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Title	Molecular phylogenetics of soricid shrews (Mammalia) based on mitochondrial cytochrome b gene sequences: with special reference to the Soricinae
Author(s)	Ohdachi, S.; Hasegawa, M.; Iwasa, M. et al.
Citation	Journal of Zoology, 270(1), 177-191 https://doi.org/10.1111/j.1469-7998.2006.00125.x
Issue Date	2006-09
Doc URL	https://hdl.handle.net/2115/14663
Rights	For full bibliographic citation, please refer to the version available at www.blackwell-synergy.com .
Type	journal article
File Information	JZ2006-270-1.pdf, 本文



**Molecular phylogenetics of soricid shrews (Mammalia) based on
mitochondrial cytochrome *b* gene sequences: with special reference to
the Soricinae**

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Short title: Molecular phylogenetics of Soricidae

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Abstract

Molecular phylogeny of soricid shrews (Soricidae, Eulipotyphla, Mammalia) was inferred by the maximum likelihood method, based on 1,140 bp mitochondrial cytochrome *b* gene (*cytb*) sequences. All 13 genera of extant Soricinae and two genera of Crocidurinae and were included in the analysis. *Anourosorex* was phylogenetically distant from both the main group of Soricinae and Crocidurinae in the present analysis, whereas the latter two formed a monophyletic group. Thus, it could not be determined to which subfamily *Anourosorex* should belong, Soricinae or Crocidurinae. We suggest that Soricinae (excluding *Anourosorex*) should be divided into four tribes (Neomyini, Notiosoricini, Soricini, and Blarinini). However, branching orders among tribes of Soricinae and those among genera of Neomyini could not be determined due to insufficient phylogenetic information of the *cytb* sequences. For water shrews of Neomyini (*Chimarrogale*, *Nectogale*, and *Neomys*), monophyly of *Neomys* and the *Chimarrogale-Nectogale* group could not be certified, which implies the possibility of multiple origins for the semi-aquatic mode of living among taxa within Neomyini. *Episoriculus* may contain several separate genera. *Blarinella* was included in Blarinini

not Soricini, based on the *cytb* sequences, but the confidence was rather low.

Furthermore, some specific problems of taxonomy were resolved in the present analysis.

In general, the *cytb* gene nucleotide sequence had enough information to resolve

phylogenetic relationships at the species level, but seems to be unreliable to determine

5 the phylogenetic relationships among higher level taxa within Soricidae.

Key words: Soricinae, Crocidurinae, shrew, taxonomy, phylogeny

INTRODUCTION

Soricidae (shrews) contains 312 species and is the second or third largest family in class Mammalia (1,326 species have been listed for Muridae and 318 species for Vespertilionidae), thus it contains about 15% of mammalian species (Wilson & Reeder, 1993). Often, they are considered to be a “primitive” eutherian group and have plesiomorphic characters (e.g., Feldhamer *et al.*, 1999; Vaughan, Ryan & Czaplewski, 2000). Their habitats, however, vary greatly from desert to wetland (even a semi-aquatic habitat), tropical rain forest to arctic tundra, ground surface to underground burrow, and lowland to highland (e.g., Abe, 1983; Churchfield, 1990). Extant Soricidae includes two subfamilies, Soricinae and Crocidurinae (Reumer, 1987; Hutterer, 1993; Wolsan & Hutterer, 1998). Soricine shrews are distributed mainly in the Holarctic region and include about 110 species, whereas crocidurine shrews are diversified primarily in Africa and southern Asia, consisting of about 200 species. At least, 11 and 12 genera are known for Soricinae and Crocidurinae, respectively (Repenning, 1967; Wolsan & Hutterer, 1998).

Phylogenetic investigations of Soricidae at higher taxonomic levels were conducted based mainly on cranial and dental morphology of fossil and/or extant specimens and several phylogenetic hypotheses among tribes and genera were proposed (e.g., Repenning, 1967; Reumer, 1987, 1989). In the last decades, molecular biological techniques have brought deep insights into phylogenetic investigations of many organisms. In Soricidae, some molecular phylogenetic investigations also were conducted within several specific groups, but not for the whole family.

In Crocidurinae, several phylogenetic hypotheses were proposed based on morphology of African (Heim de Balsac & Lamotte, 1957; Butler, Thorpe & Greenwood, 1989; McLellan, 1994) and Asian species (Heaney & Ruedi, 1994). Maddalena & Ruedi (1994) discussed karyological evolution between African and Palearctic *Crocidura* species. Also, phylogenetic relationships of *Crocidura* were explained by use of molecular techniques for some species in Africa and East Asia (Maddalena, 1989; Motokawa *et al.*, 2000; Han *et al.*, 2002; Ohdachi *et al.*, 2004). Quérrouil *et al.* (2001) analyzed the phylogeny of all genera of Crocidurinae by use of 16S rRNA sequences and proposed a hypothesis of interrelationships among African

and Eurasian crocidurine shrews.

In Soricinae, George & Sarich (1994) discussed phylogenetic relationships among some tribes based on biochemical data (see also, George, 1986). Phylogeny of species of *Sorex* has been well investigated. Phylogenetic hypotheses for some species
5 were proposed based on karyological data (e.g., Dannelid, 1991; Ivanitskaya, 1994; Zima, Lukáčova & Macholán, 1998) and molecular data (e.g., George, 1988; Ohdachi *et al.*, 1997, 2001; Fumagalli *et al.*, 1999; Demboski & Cook, 2001, 2003).

