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Phylogeny and Zoogeography of Six Squirrel Species of the Genus *Sciurus* (Mammalia, Rodentia), Inferred from Cytochrome *b* Gene Sequences

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ABSTRACT—To investigate the phylogenetic relationships between the New World *Sciurus* and the Old World *Sciurus* and their biogeographic history, the partial mitochondrial cytochrome *b* gene sequences (1,040 base pairs) were analyzed on six *Sciurus* species: *S. aberti*, *S. carolinensis*, *S. lis*, *S. niger*, *S. stramineus*, and *S. vulgaris*. Phylogenetic trees (maximum parsimony, neighbor-joining, and maximum likelihood methods) commonly showed two groups with high bootstrap values (73–100%): one consisting of the New World *Sciurus* and the other consisting of the Old World *Sciurus*. Genetic distances among the New World *Sciurus* species were remarkably larger than that between two *Sciurus* species of the Old World, suggesting the earlier radiation of the New World *Sciurus* than the Old World *Sciurus*.

INTRODUCTION

The genus *Sciurus* includes 27 extant squirrel species: 24 species of the New Continent and three species of the Old World (Corbet and Hill, 1991). These animals adapted themselves to the temperate forests, and are widely distributed in the northern parts of Eurasia, North America, Central America, and the northern to central parts of South America.

Yet despite the large amount of ecological information on *Sciurus* (e.g., Gurnell, 1987; Moncrief *et al*., 1993; Koprowski, 1996; Steele *et al*., 1998; Tamura, 1998; Lee and Fukuda, 1999), the phylogenetic relationships within this genus remain uncertain. From the paleontological study, Black (1972) described that the ancestral *Sciurus* could have been present already during the Miocene in European and North American Continents, and that some *Sciurus* species were also found in the early Pliocene in Spain and in Germany. Nadler and Sutton (1967) reported that the chromosomal constitutions are closely related within the North American species of *Sciurus*: *S. carolinensis*, *S. niger*, and *S. aberti*. In addition, the immunological analysis of serum albumin (Ellis and Maxson, 1980) and the study of protein variation (Hafner *et al*., 1994) strongly supported the close phylogenetic relationship between *S. carolinensis* and *S. niger*. Meanwhile, in the Eurasian species, the close phylogenetic relationship between *S. lis* and *S. vulgaris* was inferred from the 12S ribosomal RNA (rRNA) gene sequences (Oshida *et al*., 1996) and the chromosomal characteristics (Oshida and Yoshida, 1997). However, very few are known on the phylogenetic relationships among worldwide species of *Sciurus*.

In the present study, the phylogenetic relationships among six species of *Sciurus* from Asia and North and South America were examined based on the mitochondrial cytochrome *b* gene sequences. We here discuss the biogeographic history of *Sciurus* from the Eurasian Continent and the New Continent.

MATERIALS AND METHODS

Animals

Profile of squirrels examined in the present study is shown in Table 1. One specimen of *S. stramineus* was commercially obtained from a pet store in Japan. DNA sequence data of three North American species (*S. aberti*, *S. carolinensis*, and *S. niger*) previously reported by Thomas and Martin (1993) and Wettstein *et al*. (1995) were included for the present phylogenetic analysis (Table 1). *Tamiasciurus hudsonicus* was used for an out-group.

DNA preparation and sequencing

Total DNAs of *S. lis*, *S. vulgaris*, *S. stramineus*, and *T. hudsonicus* were extracted from muscle tissues with the phenol/proteinase K/sodium dodecyl sulfate method of Sambrook *et al*. (1989). A partial region (1,040 base pairs, bp) of the mitochondrial cytochrome *b* gene was amplified with polymerase chain reaction (PCR), using a set of primers described by Oshida *et al*. (2000): L14724 5'-GATATGAAAAACCATCGTTG-3' and H15910 5'-GATTTTTGGTTTACAAGACCGAG-3'. Primer names correspond to the light (L) or heavy (H) strand and the 3'end-position of the primers in the human mitochondrial DNA (mtDNA) sequences (Anderson *et al*., 1981). The 50 μl of reaction mixture contained 100 ng of genomic DNA, 25 picomoles 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 and the program was 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and then the extention reaction was performed at 72°C for 10 min. PCR products were puri-

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Table 1. Squirrel species used in the present study

