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Phylogenetic Relationships among Japanese Species of the Family Sciuridae (Mammalia, Rodentia), Inferred from Nucleotide Sequences of Mitochondrial 12S Ribosomal RNA Genes

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ABSTRACT—In order to investigate phylogenetic relationships of the family Sciuridae living in Japan, we sequenced partial regions (379 bases) of mitochondrial 12S rRNA genes in six species of Japanese and other Asian squirrels. Phylogenetic trees constructed by sequence data indicated that two genera of flying squirrels (Petaurista and Pteromys) were clustered in a group distinct from non-flying squirrels, suggesting a possible monophyletic relationships of these flying squirrels. The evolutionary distance between the Japanese squirrel (Sciurus lis) from Honshu island and the Eurasian red squirrel (Sciurus vulgaris) from Hokkaido island was comparable to intraspecific distances of the remaining species examined.

INTRODUCTION

In Japan, there are six native species of the family Sciuridae; the Japanese giant flying squirrel (Petaurista leucogenys), Eurasian flying squirrel (Pteromys volans), small Japanese flying squirrel (Pteromys momonga), Japanese squirrel (Sciurus lis), Eurasian red squirrel (Sciurus vulgaris), and Asiatic chipmunk (Tamias sibiricus). Based on morphological characteristics, these squirrels are generally divided into two groups: flying squirrels (Petaurista and Pteromys) with the gliding membrane and non-flying squirrels (Sciurus and Tamias) without the gliding membrane. Petaurista and Pteromys are arboreal dwellers which make use of the unique gliding membrane. Sciurus also lives in an arboreal habitat, while Tamias uses both arboreal and terrestrial habitats. The gliding membrane of flying squirrels is considered as a character acquired through adaptation to gliding behavior in the forest. With regards to phylogenetic relationships between flying squirrels and non-flying squirrels, there is still an argument as to whether flying squirrels evolved monophyletically (Thorington Jr, 1984) or polyphyletically (Black, 1963, 1972; Hight et al., 1974).

In the present study, we determined partial nucleotide sequences in mitochondrial 12S ribosomal RNA (12S rRNA) genes of several Asian squirrels including most Japanese species, and then subjected resultant data to phylogenetic analyses. Mutation in mitochondrial DNA (mtDNA) occurs more rapidly than that in the nuclear genome (Brown et al., 1979) and the sequence difference in the 12S rRNA region is generally considered to provide reliable information on phylogenetic relationships of mammalian species (Gemmell and Westerman, 1994; Springer and Kirsch, 1993). We here present molecular phylogeny of sciurid species and discuss the process of evolution of these species.

MATERIALS AND METHODS

Animals

Squirrels examined are shown in Table 1. Specimens from outside Japan represent foreign conspecifics of Japanese taxa except Petaurista petaurista. Since P. petaurista is a species of Petaurista whose distribution ranges close to Japan, it was used to investigate the origin of P. leucogenys of Japan. Corbet and Hill (1992) adopted the name Petaurista philippensis for the Taiwanese and several Southeast Asian populations of P. petaurista on the basis of morphological characteristics. But this account is not generally accepted at present. Therefore, we refer our samples from Taiwan and Laos as P. petaurista following Nowak's traditional classification (Nowak, 1991).

DNA extraction

Muscle or liver tissues were frozen at -80°C until use. From tissues crushed in liquid nitrogen using a Mikro-dismembrator II (B.Braun), total DNAs were extracted according to the phenol/proteinase K/sodium dodecyl sulfate method of Sambrook et al. (1989).

