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Short communication

Origin of *Callosciurus erythraeus* introduced into the Uto Peninsula, Kumamoto, Japan, inferred from mitochondrial DNA analysis

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Alien squirrels cause severe damage to forests and commercial tree plantations (Rowe and Gill 1985; Dagnall et al. 1998). They also cause the progressive disappearance of native squirrel species (Reynolds 1985; Gurnell and Pepper 1993; Bertolino and Genovesi 2003). To prevent these serious effects by alien squirrels, it is important to ascertain how the species was introduced to the area. Even if the species has already been introduced, further introductions can be managed if the route of introduction is identified.

The Pallas's squirrel (*Callosciurus erythraeus*) is 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 lowland and montane forests, cultivated areas, and gardens (Nowak 1991; Francis 2008). It was exported to other countries as an exotic pet. As a result of this commercial activity, *C. erythraeus* now occurs in Argentina (e.g., Aprile and Chicco 1999), France (Jouanin 1986; Mitchel-Jones et al. 1999), and Japan (Ishii 2005; Tamura 2009). It is considered serious threat to forests and tree plantations (Jouanin 1986; Setoguchi 1990; Aprile and Chicco 1999).

Callosciurus erythraeus is thought to have been introduced from Taiwan into central and southwestern parts of Japan (Tamura 2002). Study of Oshida et al. (2007) with mitochondrial DNA control region sequences shows that the *C. erythraeus* population of Japan is closely related to that of Taiwan. Several populations of *C. erythraeus* are reported in Japan (Ozaki 1986; Torii 1989, 1993; Tamura and Ohara 2005; Ishii 2005; Tamura 2009) (Fig. 1). In addition to *C. erythraeus*, Oshida et al. (2007) revealed that Finlayson's squirrel, *C. finlaysonii*, had been introduced to the Shizuoka Prefecture, Honshu Island. It is difficult to distinguish this species from *C. erythraeus* based on external characteristics such as pelage patterns. As *C. finlaysonii* occurs in only the Indochina Peninsula (Corbet and Hill 1992), its presence in Japan is clear evidence of past introductions of *Callosciurus* squirrels from the Asian Continent.

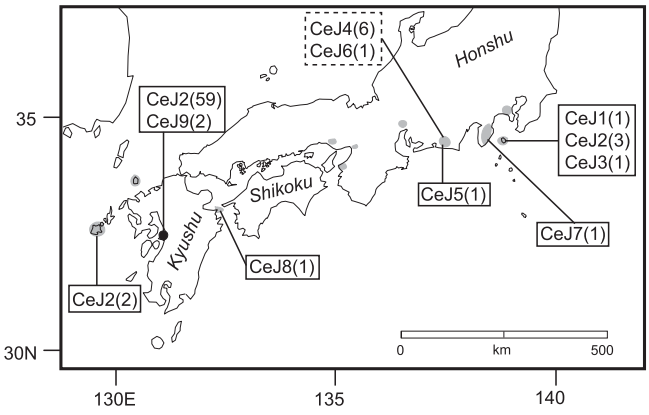

Fig. 1. Sampling localities of the Pallas's squirrel (*Callosciurus erythraeus*) and the Finlayson's squirrel (*C. finlaysonii*) in Japan. Gray areas show the distribution of *Callosciurus* squirrels (Ishii 2005; Tamura 2009). Black area shows the collecting locality in the present study (Uki, Kumamoto). Solid boxes indicate *C. erythraeus* haplotypes reported by Oshida et al. (2007) or found in the present study. The dashed box indicates *C. finlaysonii* haplotypes reported by Oshida et al. (2007).

