

Phylogenetic relationships among the Old World *Sciurus* squirrels

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A b s t r a c t. Phylogenetic relationships of *Sciurus* were examined based on the mitochondrial cytochrome *b* gene sequences of three Old World and four New World species. The purpose was to test for monophyly in Old World *Sciurus* (*S. anomalus*, *S. lis*, and *S. vulgaris*). Phylogenetic trees well supported clustering of (1) *S. anomalus*, (2) *S. lis* and *S. vulgaris*, and (3) the four New World *Sciurus* species. *Sciurus anomalus* was more closely related to the clade consisting of New World *Sciurus* species than the one consisting of *S. lis* and *S. vulgaris*, indicating a polyphyletic relationship in Old World *Sciurus*. The primary divergence of *Sciurus* may have taken place early on the Eurasian Continent. Then, the ancestral stock of modern New World *Sciurus* would have migrated from the Eurasian Continent to the North American Continent.

Key words: cytochrome *b*, molecular phylogeny, Persian squirrel, *Sciurus anomalus*

Introduction

To understand the evolutionary history of modern mammals, it is extremely important to resolve phylogeographical events that took place between the Old and New Worlds: such as movement, colonization, and diversification. Palearctic and Nearctic regions have unique mammalian fauna (e.g. Smith 1983), but both regions also have similar species and closely related species. These regions were connected by a land bridge across the Bering Sea in the Middle Cretaceous. The continents remained joined by the Bering land bridge until the Pliocene. Marinovich & Gladenkov (1999) suggested that the first opening of the Bering Strait might have occurred between 4.8 and 7.4–7.3 million years ago (Mya). The land connection between Asia and North America, however, was reestablished several times during the Pleistocene (e.g. Sanmartin et al. 2001). During the Pleistocene glaciations, several mammalian groups moved, colonized, and diversified between the Old and New Worlds. A typical filter route between Old and New Worlds, and the focus of many studies (Brunhoff et al. 2003, Cook et al. 2004, 2005, Eddingsaas et al. 2004), was Beringia, between the Alaskan Peninsula and eastern Siberia. Only mammals adapted to the colder climate of this land bridge, such as mammoth, bison, and mountain goat, could successfully cross between continents (Hopkins 1959, Cox & Moore 2005). To resolve faunal dynamics, however, we should first clearly recognize which mammals were separated by the breaking of the Bering land bridge and how they were separated.

Squirrels belonging to the genus *Sciurus* Linnaeus, 1758 are well adapted to subarctic and temperate forests and are widely distributed across the Old and New Worlds. This makes *Sciurus* a suitable taxon to study vicariance of mammals resulting from the breaking

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of the Bering land bridge. The 28 extant species in this genus show extreme bias in number of species between the Old World and the New World (Nowak 1991, Wilson & Reeder 2005). In the New World, 25 *Sciurus* species are distributed. Only three species, however, are allopatric in the Old World (Fig. 1). Based on paleontological records, Black (1972) described the ancestral *Sciurus* as living on European and North American Continents during the Miocene. European records, however, were regarded by de Bruijn (1999) as a separate genus, *Ratufa* Gray, 1867. Also, *Sciurus* species were found in both Europe and Asia in the early Pliocene (Black 1972, de Bruijn 1999). Thus, the origin and evolutionary history of the genus *Sciurus* is ambiguous. One way to resolve this ambiguity is to examine the Old World species (*S. anomalus* Gmelin, 1778, *S. lis* Temminck, 1844, and *S. vulgaris* Linnaeus, 1758) for monophyly.