Amplification of the mitochondrial 12S rRNA region

A partial region of the mitochondrial 12S rRNA gene was amplified with symmetric polymerase chain reaction (PCR) (Kocher et al., 1989) with some modifications (Masuda and Yoshida, 1994a, b). Following the published sequences of Kocher et al. (1989), two PCR primers were designed: L1091 5'-AAACTGGGATTAGATACCCCACTAT-3'
Table 1. Species of the family Sciuridae examined in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Code</th>
<th>Common name</th>
<th>Collecting locality</th>
<th>Accession No. of 12S rRNA sequences*</th>
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<tr>
<td><em>Petaurista petaurista</em></td>
<td>PPE1</td>
<td>Red giant flying squirrel</td>
<td>Laos&lt;sup&gt;a&lt;/sup&gt;</td>
<td>D50281</td>
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<td>PPE2</td>
<td></td>
<td></td>
<td>D50282</td>
</tr>
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<td><em>Petaurista leucogenys</em></td>
<td>PLE1</td>
<td>Japanese giant flying squirrel</td>
<td>Nagano Pref., Japan</td>
<td>D50279</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>D50280</td>
</tr>
<tr>
<td><em>Pteromys volans</em></td>
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<td>Eurasian flying squirrel</td>
<td>Hokkaido, Japan&lt;sup&gt;c&lt;/sup&gt;</td>
<td>D50283</td>
</tr>
<tr>
<td></td>
<td>PVO2</td>
<td></td>
<td>Hokkaido, Japan&lt;sup&gt;c&lt;/sup&gt;</td>
<td>D50284</td>
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<tr>
<td></td>
<td>PVO3</td>
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<td>China&lt;sup&gt;a&lt;/sup&gt;</td>
<td>D50285</td>
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<tr>
<td><em>Tamias sibiricus</em></td>
<td>TSI1</td>
<td>Asiatic chipmunk</td>
<td>Hokkaido, Japan&lt;sup&gt;d&lt;/sup&gt;</td>
<td>D50288</td>
</tr>
<tr>
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<td>TSI2</td>
<td></td>
<td>Korea&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Iwate Pref., Japan&lt;sup&gt;n&lt;/sup&gt;</td>
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</tr>
<tr>
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<td>SVU</td>
<td>Eurasian red squirrel</td>
<td>Hokkaido, Japan&lt;sup&gt;c&lt;/sup&gt;</td>
<td>D50287</td>
</tr>
</tbody>
</table>

<sup>a</sup>The 12S rRNA nucleotide sequences data will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with these accession numbers. a, from pet's stores in Japan, and detailed locality of sample is unknown; b, from Prof. P. C. Kuw, National Taiwan University; c, from Noboribetsu Bear Park; d, from Dr. H. Yanagawa, Obhiro University of Agriculture and Veterinary Medicine; e, from Mr. F. Sekiyama, Iwate Prefectural Museum.

and H1478 5'-GAGGGTGTACGCCGCTGTGTTG-3'. Primer names correspond to the light (L) or heavy (H) strand and the 3' end-position of the primer in the human mtDNA sequence (Anderson et al., 1981).

The reaction mixture contained 100 ng of DNA, 25 picomoles of primer, 200 µM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus). Amplification was carried out for 30 cycles using the following cycle program: 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min. The extension reaction was completed by incubation at 72°C for 10 min. To generate single-stranded DNAs, asymmetric PCR (Gyllensten and Erlich, 1988) was performed under the same conditions as the symmetric PCR, except for use of 1 µl of the symmetric PCR product as a template and a 1:100 (2.5 nM : 250 nM) ratio of the two primers.

Direct sequencing of PCR products

Single-stranded DNAs produced by asymmetric PCR were concentrated with a Centricron-30 microconcentrator (Amicon). Seven microliters of the concentrated DNA samples were applied for sequencing due to the dideoxy-nucleotide chain reaction method (Sanger et al., 1977) with the PCR primer for the light or heavy strand or one of the following internal primers. [32P]dCTP (Amersham), and T7 DNA polymerase (United States Biochemical). After the reaction, products were electrophoresed on 6% Polyacrylamide gels containing 7 M urea. Gels were dried and exposed to Fuji RX X-ray films for 1-7 days at room temperature. Internal primers for sequencing, L1281 5'-ACCGCCATCTTCAGCAAACCCTA-3' and H1260 5'-GAGGGTGACGGGCGGTGTGT-3', were newly designed based on the consensus sequence among *Petaurista leucogenys*, *Pteromys volans*, and *Sciurus vulgaris*.

Phylogenetic analysis

Sequence alignment was performed using the GeneWorks computer software (IntelliGenetics). For construction of the phylogenetic trees, we employed the UPGMA (Sokal and Michener, 1958) in the MEGA computer software (Kumar et al., 1993) and the neighbor-joining (NJ) method (Saitou and Nei, 1987) in the Clustal W computer software (Thompson et al., 1994) or MEGA. In each method, numbers of nucleotide substitutions per site were estimated for multiple sequences using the Kimura's two parameter method (Kimura, 1980). Bootstrap analyses (Felsenstein, 1985) were done (500 replications) to assess the confidence of internal branching. The 12S rRNA nucleotide sequence of *Rattus norvegicus* (Gadaleta et al., 1989) was used as an out-group for the sciurid species.

RESULTS

Using the PCR product-direct sequencing technique, partial DNA sequences (379 bp) of mitochondrial 12S rRNA genes were determined on six squirrel species (Fig. 1). Sequence alignment indicated that several gap-sites occurred among the squirrel species (Fig. 1). Table 2 shows percentage sequence differences and numbers of transitions and transversions obtained from pairwise comparison. Numbers of transitions were greater than those of transversions (Table 2).

