan with international legal and political action.

Materials and methods

Specimens, DNA extraction, amplification, and sequencing

From 2004 to 2006, 83 *Callosciurus* squirrels were collected from 19 sites in Hamamatsu, Shizuoka Prefec-

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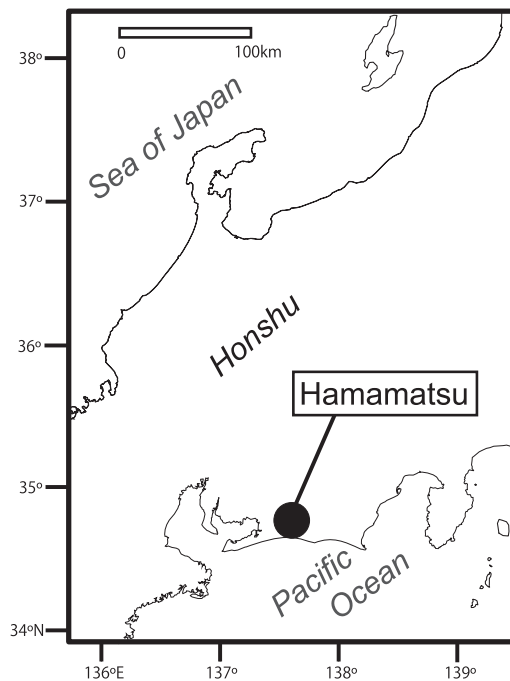


Fig. 1. Hamamatsu, Shizuoka Prefecture, Japan, where of *Callosciurus* squirrels are found.

ture, Japan (Appendix 1). One *C. finlaysonii boonsongi* was obtained from a pet store in Bangkok, Thailand. Total genomic DNA was extracted from muscle tissues using the QuiaQuick Kit (QUIAGEN K.K.).

The mtDNA control region sequence was amplified using polymerase chain reaction (PCR) with primers reported by Oshida et al. (2001a): L15933 5'-CTCTGGTCTTGTAACCAAAAATG-3' and H637 5'-AGGCCAAACCTTTGTGTTTATG-3'. Primer names cor-

respond 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 μ l reaction mixture contained 100 ng of genomic DNA, 25 pM of each primer, 200 μ M dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 2.5 units of *rTaq* 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). For sequencing, we used PCR primer L15933 and two internal primers L-cer (5'-CGGCACATACCCCATTCAGTC-3') reported by Oshida et al. (2006) and L-cfb (5'-AGTCATAAACCTTTCTTGTGCC-3') newly designed for this study. Purification of PCR products and sequencing were done by the Mission Biotech Co. Ltd. (Taipei).

Sequence and phylogeographic analyses

Sequence alignment was carried out using the software program DNASIS (Hitachi). We included control region sequences reported in Oshida et al. (2007) for two *C. finlaysonii menamicus* specimens from Laos (haplotypes Cfi1 and Cfi2), six *C. erythraeus* haplotypes from Japan (CeJ1, CeJ2, CeJ3, CeJ5, CeJ7, and CeJ8), and two *C. finlaysonii* haplotypes from Japan (CeJ4 and CeJ6) (Table 1). Gap-sites were excluded. Program Modeltest 3.06 (Posada and Crandall 1998) selected the

Table 1. Mitochondrial DNA control region sequences of *Callosciurus* squirrels used in the present study

Species	Haplotype	Locality	Accession number
<i>C. finlaysonii</i>	CeJ4*	Hamamatsu, Japan	AB259595
	CeJ6	Hamamatsu, Japan	AB259597
	CfJ1*	Hamamatsu, Japan; Thailand	AB621590
	Cfi1	Laos	AB259600
	Cfi2	Laos	AB259601
<i>C. erythraeus</i>	CeJ1	Japan	AB259592
	CeJ2	Japan	AB259593
	CeJ3	Japan	AB259594
	CeJ5	Japan	AB259596
	CeJ7	Japan	AB259598
	CeJ8	Japan	AB259599
<i>C. prevostii</i>	Cpr1	Sumatra Island, Indonesia	AB259602

Asterisks indicate haplotypes observed in Hamamatsu, Japan in the present study.