Sciurus anomalus is restricted to Turkey, Transcaucasia, northern and western Iran, Syria, Lebanon, Israel, Palestine, Jordan, and Iraq (Wilson & Reeder 2005) (Fig. 1). *Sciurus lis* is endemic to Honshu and Shikoku Islands of Japan (Abe et al. 2005) (Fig. 1). In contrast, *S. vulgaris* has the largest range of all tree squirrel species. It is found throughout the Palearctic forests from Iberia and Great Britain; east to Kamchatka Peninsula and Sakhalin of Russia and Hokkaido of Japan; south to the Mediterranean and Black Seas; and in northern Mongolia, and western and northeastern China (Mitchell-Jones et al. 1999, Wilson & Reeder 2005) (Fig. 1). If these three species are monophyletic, we can conclude two speciation events in *Sciurus*, one in the Old World and one in the New World. If, however, they are not monophyletic, a more complicated evolutionary history is implied, one that requires further study as well as consideration of multiple movements of *Sciurus* between the New and Old Worlds or early divergence of *Sciurus* before geographical separation of the New and Old Worlds.

Based on the mitochondrial cytochrome *b* gene sequences, Oshida & Masuda (2000) suggested that the cluster of *S. lis* and *S. vulgaris* was distantly related to a cluster consisting of four New World *Sciurus* species (*S. aberti* Woodhouse, 1853, *S. carolinensis* Gmelin, 1788, *S. niger* Linnaeus, 1758, and *S. stramineus* Eydoux et Souleyet, 1841). The close relationship of *S. vulgaris* and *S. lis* is supported by similarities in karyotype (Oshida & Yoshida 1997). To identify monophyly of Old World *Sciurus* species,

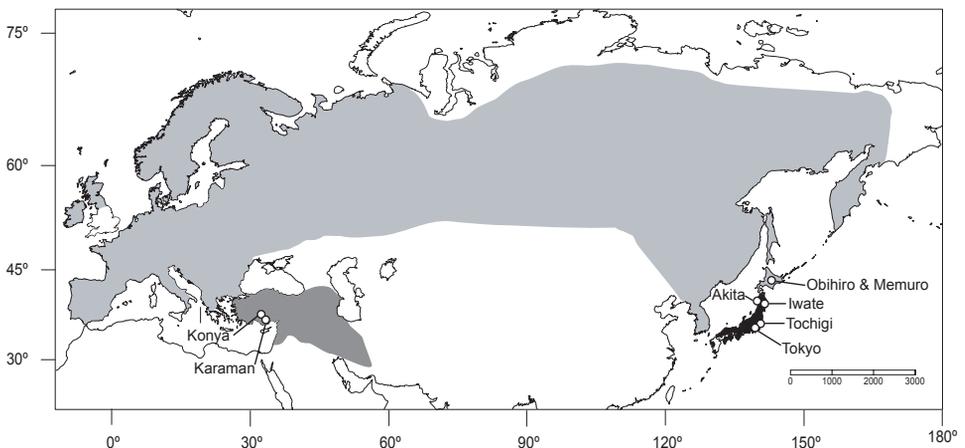


Fig. 1. Distribution and collecting localities of Palearctic *Sciurus* squirrels: *S. anomalus* in dark gray, *S. lis* in black, and *S. vulgaris* in light gray. This map is based on the descriptions in Wilson & Reeder (2005)

Table 1. Specimens of tree squirrels examined. ^aNumbers correspond to those in Table 2 and Fig. 2. ^bSequence data from the DDBJ or reference. ^cThe (NI) means no information. ^dIdentity numbers from author's private specimens.