Both phylogenetic trees of 12S rRNA sequences, constructed using the UPGMA and the NJ method, showed the same topology of branching (Fig. 2). This suggests the reliability of phylogenetic relationships between species shown in these trees. Each of genera *Petaurista*, *Pteromys*, *Sciurus*, and *Tamias* was grouped into a distinct cluster, supported with a high confidence (100% bootstrap value, Fig. 2). Moreover, the genera of flying squirrels, *Petaurista* and *Pteromys*, were collectively separated from those of non-flying squirrels, *Sciurus* and *Tamias*.

Percentage sequence differences (intragenic variations) between *Petaurista leucogenys* (PLE1 and PLE2) from two different localities (in Japan) and *Petaurista petaurista* (PPE1 from Laos and PPE2 from Taiwan) ranged from 4.8 to 5.0% (18-19/379 bases), while an intraspecific difference between PLE1 and PLE2 and that between PPE1 and PPE2 were 0.5% (2/379 bases) and 0.3% (1/379 bases), respectively (Table 2). Intraspecific differences of *Pteromys volans* among Hokkaido island populations (PVO1 and PVO2) and a Chinese population (PVO3) were 0.3-0.8% (2-3/379 bases). The difference value between *Tamias sibiricus* from Hokkaido island (TSI1) and Korea (TSI2) was 1.3% (5/379 bases). The
Fig. 1. Alignment of nucleotide sequences (379 bases) of the 12S rRNA regions. Dots show identities to nucleotides of PV01 (Pteromys volans, Eurasian flying squirrel). Sample names correspond to codes shown in Table 1.

**DISCUSSION**

Phylogenetic relationships between flying squirrels and non-flying squirrels

It is controversial whether flying squirrels form a monophyletic group descending from a common ancestor or a polyphyletic group including some independent lineages. Based on paleontological data, Black (1963, 1972) proposed a polyphyletic evolution that flying squirrels separately descended from several tree squirrels. On the other hand, Thorington Jr. (1984) suggested a monophyly of flying squirrels based on an extensive study of bone anatomy.

Phylogenetic trees of the 12S rRNA gene in the present study suggested that flying squirrels (Petaurista and Pteromys) were collectively separated from non-flying squirrels, although the branching order among genera may not be guaranteed, judging from the bootstrap values (54-61% in UPGMA tree and 36-43% in NJ tree). So far as the species examined here are concerned, our results seem to support the hypothesis of Thorington Jr. (monophyletic evolution) rather than that of Black (polyphyletic evolution). Hight et al. (1974), while also recognizing a close relationship between Petaurista and Pteromys from immunological analyses, suggested that flying squirrels evolved from more than one stock because of apparent large distances between the genus lomys from Southeast Asia and other flying squirrels. Further molecular analysis of other flying squirrels including lomys is needed for a better understanding of the phylogenetic relationships between flying and non-flying squirrels.

difference between Sciurus vulgaris (SVU) from Hokkaido island and Sciurus lis (SLI) from Honshu island (0.8%, 3/379 bases) was similar in magnitude to intraspecific differences of the other squirrels.
Zoogeography and phylogeny of flying squirrels

The genus *Petaurista* is widely distributed in the central to southern parts of the Eurasian Continent, Southeast Asia, and main islands of Japan except for Hokkaido island (Corbet and Hill, 1992; Lekagul and McNeely, 1988; Nowak, 1991). Honshu island is thus the northernmost range for *Petaurista*. Distribution of *P. leucogenys* is restricted to Japan (Honshu, Shikoku, and Kyushu islands) and central China (Imaizumi, 1960), whereas *P. petaurista* is widespread throughout the central to southern parts of the Eurasian Continent and several islands of Southeast Asia. *P. leucogenys* externally differs from *P. petaurista* mainly by hair coloration; the former’s back is gray (Imaizumi, 1960), while the latter is dark red or brown (Lekagul and McNeely, 1988). In the present study, we detected 4.8-5.0% difference in 12S rRNA sequences between these two species, and this agreed with their external distinction. Applying the available substitution rate (0.55%/million years, Myr) estimated from the feline 12S rRNA genes (Masuda and Yoshida, 1994a) to the present case, the divergence between *P. leucogenys* (PLE1 and PLE2) and *P. petaurista* (PPE1 and PPE2) was estimated to have occurred approximately 4.4-4.6 Myr ago. This age corresponds to the Pliocene. On the basis of paleontological data, Kawamura (1988, 1990) and Kawamura et al. (1989) suspected that *P.*
leucogenys emigrated to Japan through the land bridge which connected the Eurasian Continent and the main islands of Japan from the Early Pleistocene to the early Middle Pleistocene. After separation of Japanese islands from the Eurasian Continent with the glacial eustacy in the Pleistocene, Japanese populations of P. leucogenys are considered to have been isolated in this island group. Accepting Kawamura’s hypothesis and considering the occurrence of P. leucogenys populations in central China, our molecular data can be interpreted as indicating that P. leucogenys diverged from P. petaurista within the Eurasian Continent prior to its emigration to Japan.

