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Restriction fragment length polymorphism of nuclear rDNA in *Sorex caecutiens/shinto* group (Eulipotyphla, Soricidae)

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Abstract. We estimated phylogenetic relationships among shrews of the *Sorex caecutiens/shinto* group (Eulipotyphla, Soricidae) from various locations through its range, based on restriction fragment length polymorphism (RFLP) analysis of the nuclear ribosomal RNA gene (rDNA) spacer region. Seven rDNA-RFLP repetitive types (repetypes) were recognized among 15 shrews examined. Restriction patterns of *Sorex caecutiens* Laxmann, 1788 and *S. shinto* Thomas, 1905 were distinguishable from each other, but the separation was not statistically supported in the maximum parsimony analysis. The RFLP repetype from Cheju Island was close to that of *S. caecutiens* from the Eurasian continent, indicating that the shrew of Cheju should be classified as *S. caecutiens*. Within *S. caecutiens*, there were two alternative phylogenetic hypotheses. According to a parsimonious tree and a simple network, the Hokkaido population was regarded to be derived from the Sakhalin population, which in turn was derived from the continental population. Alternatively, it was inferred that the continent and Hokkaido populations were firstly separated from the ancestral population, and then shrews from both populations immigrated into Sakhalin and hybridization occurred there. The latter hypothesis seems to be more plausible because it is more congruent with a previous mitochondrial phylogeny.

Key words: Cheju Island, nuclear rDNA, RFLP, *Sorex caecutiens*.

Taxonomic ranks of *Sorex caecutiens* Laxmann, 1788 and *S. shinto* Thomas, 1905 had been debated by various authors because interpretation of their morphology is difficult (see Dokuchaev et al. 1999) and little phylogenetic information was available for these shrews. However, after phylogenetic investigations of some species of *Sorex* (George 1988; Ohdachi et al. 1997; Fumagalli et al. 1999), it is now widely accepted that these two species are distinct. Further, Ohdachi et al. (1997, 2001, 2003) revealed based on mitochondrial cytochrome *b* gene (mtDNA cyt-*b*) sequences that *S. caecutiens* and *S. shinto* form a monophyletic group (*S. caecutiens/shinto* group), and that *S. caecutiens* occurs in the Eurasian continent, Sakhalin, Hokkaido, and neighboring small islands while *S. shinto* is distributed in the southern parts of the Japanese Islands (Honshu, Shikoku, and Sado). From Cheju Island, South Korea, a *Sorex* shrew species was recently discovered (H. S. Oh, personal observation). It has not yet been formally described but initial observation suggests it belongs to the *S. caecutiens/shinto* group. Ohdachi et al. (2003) showed that the shrew on Cheju Island should be included in *S. caecutiens*, based on mtDNA cyt-*b* sequences.

According to the phylogenetic tree based on mtDNA cyt-*b*, there are two main clusters in *S. caecutiens*: the Hokkaido cluster and the Eurasian Continent-Sakhalin-

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Cheju cluster (Ohdachi et al. 2003; Ohdachi 2005). Furthermore, it was demonstrated that genetic variation was low and there was almost no local divergence of haplotype among individuals of *S. caecutiens* from the Eurasian continent and Sakhalin (Ohdachi et al. 2001, 2003). Based on these results, Ohdachi et al. (2001, 2003) suggested that the ancestral population of *S. caecutiens* were first divided into two subpopulations, one in Hokkaido and the other in the eastern part of the Eurasian continent. Then, the latter subpopulation spread rapidly throughout the continent and Sakhalin after the last glacial period.

Phylogenetic trees inferred from mtDNA, however, sometimes contradict those inferred from nuclear DNA (e.g. Mukai 2001; Sota and Vogler 2001; Iwasa and Suzuki 2002). In such cases, taxonomy and evolutionary events inferred from mtDNA alone will lead to erroneous conclusions. In the *S. caecutiens/shinto* group, phylogenetic investigations based on nuclear DNA have not been conducted. Thus, it is needed to infer the phylogeny from the information of nuclear DNA.