Species name ^a	Collecting locality	Identity number ^c	Accession No. and/or reference
<i>Sciurus aestivans</i> ^b	(NI)	T-1484	AJ389530, Montgeard et al. (2002)
<i>Sciurus anomalus</i> 1	Hadim, Konya, Turkey	T1D	AB292675
<i>Sciurus anomalus</i> 2	Akören, Konya, Turkey	T2D	AB292675
<i>Sciurus anomalus</i> 3	Ermenek, Karaman, Turkey	T3D	AB292676
<i>Sciurus anomalus</i> 4	Ermenek, Karaman, Turkey	T4D	AB292677
<i>Sciurus carolinensis</i> ^b	Alameda Co., California, USA	FC3830	Thomas & Martin (1993)
<i>Sciurus lis</i> 1 ^b	Akita, Japan	CH1	AB192892
<i>Sciurus lis</i> 2 ^b	Iwate, Japan	CH2	AB192893
<i>Sciurus lis</i> 3 ^b	Tochigi, Japan	CH9	AB192899
<i>Sciurus lis</i> 4 ^b	Tokyo, Japan	CH24	AB192914
<i>Sciurus niger</i>	(NI)	(NI)	U10180, Wettstein et al. (1995)
<i>Sciurus stramineus</i>	(NI)	ST1D	AB292678
<i>Sciurus vulgaris</i> 1	Obihiro, Hokkaido, Japan	H1D	AB292679
<i>Sciurus vulgaris</i> 2	Obihiro, Hokkaido, Japan	H2D	AB292680
<i>Sciurus vulgaris</i> 3	Memuro, Hokkaido, Japan	H3D	AB292681
<i>Sciurus vulgaris</i> 4 ^b	(NI)	(NI)	AJ238588, Reyes et al. (2000)
<i>Tamiasciurus hudsonicus</i>	Taos Co, New Mexico, USA	MSB(61555) (NK4324)	AF147643, Piaggio & Spicer (2001)

however, the phylogenetic position of *S. anomalus* must also be analyzed. Therefore, we studied the molecular phylogenetic relationships among *S. anomalus*, *S. lis*, and *S. vulgaris*, and monophyly in the Old World *Sciurus*. We also reviewed the relationships between Old World and New World *Sciurus*.

Materials and Methods

Specimens and DNA extraction

Information of squirrels examined is shown in Table 1. Four specimens of *S. anomalus* were collected from the field. We used three roadkilled *S. vulgaris* from Hokkaido, Japan. One *S. stramineus*, which occurs in northwestern Peru and southwestern Ecuador (Wilson & Reeder 2005), was purchased at a pet store in Japan. Its collecting locality was unknown. Sequences of other *Sciurus* were obtained from the previous report (Thomas & Martin 1993) and the DNA Data Bank of Japan (DDBJ). Total genomic DNA was extracted from muscle tissues using QuiaQuick kit (QUIAGEN K.K., Tokyo, Japan).

PCR and DNA sequencing

The entire mitochondrial cytochrome *b* gene (1140 bases) of squirrels was amplified with polymerase chain reaction (PCR), using a primer set (L14724 5'-GATATGAAAA CCATCGTTG-3' and H15910 5'-GATTTTTGGTTTACAAGACCGAG-3') reported by Oshida et al. (2000). Primer names are concordant with the light (L) or heavy (H) strand and the 3' end-position of the primers in human mitochondrial DNA sequences (Anderson et al. 1981). The 50 μ l of reaction mixture contained approximately 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 (ABgene, Epsom, UK). Amplification was carried out for 35 cycles; the program was 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. A final extension reaction was performed at 72°C for 10 min. The PCR products purified with the PCR Clean Up-M (Viogen, Taipei, Taiwan) were directly sequenced using an automated DNA sequencer (ABI PRISM 377-96 Sequencer, ABI PRISM 3100 Genetic Analyzer, Applied Biosystem, CA, USA) and ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1 (Applied Biosystems, CA, USA). Sequencing used the same primers used for PCR. Purification of PCR products and sequencing were carried out by Mission Biotech Co. Ltd. (Taipei, Taiwan).

Sequence analysis

Sequence data for phylogenetic analysis were from the DNA Data Bank of Japan (DDBJ) (see Table 1): one *S. aestuans* Linnaeus, 1766 (accession number AJ389530, Montgellard et al. 2002), four *S. lis* (accession numbers AB192892, AB192893, AB192899, and AB192914), one *S. niger* (accession number U10180, Wettstein et al. 1995), and one *S. vulgaris* (accession number AJ238588, Reyes et al. 2000). *Sciurus aestuans* occurs in Brazil, French Guiana, Guiana, Suriname, and Venezuela. *Sciurus niger* is distributed in Texas (USA) and adjacent Mexico, north to Manitoba (Canada), and east to the Atlantic Coast (Wilson & Reeder 2005). We also used the sequence of *S. carolinensis* reported by Thomas &

Martin (1993). This species occurs eastern Texas (USA) to Saskatchewan (Canada) and east to Atlantic Coast (Wilson & Reeder 2005).

