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Source: Zoological Science, 13(4) : 615-620

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.13.615

ZOOLOGICAL SCIENCE 13: 615–620 (1996)

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 speices 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 charactersitics. 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 mitochondorial 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

Species	Code	Common name	Collecting locality	Accession No. of 12S rRNA sequences*
Petaurista petaurista	PPE1	Red giant flying squirrel	Laos[a]	D50281
	PPE2		Taiwan[b]	D50282
Petaurista leucogenys	PLE1	Japanese giant flying squirrel	Nagano Pref., Japan	D50279
	PLE2		Wakayama Pref., Japan	D50280
Pteromys volans	PVO1	Eurasian flying squirrel	Hokkaido, Japan[c]	D50283
	PVO2		Hokkaido, Japan[c]	D50284
	PVO3		China[a]	D50285
Tamias sibiricus	TSI1	Asiatic chipmunk	Hokkaido, Japan[d]	D50288
	TSI2		Korea[a]	D50289
Sciurus lis	SLI	Japanese squirrel	Iwate Pref., Japan[e]	D50286
Sciurus vulgaris	SVU	Eurasian red squirrel	Hokkaido, Japan[c]	D50287

*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. Kuo, National Taiwan University; c, from Noboribetsu Bear Park; d, from Dr. H. Yanagawa, Obihiro University of Agriculture and Veterinary Medicine; e, from Mr. F. Sekiyama, Iwate Prefectual Museum.

and H1478 5'-GAGGGTGACGGGCGGTGTGT-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 $MgCl_2$, 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 Centricon-30 microconcentrator (Amicon). Seven microliters of the concentrated DNAs 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, [^{32}P]dCTP (Amersham), and T7 DNA polymerase (United States Biochemical). After the reaction, products were electorophoresed 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 temprature. Internal primers for sequencing, L1281 5'-ACCGCCATCTTCAGCAAACCCTA-3' and H1260 5'-TAGGGTTTGCTGAAGATGGCGGT-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 (Kumer *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 substitutions 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 (intrageneric 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