In the present paper, we investigate restriction fragment length polymorphism (RFLP) of nuclear ribosomal RNA genes (rDNA) and estimate the phylogenetic relationships between species or populations in the *S. caecutiens/shinto* group. In addition, phylogenetic status of the *Sorex* shrew from Cheju Island is investigated. Herein, we shortly summarize the nature of nuclear rDNA. The rDNA gene unit multiplies several hundred times in a nuclear genome and forms a multigene family. Thus, it is useful to estimate an overall genetic variation of a nuclear genome of an individual in a population. The rDNA gene unit is composed of the coding and spacer regions. The former regions, which encode highly conserved rDNA (18S, 5.8S, and 28S), are suitable as probe-anchoring regions (Arheim et al. 1980). The spacer regions, in contrast, are known to evolve rapidly and are useful to detect variation between and within species in phylogenetic inferences (e.g. Arheim et al. 1980; Suzuki et al. 1990; Suzuki 1994).

Materials and methods

Specimens

Eight individuals of *S. caecutiens* and five *S. shinto* were sampled from 12 localities of northern Eurasia (Table 1 and Fig. 1). Two individuals of *Sorex* shrew species (*Sorex* sp. of the *S. caecutiens/shinto* group) from Cheju Island, South Korea, were also examined.

Southern hybridization and mapping of restriction sites

Total genomic DNA was extracted from liver tissue preserved in 90% ethanol by the phenol/proteinase K/SDS method (Sambrook et al. 1989). The genomic DNA was digested with seven restriction enzymes, *Aat*I (A), *Bgl*II (B), *Dra*I (D), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P)

Table 1. *Sorex* shrew samples and their collection localities examined in this study.

Species	Repetype	Locality No.	Locality	Specimen Code
S. shinto				
	Shi-A	1	Mt. Kamegamori, Shikoku, Japan	SO97/5/25-1
	Shi-B	2	Nagano Pref., Honshu, Japan	SO96misc-13
	Shi-B	3	Ryotsu, Sado Is., Japan	HA5961
	Shi-B	3	Ryotsu, Sado Is., Japan	HA5962
	Shi-C	4	Mt. Hayachine, Honshu, Japan	SO97/8/5-1
S. caecutiens				
	Cae-A	5	Kaminokuni, Hokkaido, Japan	SO97/6/7-1
	Cae-A	6	Sarobetsu moor, Hokkaido, Japan	SO94sc1
	Cae-B	7	Trudovoe, Sakhalin, Russia	SO94sak5
	Cae-C	8	Kedrovaya, Primorye, Russia	SO96/7/16-2
	Cae-C	9	Mt. Gaya, South Korea	SO99misc-9
	Cae-C	10	Mt. Odae, South Korea	SO99/10/17-1
	Cae-C	12	Milkovo, Kamchatka, Russia	SO97misc-82
	Cae-C	13	Pallasjärvi, Lapland, Finland	VH365
Sorex sp.				
	Sp-Cheju	11	Mt. Halla, Cheju Is., South Korea	SO99/10/12-1
	Sp-Cheju	11	Mt. Halla, Cheju Is., South Korea	SO99/10/13-1

and *Pvu*II (V). After electrophoresis with 0.7% agarose gel for 17 hours at 30 V, the digested DNA was immobilized on nylon membrane (Hybond-N, Amersham) and hybridized with digoxigenin-labeled (DIG DNA Labeling Kit and Detection Kit, Roche Diagnostics) 28S probe (0.7 μg/ml) from mouse (Suzuki et al. 1994). Detection of the probe on the membrane followed the supplier's protocol. Fragment lengths were determined by comparison with a size marker (DNA Molecular Weight Marker II digoxigenin-labeled lambda *Hin*dIII). Restriction maps of the 28S downstream spacer region were constructed by referring to the standard restriction map of the 18S, 5.8S, and 28S rRNA genes that are conservative among mammalian species (Suzuki et al. 1994; Iwasa et al. 2001).