All trees were rooted using *Tamiasciurus hudsonicus* (Erxleben, 1777) sequence (accession number AF147643) reported by Piaggio & Spicer (2001). Based on the IRBP (interphotoreceptor retinoid binding protein) and 12S and 16S rRNA gene sequences, Mercer & Roth (2003) demonstrated that *Tamiasciurus* Trouessart, 1880 formed a sister group to the clade comprising *Sciurus* and *Microsciurus* J. A. Allen, 1895. This phylogenetic relationship was also supported by Steppan et al. (2004) on the basis of *c-myc* and RAG (recombination activation gene) 1 sequences. Therefore, we chose *Tamiasciurus* species as an out-group to assess phylogenetic relationships within the genus *Sciurus*, even though the phylogenetic relationship between *Sciurus* and *Microsciurus* is still unclear.

Nucleotide sequences were aligned using DNASIS (Hitachi, Tokyo, Japan). For maximum-likelihood (ML) analysis, we used the program Modeltest 3.06 (Posada & Crandall 1998) to select the most appropriate model of molecular evolution with Akaike information criterion (AIC). This test chose the general time-reversible (GTR) model of substitution (Rodríguez et al. 1990) taking into account the proportion of invariable sites (0.5491) and following a gamma distribution shape parameter (5.0791) (GTR + I + G). Base frequencies were estimated as A = 0.2658, C = 0.2949, G = 0.1367, and T = 0.3026, and the rate matrix was also estimated as A-C = 3.8716, A-G = 10.7076, A-T = 4.0846, C-G = 1.9252, C-T = 38.2007, and G-T of 1.0. We also made un-weighted maximum parsimony (MP) analysis. The ML and MP trees were constructed with the heuristic search algorithm using tree-bisection-reconnection (TBR) in PAUP4* version 4.0b10 (Swofford 2001). Neighbor-joining (NJ) analysis (Saitou & Nei 1987) was employed with the GTR + I + G model in PAUP* version 4.0b10. To assess branching confidence, bootstrap values (Felsenstein 1985) were derived from 200 replications in the ML analysis, 1000 replications in the MP analysis, and 5000 replications in the NJ analysis. A Bayesian reconstruction of the data matrix of each species was performed with MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001). We also used the best-fitting substitution model (GTR + I + G). Four Markov chain Monte Carlo (MCMC) chains ran for one million generations, sampling every 1000 generations. In Bayesian inference (BI), posterior probabilities were made: a majority-rule consensus tree was calculated based on trees remaining after the Bayesian runs.

Results

Cytochrome *b* gene sequences

Complete sequences (1140 bases) of the cytochrome *b* gene of four *S. anomalus*, three *S. vulgaris*, and one *S. stramineus* were determined. These were analyzed with previously published sequences. Base composition of these sequences was skewed toward a deficiency in guanine (12.2%). The other three nucleotides were more balanced (thymine 30.4%, cytosine 29.7%, and adenine 27.7%). Base frequency across in-group taxa was homogeneous ($\chi^2 = 6.32$, $df = 42$, $P = 1.0$). The frequency of guanine differed greatly among the three codon positions: 21.8% in the first, 14.0% in the second, and 2.8% in the third positions. The second position had more thymine (42.6%). First and third positions were more abundant in

Table 2. Pairwise comparisons of cytochrome *b* nucleotide sequences (1140 bp) between 16 *Sciurus* specimens. Data above the diagonal represent uncorrected percentage differences (*p*-distances). Data below the diagonal are numbers of nucleotide substitutions (transitions/transversions).