In 2008, a population of *Callosciurus* squirrel was found in the Uto Peninsula of Kumamoto, Kyushu Island (Yasuda 2010, Fig. 1). 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 *Callosciurus* squirrels to Japan is strictly prohibited. Therefore, it is important to identify how this population appeared in this area. With respect to the Kumamoto population, we propose three scenarios: 1) *C. erythraeus* was introduced from Taiwan or from any other area in Japan, 2) *C. erythraeus* was introduced from any original country other than Taiwan, and 3) *C. finlaysonii* was introduced from the Asian Continent or from any other area in Japan. To test these scenarios, we analyzed the mtDNA control region

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sequences of this new population and compared them to haplotype sequences reported previously from Japan and Taiwan (Oshida et al. 2006, 2007). If the haplotype sequences of the Kumamoto population were the same as or similar to those from Japan and Taiwan, the origin of this population was most likely to be Taiwan. Haplotypes from Taiwan are phylogeographically categorized into four phylogroups (eastern, northern, southern, and western) with several haplotypes not included in any phylogroup (Oshida et al. 2006). If haplotypes of the Kumamoto population were distantly related to the haplotypes from Japan and Taiwan, this new population may have been originated from an area other than Taiwan. Since this species is a common pet in Southeast Asian countries, such as Vietnam and Thailand, there are several pathways of introduction into Japan. If haplotypes of the Kumamoto population were the same as or similar to those of *C. finlaysonii* reported previously in Oshida et al. (2007), this new population is not *C. erythraeus* and could have originated from the Asian Continent. We here discuss whether the new Kumamoto population was derived from the Taiwan populations.

Materials and methods

Specimens, DNA extraction, amplification, and sequencing

For pest control, 61 *Callosciurus* specimens were collected in the Uto Peninsula: Uki, Kumamoto, Japan (Fig. 1). Total genomic DNA was extracted from muscle tissues using the QuiaQuick Kit (QUIAGEN K.K., Tokyo).

The mtDNA control region sequence was amplified using polymerase chain reaction (PCR) with primers reported by Oshida et al. (2001a): L15933 5'-CTCTG GTCTTGTAAACCAAAAATG-3' and H637 5'-AGGA CCAAACCTTTGTGTTTATG-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'-CGGCA CATACCCCATTCAGTC-3') as reported by Oshida et al. (2006) were used for sequencing. Purification of PCR products and sequencing were conducted by Mission Biotech Co. Ltd. (Taipei). We assumed that all specimens from Kumamoto were *C. erythraeus*. To root in the phylogenetic tree, we used control region sequences of two *Callosciurus finlaysonii* specimens from Laos. Their accession numbers in the DNA Data Bank of Japan (DDBJ) are AB259600 and AB259601 (Oshida et al. 2007). Phylogenetic analysis based on the cytochrome *b* gene sequence shows that this species is most closely related to *C. erythraeus* (Oshida et al. 2001b, 2010), making it a suitable out-group. If the Kumamoto population included *C. finlaysonii* specimens, we could detect presence of this species by their genetic similarity to the outgroup.

Sequence and phylogeographic analyses

Sequence alignment was carried out using the software program DNASIS (Hitachi, Tokyo, Japan). We included 43 haplotype sequences of *C. erythraeus* of Taiwan (Oshida et al. 2006) and six haplotype sequences (CeJ1, CeJ2, CeJ3, CeJ5, CeJ7, and CeJ8) from Japan (Oshida et al. 2007) (Table 1). Gap-sites were excluded. The program Modeltest 3.06 (Posada and Crandall 1998) selected the most appropriate substitution model of molecular evolution using the outgroup by the Akaike information criterion (AIC). This test selected the general time reversible model of substitution (Rodríguez et al. 1990; Yang et al. 1994), taking into account the proportion of invariable sites (0.7035), and following a gamma distribution for variable sites (0.4445) (GTR + I + G). Neighbor-joining analysis (Saitou and Nei 1987) was used with this model in PAUP* 4.0b10 (Swofford 2001). To assess nodal supports, we used bootstrapping (Felsenstein 1985) with 10,000 replicates.