Phylogenetic analyses

All restriction sites were numbered from 1 to 13 (Fig.

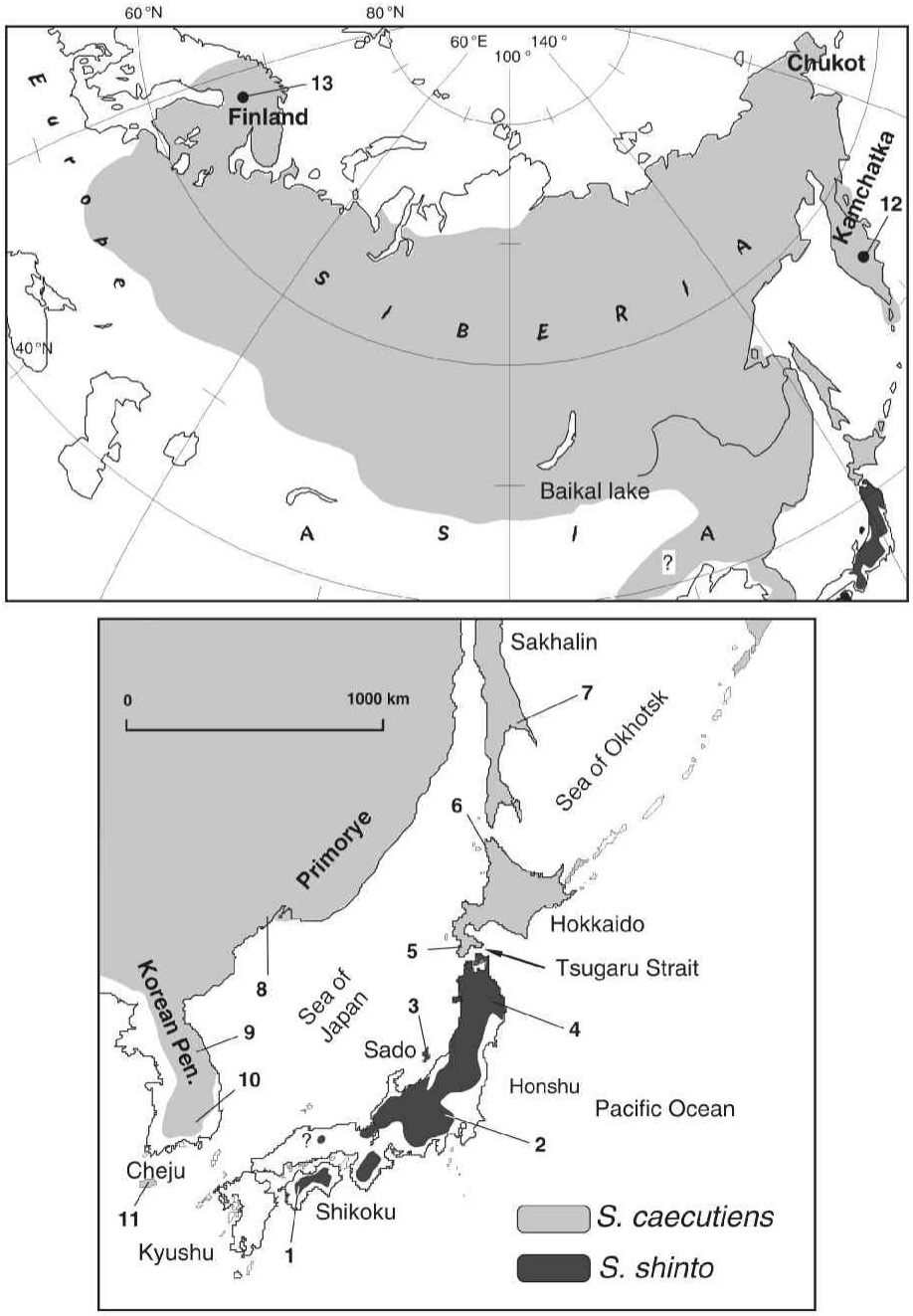

Fig. 1. Collection localities of shrew samples. Locality numbers correspond with those of Table 1.