	<i>S. aestuans</i>	<i>S. anomalous 1,2</i>	<i>S. anomalous 3</i>	<i>S. anomalous 4</i>	<i>S. carolinensis</i>	<i>S. lis 1</i>	<i>S. lis 2</i>	<i>S. lis 3</i>	<i>S. lis 4</i>	<i>S. niger</i>	<i>S. stramineus</i>	<i>S. vulgaris 1</i>	<i>S. vulgaris 2</i>	<i>S. vulgaris 3</i>	<i>S. vulgaris 4</i>
<i>S. aestuans</i>		16.75	16.58	16.40	14.21	17.63	17.63	17.90	17.72	14.21	11.14	16.84	17.19	17.02	17.02
<i>S. anomalous 1, 2</i>	138/53		0.53	0.53	16.75	16.34	16.49	16.67	16.40	15.53	16.32	16.05	16.23	16.23	16.05
<i>S. anomalous 3</i>	136/53	4/2		0.70	15.70	16.05	16.23	16.40	16.14	15.26	16.14	16.00	16.14	16.14	16.00
<i>S. anomalous 4</i>	134/53	6/0	6/2		16.75	16.40	16.58	16.75	16.49	15.53	16.40	16.14	16.32	16.32	16.14
<i>S. carolinensis</i>	137/25	135/56	134/56	135/56		14.49	16.58	16.75	16.49	13.51	13.60	16.05	16.40	16.23	16.14
<i>S. lis 1</i>	146/55	130/56	129/54	131/56	130/58		0.26	0.35	0.26	14.49	16.05	4.47	4.56	4.65	4.47
<i>S. lis 2</i>	144/57	130/58	129/56	131/58	129/60	1/2		0.53	0.53	16.49	16.05	4.65	4.74	4.83	4.65
<i>S. lis 3</i>	144/58	131/59	130/57	132/59	130/61	1/3	1/5		0.61	16.75	16.32	4.83	4.91	5.00	4.83
<i>S. lis 4</i>	147/55	131/56	130/54	132/56	130/58	3/0	4/2	4/3		16.49	16.14	4.74	4.83	4.91	4.74
<i>S. niger</i>	129/33	119/58	116/58	119/58	116/38	134/54	132/56	134/57	134/54	118/29	12.90	15.53	15.70	15.70	15.61
<i>S. stramineus</i>	113/14	139/47	137/47	140/47	134/21	136/47	134/49	136/50	137/47	118/29	16.23	16.23	16.58	16.40	16.40
<i>S. vulgaris 1</i>	139/53	127/56	128/54	128/56	125/58	45/6	45/8	46/9	48/6	125/52	142/43	0.61	0.61	0.12	0.35
<i>S. vulgaris 2</i>	143/53	129/56	130/54	130/56	129/58	46/6	46/8	47/9	49/6	127/52	146/43	7/0	7/0	0.61	0.44
<i>S. vulgaris 3</i>	141/53	129/56	130/54	130/56	127/58	47/6	47/8	48/9	50/6	127/52	144/43	2/0	7/0	0.61	0.35
<i>S. vulgaris 4</i>	140/54	126/57	127/55	127/57	125/59	44/7	44/9	45/10	47/7	125/53	143/44	3/1	4/1	3/1	3/1