most appropriate substitution model of molecular evolution using the outgroup by the Akaike information criterion (AIC) in PAUP*, version 4.0 beta10 (Swofford 2001). This test selected the Tamura-Nei (TrN) model (Tamura and Nei 1993), taking into account the proportion of invariable sites ($I = 0.615$). Bayesian reconstruction was carried out using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Bayesian analysis was conducted using the TrN substitution model selected by Modeltest with outgroup. Analysis involved two runs of one million iterations using four Markov chain Monte Carlo (MCMC) chains sampling every 100 generations and a burn-in of 20%. A 50% majority rule consensus tree was generated based on the remaining trees. Posterior probabilities assessed nodal support of the Bayesian tree. Maximum parsimony analysis was conducted with the heuristic search approach using the tree-bisection-reconnection (TBR) swapping algorithm, random addition, and 10 replicates in the PAUP*. All characters were equal-weighted. Bootstrapping (Felsenstein 1985) was performed with 10,000 replicates for MP reconstruction. To root both Bayesian and MP trees, we used the Prevost's squirrel (*C. prevostii*) as outgroup. Phylogenetic analysis based on the cytochrome *b* gene sequence shows that not only *C. erythraeus* and *C. finlaysonii* are closely related, but they are also commonly distant from *C. prevostii* (Oshida et al. 2001b, 2010). All accession numbers are in the DNA Data Bank of Japan (DDBJ) and are shown in Table 1.

Results

Complete sequences (1,078 bases) of the mtDNA control region were obtained from 83 *Callosciurus* squirrels from Hamamatsu and one *C. finlaysonii boonsongi* from Thailand. All squirrels from Hamamatsu had *C. finlaysonii* haplotypes. There were no *C. erythraeus* sequences in this population. We identified two haplotypes in the Hamamatsu population (Table 1): 61 individuals had haplotype CeJ4 reported by Oshida et al. (2007) and 22 individuals had haplotype CfJ1. Sequence for the newly identified CfJ1 was deposited in the DDBJ (Table 1). The *C. finlaysonii boonsongi* from Thailand had haplotype CfJ1. We did not find the *C. finlaysonii* haplotype CeJ6 reported by Oshida et al. (2007). Among all haplotypes, including the outgroup (*C. prevostii*), there were 790 constant sites, 133 parsimony-uninformative variable sites, and 132 parsimony-informative sites.

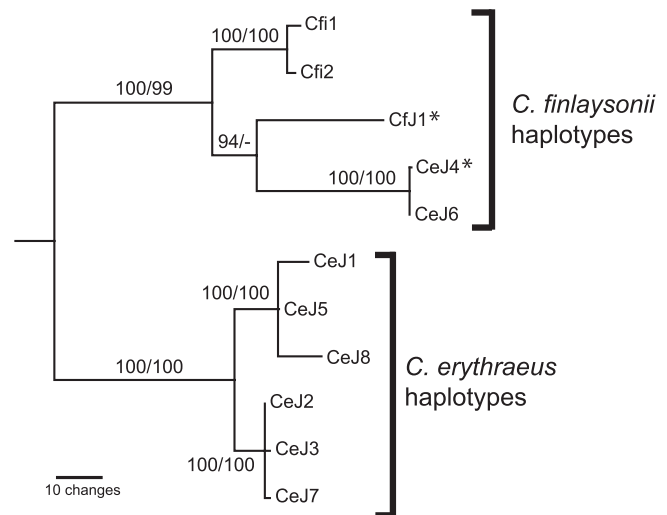


Fig. 2. Bayesian tree showing phylogenetic relationship among mtDNA control region haplotypes identified from *Callosciurus* squirrels based on the TrN + I model. Posterior probability supports in Bayesian analyses (left of slash) and bootstrap support in MP analyses (right of slash) are given on branches; hyphen indicates no data. Bar under tree correlates the length of branch to number of substitutions (changes). Asterisks indicate haplotypes found in Hamamatsu, Japan, in the present study.