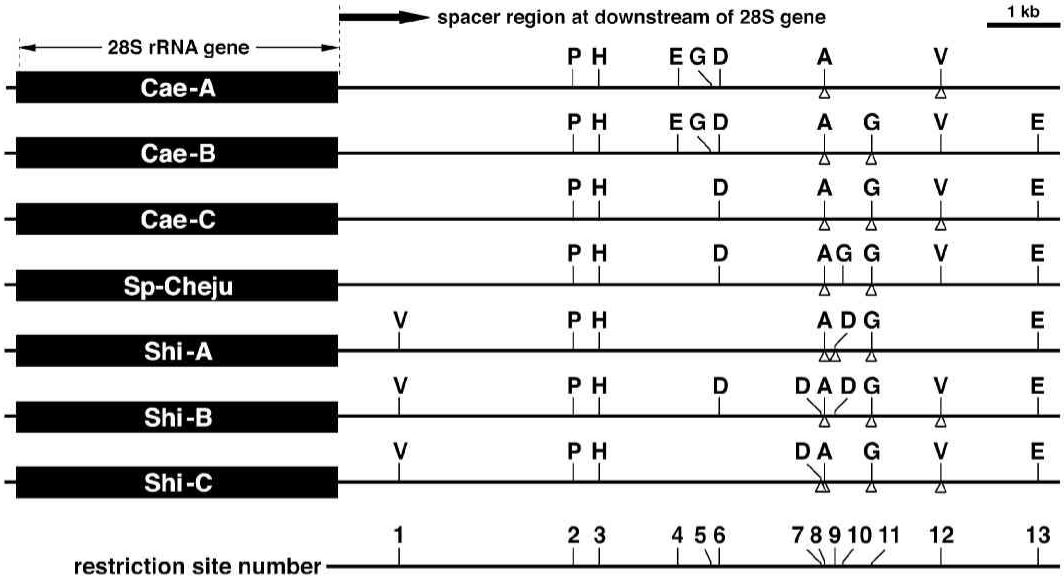

Fig. 2. Restriction maps of the major repeating unit types of the ribosomal RNA gene downstream of the 28S coding region in the *Sorex caecutiens/shinto* group from various localities of Eurasia. Abbreviations of restriction enzymes: A, *Aat*I; B, *Bgl*II; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; V, *Pvu*II. Among 15 individuals examined, some had identical repetitive types (repetypes). See Table 1 for individuals examined and their RFLP repetyes. Open triangles indicate polymorphic sites as size variation within a genome of an individual.

2) and converted into a 0/1 data matrix, where a 0 indicates absence of the band and a 1 indicates presence. Phylogenetic analyses were conducted using the maximum parsimony method, applied using PAUP ver. 4.0b10 (Swofford 2000) with the exhaustive search method and Dollo's criterion. The cost for gain of a restriction site was six times higher than for loss of a site because the restriction enzymes used all had a six base recognition sequence (Swofford and Olsen 1990). Nodal support was evaluated by 10,000 bootstrap replicates with 50% majority rule consensus. A parsimonious network was also constructed according to Bandelt (1994). This method indicates every change of state in a network. Thus, when the number of informative sites is small, this method is more straightforward than other methods of phylogeny reconstruction.