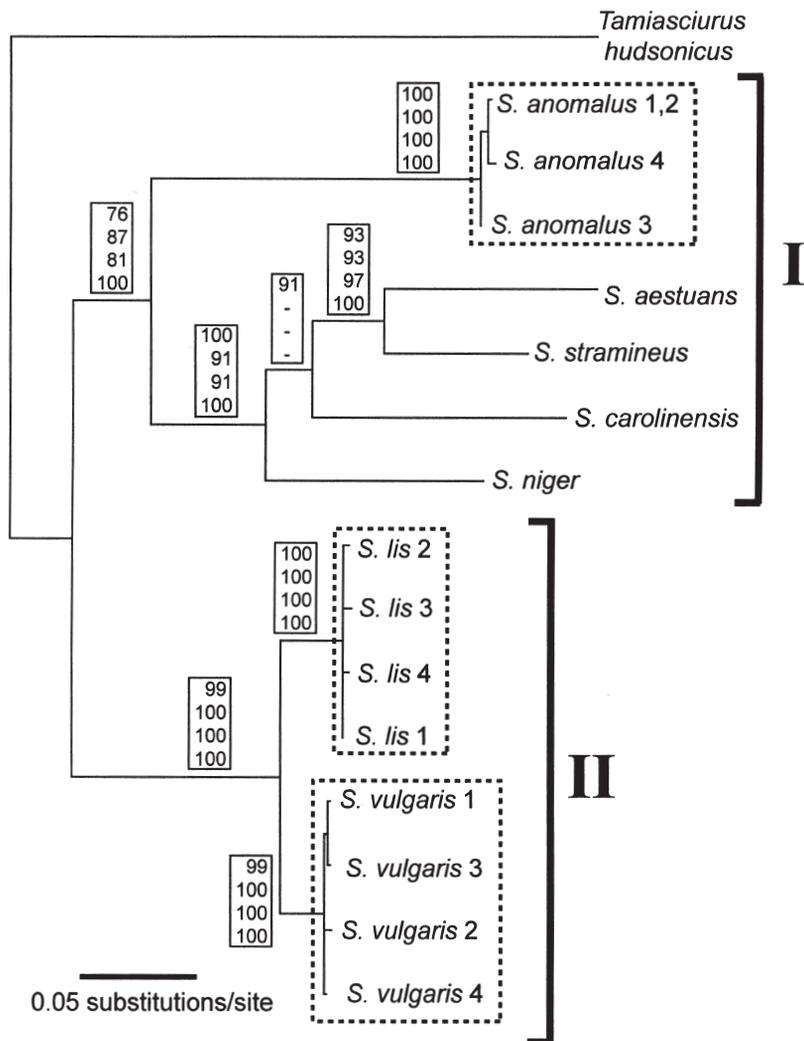


Fig. 2. A maximum-likelihood (ML) phylogenetic reconstruction of seven *Sciurus* species using a heuristic search algorithm with tree-bisection-reconnection (TBR) assuming the GTR + I + G model of evolution, showing two clades: I) *S. anomalus* and New World *Sciurus* and II) *S. lis* and *S. vulgaris*. Tree was rooted with *Tamiasciurus hudsonicus*. Number in boxes above branches represent, from the top: bootstrap values from 200 replicates of ML analysis and from 1000 replicates of un-weighted maximum parsimony (MP) analysis using heuristic search algorithm with TBR, bootstrap values from 5000 replicates of the neighbor-joining (NJ) analysis under the GTR + I + G model, and posterior probability of Bayesian inference (BI); hyphens mean no data, because the clade was absent. The three Old World *Sciurus* species indicated by dotted boxes.

adenine (27.6% and 32.7%, respectively). These values were similar to base compositional patterns found in other mammalian taxa (I r w i n et al. 1991, L a r a et al. 1996, M a r t i n et al. 2000, O s h i d a et al. 2004). The bias of base composition calculated according to the formula in I r w i n et al. (1991) was smaller at the first and second positions (0.04 and 0.24, respectively) than at the third positions (0.29). Therefore, the first and second positions showed less variability than the third codon position. This matches variability in cytochrome *b* genes reported for other mammals (I r w i n et al. 1991).

Uncorrected percentage sequence divergences (*p*-distances) and nucleotide substitutions among the 16 specimens of *Sciurus* squirrels are shown in Table 2. The *p*-distances among *Sciurus* species examined ranged from 4.47 to 17.90%. The *p*-distances between *S. anomalus* from Turkey and *S. lis* and *S. vulgaris* were 16.05–16.75% and 16.00–16.32%, respectively. Those between *S. anomalus* and the four New World *Sciurus* species were 15.26–6.75%. The *p*-distances between *S. lis* and *S. vulgaris* were 4.47–5.00%. The *p*-distances among the New World *Sciurus* species were 12.90–14.21%.