Species or subspecies name	Code	Common name	Collecting locality or distribution	Supplier of sample	Accession No. of sequence* or reference
Sciurus aberti aberti	SAB	tassel-eared squirrel	Arizona, U.S.A.		Wettstein *et al*. (1995)
Sciurus carolinensis	SCA	eastern gray squirrel	California, U.S.A.		Thomas and Martin (1993)
Sciurus niger	SNI	eastern fox squirrel	Iowa, U.S.A.		Wettstein *et al*. (1995)
Sciurus stramineus	SST	Guayaquil squirrel	Peru		AB030025
Sciurus lis	SLI1	Japanese squirrel	Iwate, Japan	Iwate Prefectual Museum, Morioka	AB030024
Sciurus lis	SLI2	Japanese squirrel	Nagano, Japan	Obihiro University of Agriculture and Veterinary Medicine, Obihiro	AB030023
Sciurus vulgaris orientis	SVU1	Eurasian red squirrel	Hokkaido, Japan	Noboribetsu Bear Park, Noboribetsu	AB030026
Sciurus vulgaris koreae	SVU2	Eurasian red squirrel	Korea	Kobe Municipal Oji Zoo, Kobe	AB030027
Sciurus vulgaris	SVU3	Eurasian red squirrel	Transbaikalia, Rusia	Zoological Institute, Russian Academy of Sciences, Saint-Petersburg	AB030028
Out group					
Tamiasciurus hudsonicus	THU	American red squirrel	U.S.A.	Awaji Farm Park, Mihara-gun	AB030029

*Sequence data will appear in the DDBJ nucleotide sequence databases with accession numbers.

fied with the Qia-quick PCR purification kit (QIAGEN) and directly sequenced using an automated DNA sequencer (SQ5500L, Hitachi).

Phylogenetic analysis

All sequences were aligned using a computer software Gene Works (Intelligenetics). The phylogenetic trees were constructed with the maximum parsimony (MP) method using the branch and bound search algorithm (Hendy and Penny, 1982) with the 50% majority-rule consensus in PAUP (Swofford, 1993), with the neighbor-joining (NJ) method (Saitou and Nei, 1987) in Clustal W (Thompson *et al*., 1994), and with the maximum likelihood (ML) method using DNAML in PHYLIP package program (Felsenstein, 1993). In NJ and ML methods, numbers of nucleotide substitutions per site were estimated for multiple substitutions by the Kimura's (Kimura, 1980) two-parameter method. MP tree was produced by unweighted parsimony. To assess the branching confidence, bootstrap values (Felsenstein, 1985) were derived from 1,000 replications in MP and NJ methods and 100 replications in ML method.

To estimate the divergence time between *Sciurus* species, the transversional substitution rate (0.5% / million years, Myr) at the third codon positions of mammalian cytochrome *b* gene (Irwin *et al*., 1991) was employed.

RESULTS

The partial sequences (1,040 bp) of the cytochrome *b* gene were successfully determined for two *S. lis*, three *S. vulgaris*, one *S. stramineus*, and one *T. hudsonicus*. Percentage differences corrected by Kimura's two-parameter model and numbers of transitions and transversions obtained from pairwise comparison are shown in Table 2. The transversional substitutions at the third codon positions (Table 3) were used for estimation of the divergence time.

Maximum parsimony (MP), neighbor-joining (NJ), and maximum likelihood (ML) analyses yielded similar branching in trees, all of which contained the same two major groups with high bootstrap values: the New World groups consisting of *S. aberti*, *S. carolinensis*, *S. niger*, and *S. stramineus* (84% in MP tree of Fig. 1a, 80% in NJ tree of Fig. 1b, and 73% in ML tree of Fig. 1c); the Old World groups consisting of *S. lis* and *S. vulgaris* (100% all in MP, NJ, and ML trees, Fig. 1). In MP analysis, only one most-parsimonious phylogenetic tree was obtained by unweighted parsimony, with a consistency index of 0.688.

Although the groups consisting of four *Sciurus* species from the New World were supported with high bootstrap values (73–84%), the phylogenetic relationships within the New World *Sciurus* analyzed in the present study were unclear because of the polychotomy found in MP tree (Fig. 1a) and the low bootstrap values on NJ tree (51–77% in NJ tree, Fig.