Results

Complete sequences (1,081 bases) of the mtDNA control region were successfully obtained from 61 *Callosciurus* squirrels from Kumamoto. All individuals were identified as *C. erythraeus*. There were no *C. finlaysonii* sequences in this population. We identified two haplotypes (Table 1): 59 individuals had haplotype CeJ2 reported previously from Izu-Oshima, Tokyo and Fukuejima, Nagasaki by Oshida et al. (2007) and 2 individuals had haplotype CeJ9. The sequence for the

Table 1. Haplotypes of *Callosciurus erythraeus* used in the present study; except for CeJ9, all haplotype sequences were from Oshida et al. (2006, 2007). Asterisks indicate haplotypes found in Kumamoto, Japan

Haolotype	Phylogroup	Locality	Accession number in DDBJ
CeJ1	Eastern	Izu-ohshima Island	AB259592
CeJ2*	Japan	Izu-ohshima Island, Fukue Island, Kumamoto	AB259593
CeJ3	Japan	Izu-ohshima Island	AB259594
CeJ5	Eastern	Hamamatsu	AB259596
CeJ7	Japan	Izu Peninsular	AB259598
CeJ8	Eastern	Miyazaki	AB259599
CeJ9*	Japan	Kumamtoto	AB576365
Ce01	Northern	Wufeng	AB181249
Ce02	Northern	Shuanglianpi	AB181250
Ce03	Northern	Fushan	AB181251
Ce04	Northern	Tunglou	AB181252
Ce05	Northern	Wufeng	AB181253
Ce06	Northern	Shueili	AB181254
Ce07	Northern	Longtan	AB181255
Ce08	Northern	Wufeng	AB181256
Ce09	Northern	Dadushan, Wufeng	AB181257
Ce10	Northern	Chilan, Paoshan Dam	AB181258
Ce11	Northern	Tunglou	AB181259
Ce12	Northern	Shinshe, Wufeng	AB181260
Ce13	Western	Neimen	AB181261
Ce14	Western	Alishan, Tsaolian	AB181262
Ce15	Western	Baolai	AB181263
Ce16	Western	Baolai	AB181264
Ce17	Western	Shitou	AB181265
Ce18	–	Fangliau	AB181266
Ce19	Western	Lugu	AB181267
Ce20	Eastern	Kuanfu	AB181268
Ce21	Eastern	Kuanfu	AB181269
Ce22	–	Kenting	AB181270
Ce23	Eastern	Rentze	AB181271
Ce24	Western	Lugu	AB181272
Ce25	–	Shizi	AB181273
Ce26	Eastern	Datong, Wuling Farm	AB181274
Ce27	Eastern	Kuanfu	AB181275
Ce28	Eastern	Kuanfu	AB181276
Ce29	Eastern	Dongshan	AB181277
Ce30	Eastern	Neipu	AB181278
Ce31	–	Dunghe	AB181279
Ce32	Eastern	Kuanfu	AB181280
Ce33	Eastern	Kuanfu	AB181281
Ce34	–	Jialeshuei, Kenting	AB181282
Ce35	Eastern	Fangliau	AB181283
Ce36	Southern	Fangliau, Shizi	AB181284
Ce37	Southern	Fangliau	AB181285
Ce38	Eastern	Dongshan	AB181286
Ce39	Western	Tsaolian	AB181287
Ce40	Eastern	Shinbaiyang	AB181288
Ce41	Eastern	Shinbaiyang	AB181289
Ce42	Western	Sandimen	AB181290
Ce43	Western	Sandimen	AB181291