Molecular phylogenetic analysis

The ML tree constructed under the GTR + I + G model is shown in Fig. 2. Phylogenetic trees constructed by un-weighted MP, NJ, and BI analyses were not shown, as they were essentially similar to ML tree. The bootstrap values for ML, MP and NJ trees and posterior probability for BI tree are also given in Fig. 2. *Sciurus* squirrels were split into two clades: clade I consisting of *S. aestuans*, *S. anomalus*, *S. carolinensis*, *S. niger*, and *S. stramineus* and clade II consisting of *S. lis* and *S. vulgaris*. Bootstrap values supporting clade I were somewhat low (76% in ML, 87% in MP, and 81% in NJ), although posterior probability of BI was high (100%). Clade II was well supported with bootstrap values of 99% in ML and 100% in MP and NJ and with posterior probability of 100% in BI. In clade I, *S. anomalus* formed a sister group with four New World species (*S. aestuans*, *S. carolinensis*, *S. niger*, and *S. stramineus*). The New World species group within clade I was well supported (100% nodal support in ML and BI and 91% in MP and NJ). In this group, the two South American species (*S. aestuans* and *S. stramineus*) were clustered together (93% nodal support in ML and MP, 97% in NJ, and 100% in BI). In the MP and NJ trees, *S. carolinensis*, *S. niger*, and the group consisting of *S. aestuans* and *S. stramineus* showed a polytomic relationship (data not shown). Unlike ML tree, in BI tree, *S. carolinensis* first diverged from other three species (data not shown).

Discussion

Analysis of the three Old World *Sciurus* species (*S. anomalus*, *S. lis*, and *S. vulgaris*) did not reveal a monophyletic relationship. *Sciurus lis* and *S. vulgaris* were closely related, but *S. anomalus* was clustered with the New World *Sciurus* species (Fig. 2). Bootstrap values supporting this clustering, however, were somewhat low. The genetic distances between *S. anomalus* and other Old World species were very similar to those between *S. anomalus* and the New World species (Table 2). Therefore, it is possible to recognize three major clades in this genus: (1) *S. anomalus*, (2) *S. lis*, and *S. vulgaris*, and (3) the New World *Sciurus* species. This suggests that divergence of these three clades took place early in the evolutionary history of *Sciurus*.

Phylogenetic relationship between *S. lis* and *S. vulgaris*

The shortest genetic distances (4.47–5.00%) were between *S. lis* and *S. vulgaris* (Table 2). Partial 12S rRNA gene sequences (Oshida et al. 1996) and partial cytochrome *b* gene sequences (Oshida et al. 2000) also demonstrate the close relationship between these species. Moreover, their karyotypes are similar, differing only in the number of constitutive heterochromatin blocks and nucleolus organizer regions (Oshida & Yoshida 1997). Our results strongly support these conclusions of a close evolutionary relationship between

S. lis and *S. vulgaris*. Based on fossil records, Kawamura (1988) and Kawamura et al. (1989) considered *S. vulgaris* ancestral to *S. lis*. Since *S. lis* exists in the Japanese Islands on the periphery of *S. vulgaris*, it may have recently diverged from *S. vulgaris*, making it another example of peripatric speciation as proposed by Mayr (1982).

Phylogenetic relationships among New World *Sciurus* species

Hafner et al. (1994) used protein variation to reveal that *S. stramineus* from South America is distantly related to *S. carolinensis* and *S. niger*. We found a close relationship between the two South American species (*S. aestuans* and *S. stramineus*) and clear separation from *S. carolinensis* and *S. niger* (Fig. 2). Therefore, the speciation may have occurred in South America. Serum albumin (Ellis & Maxson 1980) and protein variation (Hafner et al. 1994) show a close relationship between *S. carolinensis* and *S. niger*. Partial cytochrome *b* sequences (Oshida et al. 2000) also show the same close relationship. Also, *S. carolinensis* and *S. niger* share similar karyotypes (Nadler & Sutton 1967). In our analyses, however, these two species did not form a single sub-clade similar to the one for *S. aestuans* and *S. stramineus* (Fig. 2). In the New World, there are 25 *Sciurus* species, but we only included four in our analyses. To resolve the relationship of New World *Sciurus*, phylogenetic analysis of all species is needed.