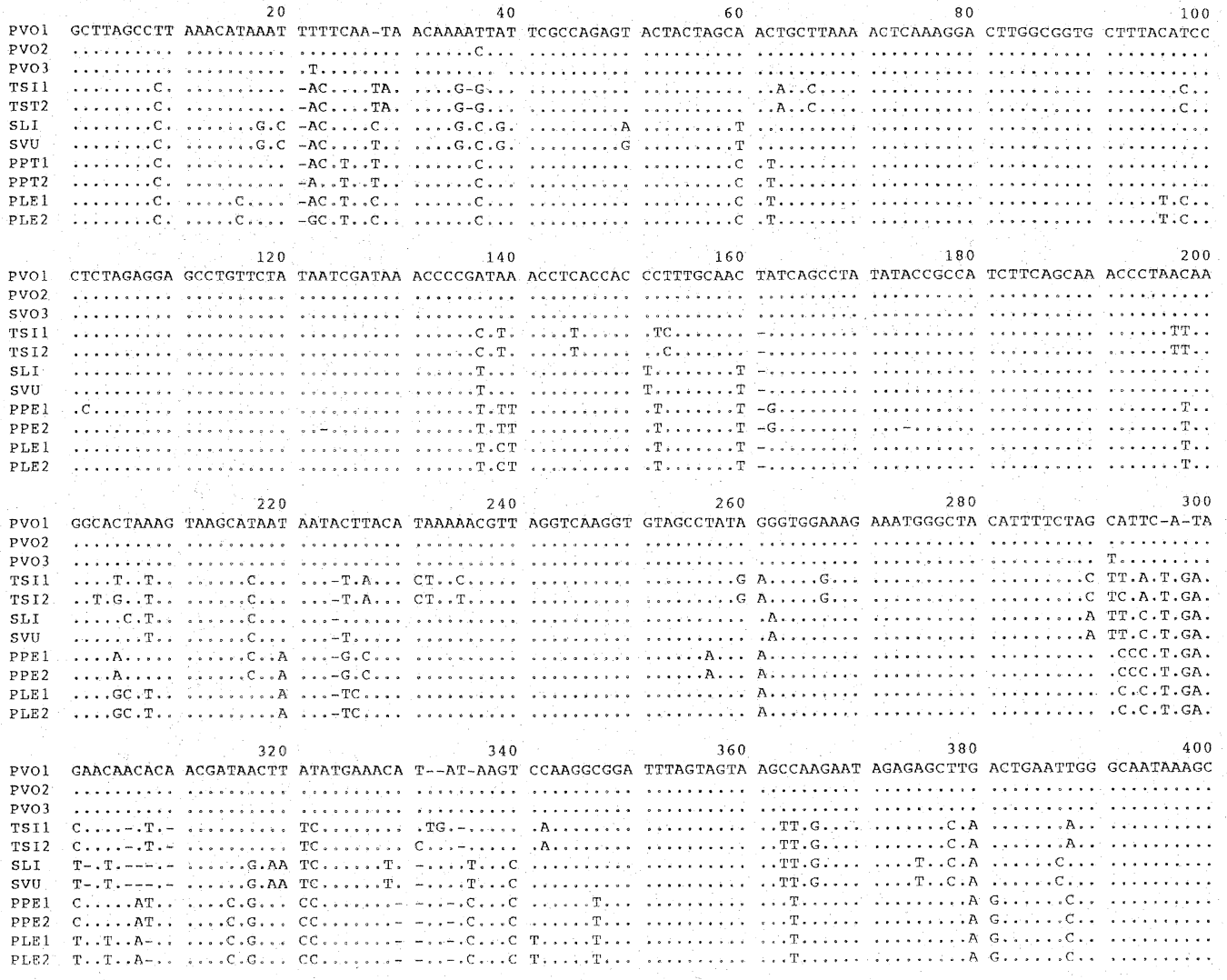

Fig.1. Alignment of nucleotide sequences (379 bases) of the 12S rRNA regions. Dots show identities to nucleotides of PVO1 (*Pteromys volans*, Eurasian flying squirrel). Sample names correspond to codes shown in Table 1.

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.

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 *Iomys* from Southeast Asia and other flying squirrels. Further molecular analysis of other flying squirrels including *Iomys* is needed for a better understanding of the phylogenetic relationships between flying and non-flying squirrels.