Results

Restriction map

Seven rDNA-RFLP repetitive types (repetypes) were recognized among 15 shrews examined (Fig. 2). The restriction patterns of *S. shinto* and *S. caecutiens* were distinguished by site 1 (Fig. 2). Four individuals of *S. shinto* showed three rDNA-RFLP repetitive type: Shi-A, Shi-B and Shi-C (Table 1, Fig. 2). In contrast, five individuals of *S. caecutiens* from several localities on the Eurasian continent including the Korea Peninsula shared only one repetype (Cae-C; Table 1, Fig. 2). Two *S. caecutiens* individuals from Hokkaido had a single repetype (Cae-A; Table 1, Fig. 2), and sites 4 and 5 were not observed in Cae-C while sites 11 and 13 shown in Cae-C were absent in Cae-A (Fig. 2). The repetype from Sakhalin (Cae-B) had sites 4, 5, 11, and 13 (Fig. 2), demonstrating it contained characteristics of repetypes both from the continent (Cae-C) and Hokkaido (Cae-A). Two individuals from Cheju Island had a single repetype (Sp-Cheju), which showed a unique restriction site (site 10) but other sites were identical to the repetype (Cae-C) from the continent (Fig. 2).

Cladogram and network

One shortest tree (tree length = 16) was obtained by the maximum parsimony method (Fig. 3). *Sorex shinto* and *S. caecutiens* were distinguished in the cladogram (Fig. 3) but the confidence of this division was statistically low (bootstrap values, <50%). The repetype from Sp-Cheju (Cheju Island) was included in the *S. caecutiens* clade and formed a subclade with Cae-C (Eurasian continent). The repetypes for Cae-A (Hokkaido) and Cae-B (Sakhalin) formed a second subclade within the *S. caecutiens* clade (Fig. 3).

In the phylogenetic network (Fig. 4A), *S. caecutiens* and *S. shinto* were distinguished by restriction site 1, and

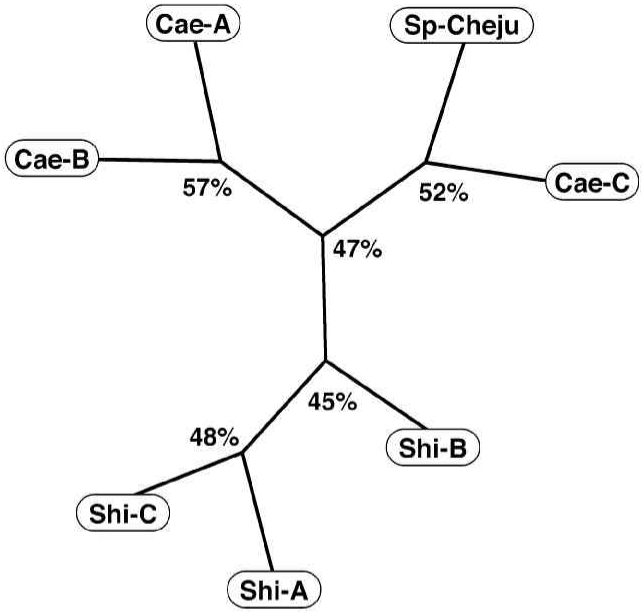

Fig. 3. Unrooted maximum parsimony tree based on nuclear rDNA-RFLP repetypes in the *Sorex caecutiens/shinto* group. Percent values are the bootstrap values after 10,000 replications. See Table 1 for acronyms of the rDNA-RFLP repetypes.

the shrews from Cheju Island (Sp-Cheju) were most closely connected with *S. caecutiens* from the continent, as in the maximum parsimony tree. The phylogenetic network suggests that the repetype of Hokkaido (Cae-A) was derived from that of Sakhalin (Cae-B), which was in turn derived from that of the Eurasian continent (Cae-C). The divergence pattern within *S. shinto* was unclear (Fig. 4A).

In addition, it was observed that the mapping pattern of the restriction sites of Sakhalin (Cae-B) was the sum of the continental (Cae-C) and Hokkaido (Cae-A) types (Fig. 2); i.e., Cae-B was the intermingled type of Cae-C and Cae-A. Thus, allowing hybridization of the rDNA-RFLP pattern, an alternative network has been reconstructed (Fig. 4B).