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

Nadler & Hoffmann (1970) reported that diploid number ($2n = 40$) and autosomal arm numbers ($FN = 76$) of *S. anomalus* are similar to those of *S. niger* and *S. carolinensis*, but the Y chromosome of *S. anomalus* differs in shape. The FN and Y chromosome of *S. anomalus*, however, are distinctly different from those of *S. vulgaris* ($FN = 74$) (Sasaki et al. 1968, Oshida et al. 1993). From the cytogenetical point of view, therefore, *S. anomalus* is more closely related to *S. carolinensis* and *S. niger*, than it is to *S. vulgaris*. Our phylogenetic results also support this relationship, although the nodal supports were somewhat low. We did, however, find deep phylogenetic divergences between *S. anomalus* and the other New World species. Therefore, *S. anomalus* could have a unique evolutionary history.

Considering the modern distribution of *S. anomalus*, *S. lis*, and *S. vulgaris* (Fig. 1), and the phylogenetic position of *S. anomalus* (Fig. 2), we conclude the primary divergence in the genus *Sciurus* was on the Eurasian Continent. First, however, we review and reject hypotheses of primary divergence on North America and colonization of Eurasia from North America through Beringia or the North Atlantic.

Current geographic distributions may support the hypothesis of the New World origin and original invasion of Asia by *S. anomalus* through Beringia, since *S. anomalus* is currently more distantly located from Beringia. After this invasion through Beringia, drastic speciation of *Sciurus* would have occurred in North America and *S. vulgaris* would have invaded Asia. Our molecular results, however, indicated that *S. anomalus* is more closely related to New World *Sciurus* than to *S. vulgaris*.

Our molecular data and the current geographic distribution do not eliminate the hypothesis of the New World origin followed by an invasion of *S. anomalus* and *S. vulgaris* into the Old World through a North Atlantic land bridge. Although the latest land bridge

between Europe and eastern North America (the Greenland-Faeroes Bridge) persisted from the Late Eocene to the Miocene, it is not considered an important dispersal route because it was probably a chain of islands (McKenna 1983, Tiffney 1985, Sanmartín et al. 2001). This makes it unlikely that *S. anomalus* and *S. vulgaris* moved from eastern North America to Europe through the North Atlantic.

Therefore, we conclude the primary divergence in the genus *Sciurus* was on the Eurasian Continent and that an ancestral *Sciurus* moved from the Old World through Beringia to the New World. After isolation resulting from opening of the Bering Strait, there would have been explosive speciation (adaptive radiation) in the North America during the Pliocene and Pleistocene. This is because this ancestral *Sciurus* would have been highly adapted to North America's abundant, subarctic and temperate forests. As its distribution expanded to include Central and South America, more speciation would have occurred. Meanwhile, in the Old World, speciation would have produced *S. anomalus* and *S. vulgaris*. By the Pliocene, these two species would have allopatric distribution. Then, in the Pleistocene, and on periphery of the range of *S. vulgaris*, *S. lis* would have evolved from *S. vulgaris*.

The place of origin of the modern *Sciurus* is not known (e.g. Gurnell 1987), even though fossil records of this genus are found from the early Miocene in North America (Black 1972) and from the Pliocene in North America, Europe, and Asia (Black 1972, de Bruijn 1999). Mercer & Roth (2003) demonstrated that the divergence within the genus *Sciurus* occurred approximately 8.6 ± 1.3 Mya. Therefore, the primary divergence of *Sciurus* probably occurred before the opening of the Bering Strait. To fully test the phylogeographical scenarios outlined here, a complete phylogenetic analysis of all modern *Sciurus* species is needed.

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