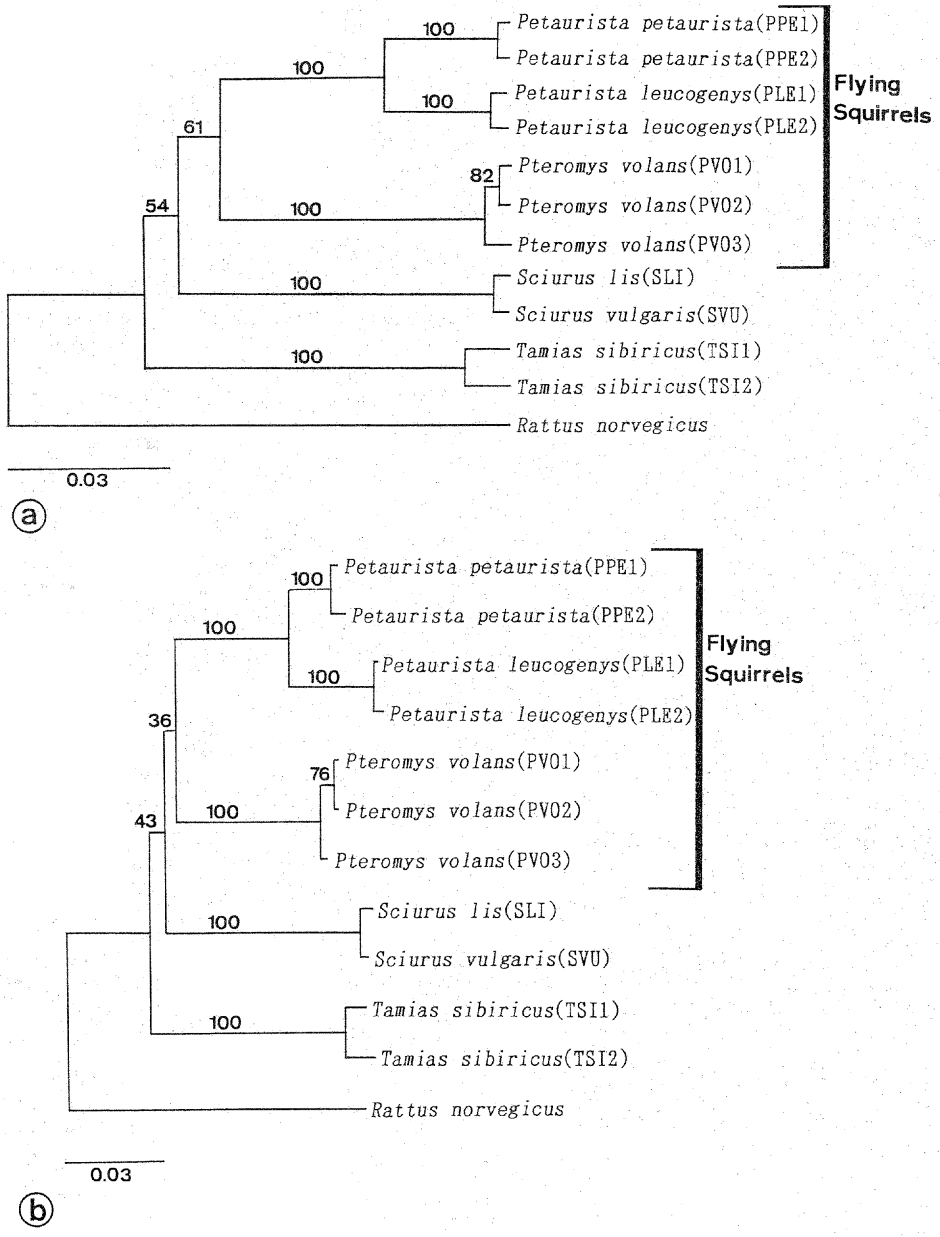

Fig.2. Phylogenetic trees of 12S rRNA nucleotide sequences reconstructed by UPGMA (a) and the NJ method (b). Alphabetical abbreviations correspond to codes shown in Table 1. Rat sequences was used as an outgroup. Bars indicate evolutionary distance of 0.03 substitution per site. Distance estimation employed the Kimura's two-parameter method. Numbers above branches are bootstrap values (%) derived from 500 replications.

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 difference 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) suspected 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

	PPE1	PPE2	PLE1	PLE2	PVO1	PVO2	PVO3	TSI1	TSI2	SLI	SVU
PPE1		0.5	4.8	5.0	9.2	9.0	9.5	11.6	11.9	11.4	11.1
PPE2	2/0		4.8	5.0	9.8	9.5	10.0	11.6	11.9	11.4	11.1
PLE1	15/3	15/3		0.3	10.3	10.0	10.6	12.1	12.1	10.6	10.6
PLE2	16/3	16/3	1/0		10.3	10.0	10.6	12.4	12.4	10.8	10.8
PVO1	22/13	24/13	27/12	27/12		0.3	0.5	11.1	10.6	10.0	10.0
PVO2	21/13	23/13	26/12	26/12	1/0		0.8	11.1	11.1	9.8	9.8
PVO3	23/13	25/13	28/12	28/12	2/0	3/0		10.8	10.8	9.8	9.8
TSI1	26/18	26/18	31/15	32/15	23/19	23/19	22/19		1.3	11.4	10.8
TSI2	28/17	28/17	32/14	33/14	20/20	22/20	21/20	4/1		11.6	11.6
SLI	32/11	32/11	32/8	33/8	26/12	25/12	25/12	26/17	26/18		0.8
SVU	31/11	31/11	32/8	33/8	26/12	25/12	25/12	24/17	26/18	3/0	

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.

ACKNOWLEDGMENTS

We would like to thank Dr. P. C. Kuo, Dr. L. J. Liao, and Miss H. L. Li (National Taiwan University), Dr. H. Yanagawa and Miss K. Takedazu (Obihiro University of Agriculture and Veterinary Medicine), Mr. Y. Yamaguchi (Kyushu University), Dr. K. Gouda and Dr. M. Satoh (Noboribetsu Bear Park), and Mr. F. Sekiyama (Iwate Prefectual Museum) for supplying specimens. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, and a grant from the Hokkaido-shimbun Press.

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(Received September 27, 1995 / Accepted April 11, 1996)