Branching patterns of the Bayesian tree (Fig. 2) were very similar to those of the MP tree (data not shown). In the MP analysis, the most parsimonious tree had the length, consistency index, retention index, and homoplasy index of 335, 0.863, 0.879, and 0.137, respectively. In both Bayesian and MP trees, there were two main clusters: a *C. erythraeus* cluster consisting of haplotypes CeJ1, CeJ2, CeJ3, CeJ5, CeJ7, and CeJ8 and a *C. finlaysonii* cluster consisting of haplotypes Cfi1, Cfi2, CfJ1, CeJ4, and CeJ6 (Fig. 2). Bayesian analysis supported these clusters with high posterior probabilities (100%). The high bootstrap values also supported a *C. finlaysonii* cluster (99%) and a *C. erythraeus* cluster (100%) in MP analysis (Fig. 2). In the *C. finlaysonii* cluster, the two haplotypes from Laos clustered together with high nodal supports (100% in both trees). The Bayesian tree showed a cluster of three haplotypes from Japan and Thailand with a high posterior probability (94%). The MP tree, however, showed a polytomic relationship consisting of three groups: 1) Cfi1 and Cfi2, 2) CfJ1, and 3) CeJ4 and CeJ6.

Discussion

Unlike results report by Oshida et al. (2007), there was no *C. erythraeus* haplotype in Hamamatsu. Also,

we did not find *C. finlaysonii* haplotype CeJ6. Squirrels with *C. finlaysonii* haplotype CeJ4 accounted for more than 70% of the Hamamatsu population. Newly found *Callosciurus finlaysonii* haplotype CfJ1 was present in 25% of the specimens. Almost *Callosciurus* squirrels living in Hamamatsu probably have *C. finlaysonii* mtDNA sequences, but we cannot conclude that all *Callosciurus* squirrels analyzed in this study were *C. finlaysonii*. Timmins and Duckworth (2008) suggested that *C. finlaysonii* may hybridize with *C. erythraeus*. The hybridization between two species may more frequently happen in cage of pet stores. In addition, mtDNA introgression is known in other squirrels (Wettstein et al. 1995; Good et al. 2008; Hird and Sullivan 2009). Therefore, mtDNA introgression from *C. finlaysonii* to *C. erythraeus* may have meant we only detected *C. finlaysonii* sequences, and we cannot conclude that the *Callosciurus* squirrels found in Hamamatsu are *C. finlaysonii*. We, however, found exact molecular evidences of *C. finlaysonii*. We may need to consider treating *C. finlaysonii* as an invasive alien species in Japan.

Results of our Bayesian analysis show that haplotypes CeJ4 and CeJ6 are more closely related to CfJ1 rather than to haplotypes Cfi1 and Cfi2 from Laos, although this relationship was not supported in the MP analysis (Fig. 2). The Hamamatsu population may have originated Thailand because haplotype CfJ1 was also found in *C. finlaysonii boonsongi* from Thailand. This subspecies is confined to the northeastern part of Thailand (Francis 2008). Therefore, squirrels might have been imported through Bangkok into Japan. To avoid further introduction of *C. finlaysonii* in Japan, we recommend focusing attention on routes of introduction from Thailand.

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Appendix 1

Localities and haplotypes of *Callosciurus* squirrels captured in Hamamatsu, Shizuoka Prefecture, Japan

Locality	Haplotype	ID number
Hamamatsu Bungeikan	CeJ4	95, 96, 109, 128, 133, 142, 152, 154, 161, 164, 167, 179, 180, 184, 219, 227
	CfJ1	86, 101, 102, 134, 140, 143, 147, 155, 159, 185, 188, 211, 221, 300
Shijimizuka Park	CeJ4	136
	CfJ1	292, 308
Grand Hotel Hamamatsu	CeJ4	105
nearby Grand Hotel Hamamatsu	CeJ4	253
Sanaru Lake Park	CeJ4	148, 169, 182, 186, 191
	CfJ1	146
east side of Sanaru Lake	CeJ4	270
west side of Sanaru Lake	CeJ4	208, 265, 295
Izumi Park	CeJ4	197
Ryuun-ji Temple	CeJ4	198, 201, 203, 233, 258, 277
Agatai Shrine	CeJ4	216, 220, 235, 275, 294, 323
	CfJ1	271
Tomizuka Park	CeJ4	236, 267
Nishimi Temple	CeJ4	243, 330
Syorin-ji Temple	CeJ4	314, 319, 332
	CfJ1	245, 246, 250, 331
Yotsuie Park	CeJ4	249, 278, 281, 285
Doun-ji Temple	CeJ4	257, 260
Sui Shrine	CeJ4	261, 326
Wago-aikoen	CeJ4	276, 283
Ushiyama Park	CeJ4	282, 301
Hamamatsu Hachimangu Shrine	CeJ4	327