Discussion

Previous phylogenetic studies based on mtDNA cyt-*b* sequences (Ohdachi et al. 1997, 2001, 2003; Fumagalli et al. 1999) and allozymes (George 1988) demonstrated

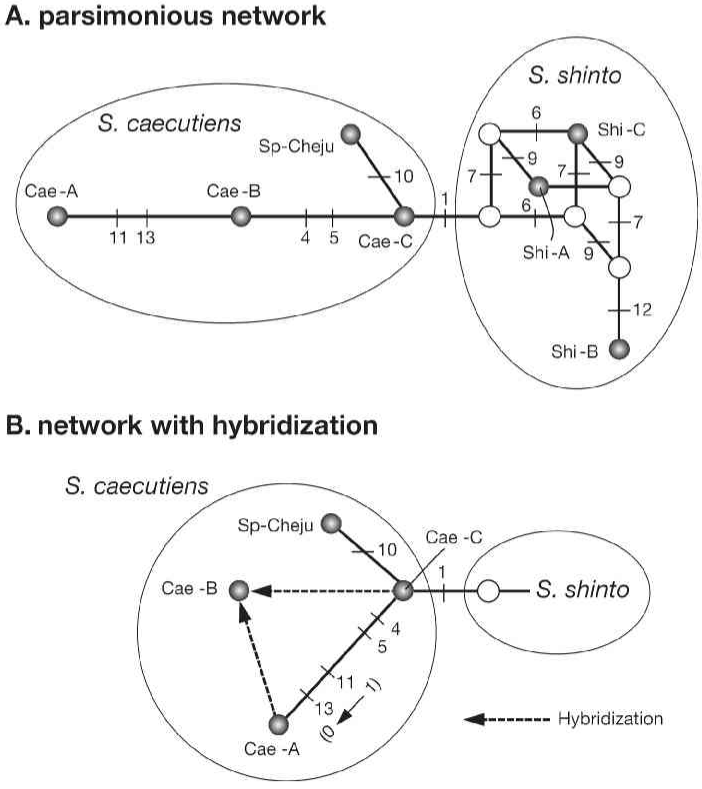

Fig. 4. Parsimonious network based on rDNA-RFLP repetypes (A) and a phylogenetic network allowing hybridization (B), based on rDNA-RFLP repetypes in the *Sorex caecutiens/shinto* group. Numbers near short bars denote those of the change of restriction sites. Site numbers correspond with those of Fig. 2. An open circle is a hypothetical taxonomic unit. Intraspecific relationships of *S. shinto* were omitted in the networks. See Table 1 for acronyms of the rDNA-RFLP repetypes.