Table 2. Pairwise comparison of cytochrome *b* nucleotide sequences (1,040 bp) between ten squirrel specimens

	SAB	SCA	SNI	SST	SLI1	SLI2	SVU1	SVU2	SVU3	THU
SAB		18.3	19.8	18.7	20.4	19.8	20.3	20.1	21.3	23.2
SCA	136/27		14.7	16.3	19.0	20.0	19.6	19.2	20.6	21.9
SNI	145/29	106/30		15.5	19.5	19.1	18.2	18.0	19.5	22.1
SST	142/24	128/19	113/29		19.7	19.4	20.6	20.0	21.2	22.8
SLI1	131/50	128/49	122/53	128/48		0.8	6.2	5.4	6.8	21.0
SLI2	128/49	130/48	122/50	128/45	5/3		5.7	4.9	6.3	21.0
SVU1	132/48	126/49	116/49	140/42	51/10	49/7		1.3	2.4	22.2
SVU2	132/47	124/48	115/48	136/38	45/9	43/6	12/1		1.5	21.8
SVU3	134/54	130/53	120/55	139/48	49/18	47/15	14/10	6/9		23.0
THU	132/72	116/79	114/83	122/80	112/76	113/75	121/76	119/75	121/82	

Data above the diagonal represent percentage differences corrected by Kimura's two-parameter model (Kimura, 1980).
Data below the diagonal are the numbers of nucleotide substitutions (transition/transversion).

Table 3. Pairwise comparison of transversional substitutions at the third codon positions of cytochrome *b* gene between nine specimens of *Sciurus*

	SAB	SCA	SNI	SST	SLI1	SLI2	SVU1	SVU2	SVU3
SAB		5.5	6.1	4.9	13.3	13.3	13.0	12.7	13.3
SCA	19		7.2	3.7	12.7	12.1	13.3	13.0	12.7
SNI	21	25		6.3	13.8	13.0	11.2	12.1	12.7
SST	17	13	22		12.4	11.8	11.2	11.0	11.5
SLI1	46	44	48	43		0.6	2.3	2.0	2.6
SLI2	46	42	45	42	2		2.3	2.0	2.6
SVU1	45	46	39	39	8	8		0.3	1.4
SVU2	44	45	42	38	7	7	1		1.2
SVU3	46	44	44	40	9	9	5	4	

Data above the diagonal represent transversional percentage differences between specimens.
Data below the diagonal are the numbers of transversions.