Another flying squirrel, the genus Pteromys, is widely distributed in the central to northern parts of the Eurasian Continent and main islands of Japan (Nowak, 1991); Japanese main islands are the southernmost range for Pteromys. The sequence divergence within the Hokkaido island population (PVO1 and PVO2) and that between the Hokkaido island population and the Chinese population (PVO3) were similar to the intraspecific differences observed in Petaurista. The divergence time between the Hokkaido island and the Chinese populations was estimated to be 0.5-0.7 Myr ago. This age is included in the Pleistocene. Kawamura (1988, 1990) suggested that P. volans emigrated from the Eurasian Continent to Hokkaido island through Sakhalin in the Middle Pleistocene. The divergence time estimated in the present study was not in conflict with his hypothesis.

Phylogeny of the Asiatic chipmunk

The genus Tamias is widely distributed in the central to the northern parts of the Eurasian Continent, Hokkaido island, and North America. Although Tamias inhabits both the tree and the ground and is considered to have diverged early from a ground squirrel lineage (Black, 1963, 1972; Hight et al., 1974), evolution and the dispersal pattern of Tamias still remains unsettled. Molecular phylogenetic trees (Fig. 2) suggested that the Asiatic chipmunk Tamias sibiricus diverged first from other squirrels, and that evolutionary distances between Tamias and the flying squirrels (Petaurista or Pteromys) is similar to those between Tamias and Sciurus (Table 2). To elucidate the precise phylogenetic status of Tamias in the sciurid, it is needed to conduct further extensive analyses involving the other species of the genus Tamias.

The divergence time between T. sibiricus (TSI1) from Hokkaido island and Korea (TSI2) was estimated to be 1.2 Myr ago. Tamias may have emigrated from the Eurasian Continent to Hokkaido island in a way similar to Pteromys as Kawamura supposed previously (1990).

Controversial classification of the genus Sciurus living in Japan

Sciurus vulgaris is distributed throughout the northern part of the Eurasian Continent including Hokkaido island, while S. lis is endemic to Honshu, Shikoku, and Kyushu islands of Japan. Imaizumi (1960) classified S. lis as a distinct species on the basis of differences in tail hair color, body size, and cranial characteristics, whereas Okada et al. (1973) considered this squirrel as a subspecies (S. vulgaris lis) of S. vulgaris also based on morphological characteristics. At present, Imaizumi’s classification is widely accepted with an additional support from paleontological data (Kawamura, 1988), and S. lis and S. vulgaris are categorized as different species. The sequence difference between S. lis and S. vulgaris examined in the present study is, however, similar to those of the intraspecific level in other squirrel species including Petaurista petaurista, P. leucogenys, Tamias sibiricus, and Pteromys volans (Table 2). It may, therefore, be reasonable to regard S. lis and S. vulgaris as conspecific taxa rather than remaining them in separate species status (Okada et al., 1973). In addition, the estimated divergence time between S. lis and S. vulgaris was approximately 0.7 Myr ago. Kawamura (1988, 1990) and Kawamura et al. (1989) considered from fossil data that Sciurus emigrated from Hokkaido island to Honshu island in the Middle Pleistocene. Thus, the divergence date estimated in the present study is not in discordance with paleontological data.

This is the first report on molecular phylogeny of Japanese squirrels including flying and non-flying species. Our results provided an insight for a better understanding of evolution and phylogeny of squirrel species.

Table 2. Pairwise comparisons of 12S rRNA nucleotide sequences (379 bases) between 6 species of squirrels

<table>
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<tr>
<th></th>
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<th>PPE2</th>
<th>PLE1</th>
<th>PLE2</th>
<th>PVO1</th>
<th>PVO2</th>
<th>PVO3</th>
<th>TSI1</th>
<th>TSI2</th>
<th>SLI</th>
<th>SVU</th>
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<td>9.8</td>
<td>9.5</td>
<td>10.0</td>
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<td>11.9</td>
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Data above the diagonal represent percentage differences between species. Data below the diagonal are the numbers of nucleotide substitutions (transitions/transversions). Gaps in sequence alignment were eliminated from comparison.
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