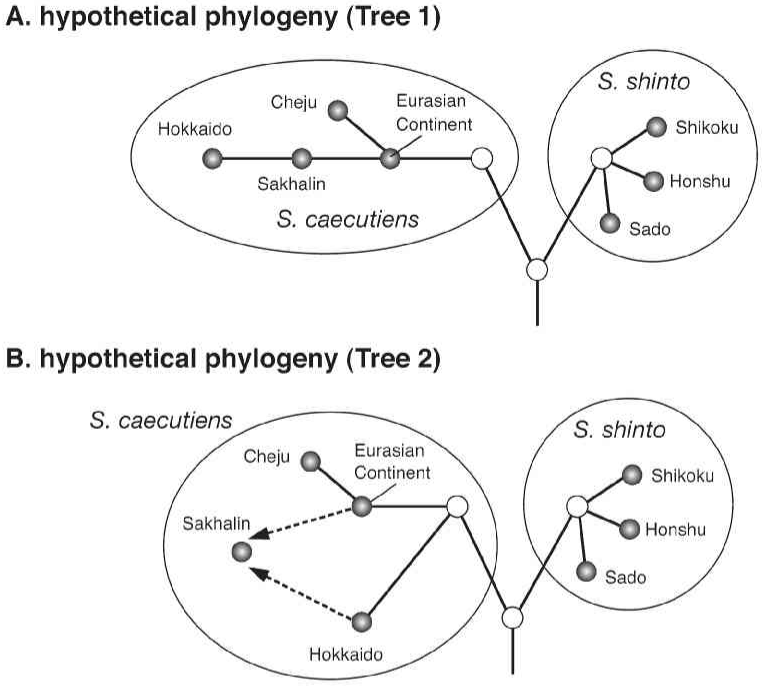

Fig. 5. Two hypothetical phylogenies of nuclear DNA in the *Sorex caecutiens/shinto* group, based on the rDNA-RFLP repetypes. Tree 1 (A) was inferred from the parsimonious network (Fig. 4A) and tree 2 (B) from the network allowing hybridization (Fig. 4B). Arrows with broken lines indicate hybridization.

that *S. caecutiens* and *S. shinto* should be treated as two separate species. In the present study, it was not statistically supported, although topology showed these two were separated (Fig. 3). However, the shrew from Cheju Island was most close to *S. caecutiens* in the Eurasian continent and it should be recognized as *S. caecutiens* (Fig. 3). In addition, although sampling locations from the Eurasian continent were greatly scattered, there was no variation in the rDNA-RFLP repetype within *S. caecutiens* over this range (Table 1, Fig. 2). The same tendency was observed in the genetic variation of mtDNA cyt-*b* (Ohdachi et al. 2001, 2003; Ohdachi 2005). These findings from both nuclear DNA and mtDNA suggest that *S. caecutiens* has gone through a recent and rapid range expansion in the Eurasian continent. Patterns of genetic variation were largely different between Hokkaido and the continent both in rDNA-RFLP repetypes (Fig. 4) and mtDNA haplotypes (Ohdachi et al. 2001, 2003; Ohdachi 2005). Therefore, the range expansion of *S. caecutiens* in the continent must have occurred after Hokkaido and the continent were geologically separated after the last glacial period. It is estimated that Hokkaido and Sakhalin+Eurasian continent were separated ca. 12,000 years ago (Ohshima 1990). Thus, the great range expansion in the continent presumably began after 12,000 years ago.

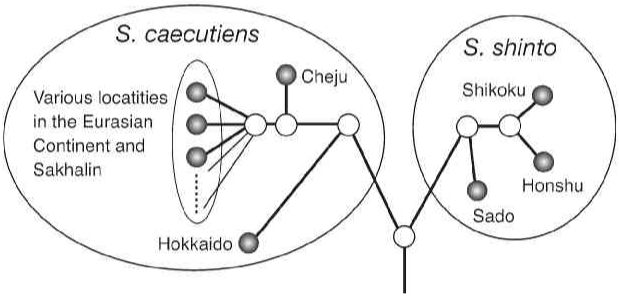

Fig. 6. Schematic tree of the mitochondrial DNA phylogeny in the *Sorex caecutiens/shinto* group, based on mtDNA cytochrome *b* gene sequences (Ohdachi et al. 2003; Ohdachi in press). An open circle is a hypothetical taxonomic unit.

The maximum parsimony tree (Fig. 3) and the phylogenetic network (Fig. 4A) were used to construct a hypothetical phylogeny of the nuclear rDNA (Tree 1, Fig. 5A). On the other hand, allowing hybridization between the rDNA-RFLPs, an alternative network was reconstructed (Fig. 4B), and from this phylogenetic network with hybridization, another hypothetical phylogeny was inferred (Tree 2, Fig. 5B).

We can not conclude which phylogeny (Fig. 5: trees 1 and 2) is more appropriate as an evolutionary process of

the nuclear rDNA. A mtDNA phylogeny of the *S. caecutiens/shinto* group, was inferred (Fig. 6) based on Ohdachi et al. (2003) and Ohdachi (2005), where *S. caecutiens* was divided into Hokkaido and Continent-Sakhalin-Cheju genealogies after *S. caecutiens* and *S. shinto* diverged. Hence, tree 2 is more congruent with the mtDNA phylogeny than tree 1 and for this reason seems the more appropriate phylogenetic tree (network) of the nuclear rDNA.

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