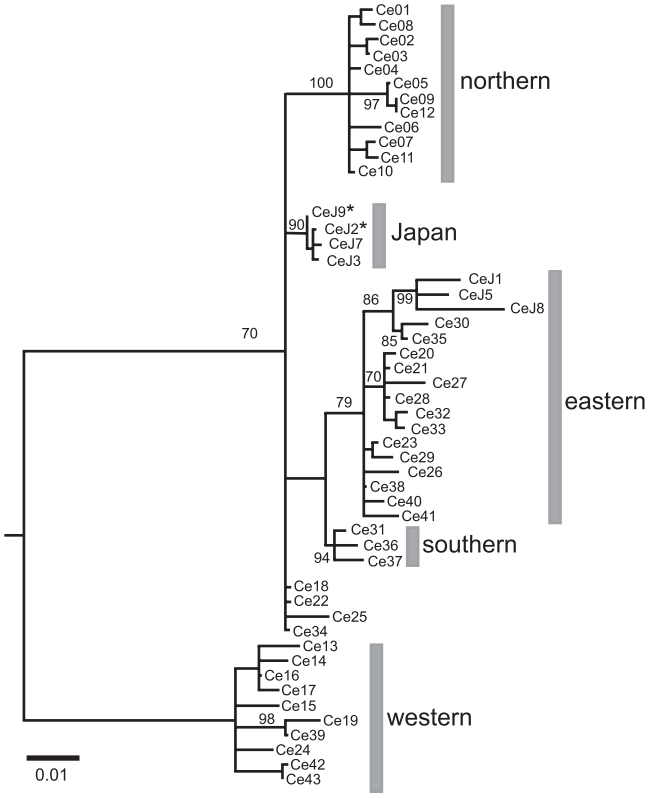

Fig. 2. Neighbor-joining tree showing phylogenetic relationships among the 50 mtDNA control region haplotypes of Pallas's squirrels (*Callosciurus erythraeus*) from Taiwan and Japan. Tree is based on the general time reversible model with gamma shape parameter and proportion of invariable sites. Bootstrap supports (70% ≤) are given on branches. Four mtDNA phylogroups from Taiwan are indicated: northern, western, southern, and eastern. A phylogroup found in Japan is also indicated. Bar under tree shows genetic distances (substitutions/site) correlated by the substitution model. Asterisks indicate the haplotypes found in Kumamoto, Japan.

newly identified CeJ9 was deposited in the DDBJ (Table 1). Among all haplotypes, including the out-group (*C. finlaysonii*), there were 899 constant sites, 40 parsimony-uninformative variable sites, and 136 parsimony-informative sites.

In the rooted neighbor-joining tree using the GTR + I + G model including out-group (*C. finlaysonii*), seven haplotypes found in Japan (including CeJ2 and CeJ9 found in Kumamoto) were separated into two clusters (Fig. 2). Haplotypes CeJ2, CeJ3, CeJ7, and CeJ9 were clustered together with high bootstrap value (90%). This cluster was not included in any of Taiwan's four phylogroups. Three Japanese haplotypes, CeJ1, CeJ5, and CeJ8, were included in the Taiwan's eastern phylogroup that is distributed in northeastern part of Taiwan (Oshida et al. 2006). These three haplotypes form a single cluster with 99% nodal support (Fig. 2).

Discussion

In Japan, there were at least two mtDNA lineages of *C. erythraeus* (Fig. 2). One lineage (CeJ1, CeJ5, and CeJ8) could have been introduced into Japan from the northeastern part of Taiwan. Although the cluster including two haplotypes from Kumamoto (CeJ2 and CeJ9) was not included in any Taiwan's phylogroups, it was grouped with Taiwan's phylogroups (Fig. 2). Therefore, haplotypes found in Kumamoto were probably derived from the Taiwan populations. In Taiwan, there are at least four phylogroups. Several haplotypes (Ce18, Ce22, Ce25, and Ce34), however, did not belong to any Taiwan's four phylogroups (Oshida et al. 2006). Further examination of DNA sequences of *C. erythraeus* from Taiwan may reveal a new phylogroup. Although the cluster consisting of CeJ2, CeJ3, CeJ7, and CeJ9 indicates a unique phylogroup, it has not ever been found in Taiwan. Individuals with these haplotypes may have been introduced to Japan from the same geographical region in Taiwan. Although we cannot clearly identify the origin of the Kumamoto population, we do discard the hypothesis that this new population was introduced from an area outside of Japan and Taiwan. To avoid further population expansion of *C. erythraeus* in Japan, attention should focus on routes of introduction within Japan and from Taiwan.

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