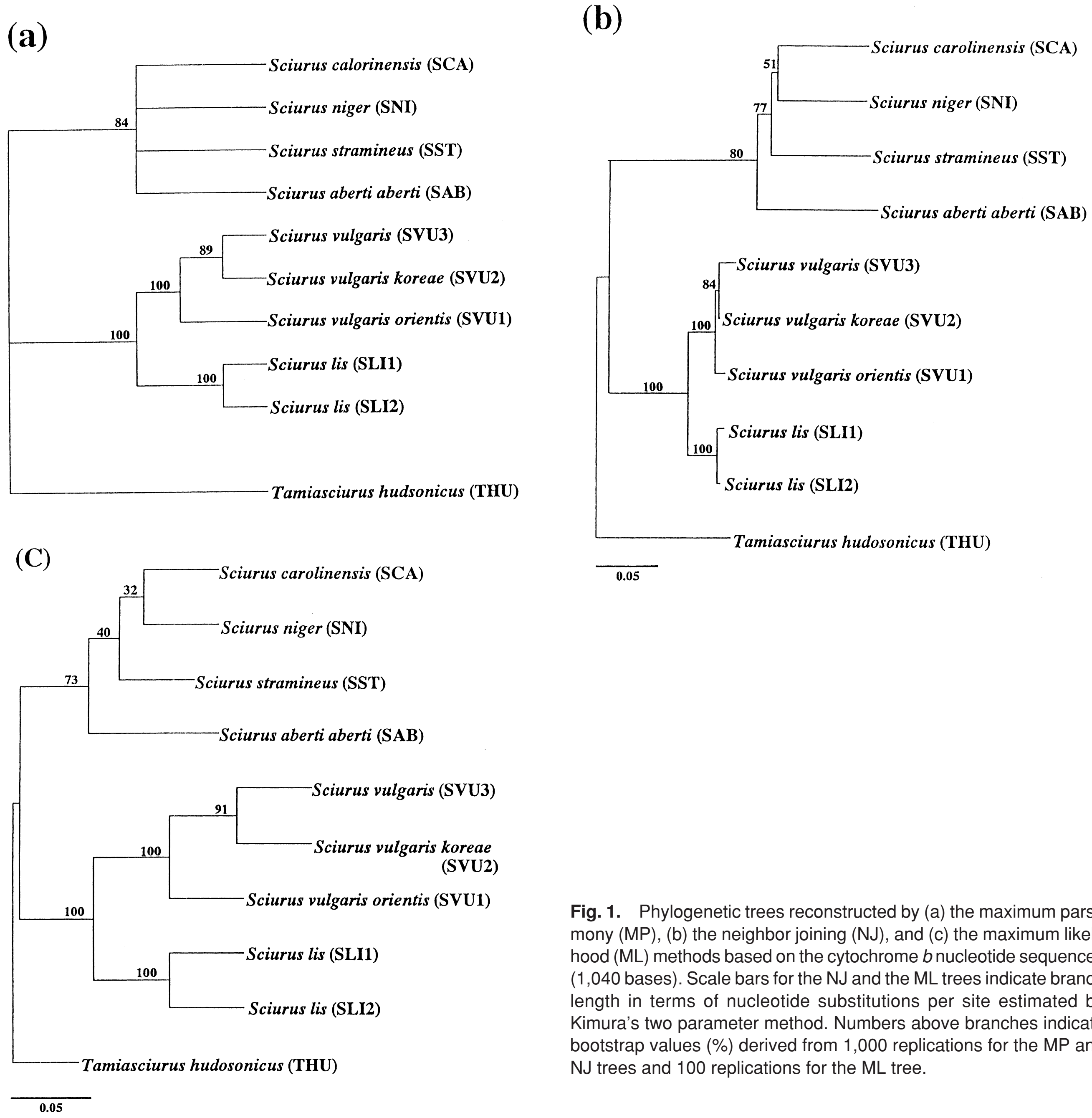

Fig. 1. Phylogenetic trees reconstructed by (a) the maximum parsimony (MP), (b) the neighbor joining (NJ), and (c) the maximum likelihood (ML) methods based on the cytochrome *b* nucleotide sequences (1,040 bases). Scale bars for the NJ and the ML trees indicate branch length in terms of nucleotide substitutions per site estimated by Kimura's two parameter method. Numbers above branches indicate bootstrap values (%) derived from 1,000 replications for the MP and NJ trees and 100 replications for the ML tree.

1b) and ML tree (32–40% in ML tree, Fig. 1c). Genetic distances were 14.7–19.8% among the New World *Sciurus* (Table 2). On the other hand, the monophyly of two Old World *Sciurus* species was clearly shown with very high bootstrap values (100% all in MP, NJ, and ML trees, Fig. 1) and the genetic distances between two species were 4.9–6.8% (Table 2). In addition, genetic distances between the New World *Sciurus* and the Old World *Sciurus* were 18.0–21.3% (Table 2).

DISCUSSION

Phylogeny of the New World *Sciurus*

Sciurus aberti, *S. carolinensis*, and *S. niger* are currently distributed in the North American Continent, while *S. stramineus* occurs in the South American Continent. Although the distribution area of *S. stramineus* is separated from those of the former three species, the phylogenetic position of *S. stramineus* was included in their group (Fig. 1) and did not reflect the geographically expected position. Ellis and Maxson (1980) and Hafner *et al*. (1994) described the close relationship between *S. carolinensis* and *S. niger*, based on the serum albumin and the protein variation, respectively. Our results agreed with their opinions. Moreover, genetic distances among species in NJ and ML trees and the polycotomy in MP tree suggest that the radiation of *Sciurus* might have explosively occurred in the New World (Fig. 1) Based on the fossil records, Black (1972) reported that the ancestral *Sciurus* had already been distributed in North America during the Miocene. Kurtén and Anderson (1980) described that four species of *Sciurus* (*S. alleni*, *S. arizonensis*, *S. carolinensis*, and *S. niger*) were identified in Pleistocene deposits of the New World. From the transversional substitutions at the third codon, the divergence time among the New World *Sciurus* was estimated to be approximately 9.8–14.4 Myr ago. Therefore, the radiation of the New World *Sciurus* might have occurred during the Miocene.

Phylogeny of the Old World *Sciurus*

Sciurus vulgaris is widespread throughout the northern parts of the Eurasian Continent and this species is divided into several subspecies (Sidorowicz, 1971; Wilson and Reeder, 1993). However, the phylogenetic relationships among subspecies have not ever been studied. In three specimens of *S. vulgaris* examined here, one individual from Russia (SVU3) seemed to be closer to *S. v. koreae* from Korea (SVU2) than to *S. v. orientis*, which is endemic to Hokkaido in Japanese islands (SVU1), with high bootstrap values (89% in MP tree, Fig. 1a; 84% in NJ tree, Fig. 1b; 91% in ML tree, Fig. 1c). Ohshima (1990, 1991) suggested that Hokkaido was separated from the Eurasian Continent and Sakhalin during the late Pleistocene via the formation of the straits. *Sciurus v. orientis* may be a population which has been geographically isolated in Hokkaido since then. However, so as to consolidate this hypothesis, further analysis of geographic variation of *S. vulgaris* is required.

The phylogenetic relationship and classification between *S. vulgaris* and *S. lis* are controversial. *S. lis* is endemic to Honshu, Shikoku, and Kyushu islands of Japan (Corbet and Hill, 1991). Imaizumi (1960) classified *S. lis* as an independent species on the basis of differences in tail hair color, body size, and cranial characteristics, while Oshida *et al.* (1996) pointed out that the sequence difference of mitochondrial 12S rRNA gene between *S. lis* and *S. vulgaris* corresponded to intraspecific differences of the genera *Petaurista* and *Tamias*. In addition, Oshida and Yoshida (1997) reported the karyotypic similarity between *S. lis* and *S. vulgaris*. In the present study, genetic distance between two species was 4.9–6.8% (Table 2), and referred to interspecific differences of other squirrel genera, *Glaucomys* (Arbogast, 1999) and *Petaurista* (Oshida *et al*., 1999). Since intraspecific cytochrome *b* differences of other squirrels reported heretofore are <3.0% (Wettstein *et al*., 1995; Arbogast, 1999; Oshida *et al*., 2000), our results of cytochrome *b* are not discordant with that *S. lis* and *S. vulgaris* are regarded as an independent species. In general, the substitution rate of cytochrome *b* gene is more rapid than that of 12S rRNA gene (Irwin *et al*., 1991). The substitution rate differences might be responsible for the conflict between the 12S rRNA phylogeny (Oshida *et al*., 1996) and the cytochrome *b* phylogeny of the present study. Our results also indicate that cytochrome *b* is a more suitable marker to perceive the phylogenetic relationships between closely related squirrel species.

Based on fossil records, Kawamura (1988, 1990) and Kawamura *et al*. (1989) considered that an ancestral *S. vulgaris* immigrated from Hokkaido to Honshu through the land bridge formed during the middle Pleistocene, and then *S. vulgaris* had diverged to *S. lis*. However, in the present study, divergence time between the two species estimated using the transversional substitution rate at the third codon positions of mammalian cytochrome *b* gene was approximately 4.0–5.2 Myr ago. Accordingly, the divergence period between the two species may be earlier than the estimation of Kawamura (1988, 1990) and Kawamura *et al*. (1989).

Phylogenetic relationships between the New World and the Old World *Sciurus*

The present study revealed the phylogenetic differentiation of *Sciurus* between the New World and the Old World (Fig. 1). Fossils of the ancestral *Sciurus* are found in the deposits of the Miocene in Europe and in North America (Black, 1972). In addition, *Sciurus* species are seen in the early Pliocene in Europe (Mein, 1970; Black, 1972). Although the origin of the genus *Sciurus* is currently ambiguous, judging from the information about fossil remains, it is obvious that ancestral *Sciurus* were widespread in the New World and in the Old World during the Miocene. At present, the range of the genus *Sciurus* are absolutely separated by the Bering Strait. Recently, based on the paleontological records, Marincovich Jr. and Gladencov (1999) suggested that the first opening of the Bering Strait might have occurred between 4.8 and 7.3–7.4 Myr ago. The divergence time (approximately

22.4–26.6 Myr ago) between the two *Sciurus* groups estimated in the present study was older than the first opening time of the Bering Strait reported by Marincovich Jr. and Gladencov (1999). Therefore, it may be reasonable that the geographic isolation by the opening of the Bering Strait was not the main cause of the divergence between the two *Sciurus* groups. However, it is likely that the absolute geographic isolation by the opening of the Bering Strait during the Pliocene or the Miocene influenced secondarily the independent evolution of the two *Sciurus* groups.

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