In contrast, information pertaining to the phylogeny of non-*Sorex* species of Soricinae is scarce. Ohdachi *et al.* (1997) examined several non-*Sorex* species of
10 soricine shrews in Eurasia and Brant & Ortí (2002) studied North American *Blarina* and *Cryptotis* species. However, phylogenetic information for other non-*Sorex* genera, such as *Blarinella*, *Nectogale*, and *Megasorex*, has not been reported yet. As a result, molecular phylogenetics at higher levels of taxonomy within Soricidae is not well understood.

15 In addition, there is disagreement regarding the subfamily rank of *Anourosorex*. *Anourosorex* was placed in Crocidurinae by Simpson (1945) whereas

Reumer (1984) placed it in Soricinae. This disagreement mainly is due to the difference in interpreting morphology, thus molecular data might resolve this taxonomic problem.

Herein, we estimated phylogenetic relationships among all genera of Soricinae, two genera of Crocidurinae, and *Anourosorex* based on full sequences (1,140 bp) of the mitochondrial cytochrome *b* gene (*cytb*). Based on the phylogenetic trees obtained, we determined the taxonomic status of some species and proposed some hypotheses for the systematics of higher level taxonomy in Soricidae.

MATERIALS AND METHODS

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Samples of DNA analysis

Full nucleotide sequences (1,140 bp) of mitochondrial DNA (mtDNA) *cytb* gene of 124 individuals of soricid shrews (Soricidae, Eulipotyphla) were used for phylogenetic analyses (Appendix). Two individuals each from *Mogera wogura* and *Talpa europaea* (Talpidae, Eulipotyphla) were used as an outgroup to Soricidae, because Talpidae is closely related to Soricidae (Murphy *et al.*, 2001a, b; Douady *et al.*, 2002).

Among 126 individuals (124 shrews + two moles), DNA sequences of 49 individuals were cited from DNA databases (DDBJ/EMBL/GenBank); those of the remaining 77 were determined in the present study (Appendix). For the 77 samples that were sequenced for this research, collection numbers or specimen codes were explicitly indicated in the DNA databases.

Fundamentally, we followed the nomenclature system for the order Eulipotyphla (= a part of the order Insectivora according to Douady *et al.*, 2002, or Soricomorpha) as presented by Hutterer (1993) and Wolsan & Hutterer (1998). Differences are: following Repenning (1967), we treated *Soriculus*, *Episoriculus*, and *Chodsigoa* as three separate genera although Hutterer (1993) treated them as subgenera within the genus *Soriculus* (Table 1). In addition, *Chodsigoa sodalis* and *Anourosorex yamashinai* (Motokawa *et al.*, 1997, 2004, respectively) as well as *Crocidura tadae* (ssp. *kurodai*), *C. shantungensis*, and *C. watasei* (Motokawa, 1999; Fang & Lee, 2002) were regarded as valid species. We used subspecies names of *Episoriculus caudatus caudatus* and *E. c. soluensis* in Nepal tentatively according to Abe (1977), although he originally used the genus *Soriculus* instead of *Episoriculus*. For the other species of *Soriculus* and

Episoriculus in Nepal, we followed the taxonomic treatment by Hoffmann (1985), although he treated the genus *Episoriculus* as a subgenus of *Soriculus*. We followed Lunde & Musser (2002) and Lunde, Musser & Son (2003) for taxonomy of soricid shrews in Vietnam and Cheng, Chengchien & Chang (2000) and Ci (1998) for shrews in Taiwan, although we used the names *Anourosorex yamashinai*, *Episoriculus fumidus*, *Chodsigoa sodalis*, and *Crocidura tadae kurodai* instead of *A. squamipes yamashinai*, *Soriculus fumidus*, *S. sodalis* and *C. kurodai*, respectively. *Sorex antinorii* was regarded as an independent species according to Brünner *et al.* (2002). As a result, 76 species (78 subspecies) of Soricidae and two species of moles were used in analyses (Appendix).

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DNA analysis

Total DNA of the 77 individuals whose nucleotide sequences were determined herein were extracted by the phenol/proteinase K/sodium dodecyl sulphate method (Sambrook, Fritsch & Maniatis, 1989) or by use of the DNeasy Tissue Kit (Qiagen) from liver or muscle tissues preserved in 70-100% ethanol (ca. 27 mm³) or a dried foot or muscles. The region of the mtDNA *cytb* gene (1,140 bases) was amplified by

polymerase chain reaction (PCR) using *rTaq* DNA polymerase (Takara Bio), KOD-plus DNA polymerase (Toyobo), or AmpliTaq (ABI). When a single PCR could not amplify a region that included the whole region of the *cytb* gene, several PCRs were conducted until the complete 1,140-bp sequence was obtained. Primer sets for PCR varied

5 depending on species and conditions of samples (Table 2, Appendix). PCR conditions also varied depending on situation; annealing temperature varied from 49 to 55°C and PCR cycles from 35 to 40 cycles. After the PCR products were purified by the PEG (polyethylene glycol) precipitation method, the purified products were directly sequenced using BigDye Terminator kit ver. 3.1 (ABI) by an autosequencer (ABI

10 PRISM 310 or 3100 Avant Genetic Analyzer). When needed internal primers were used and both forward and reverse sequencing was conducted (Ohdachi *et al.*, 2001). Sequences obtained herein were registered in DNA databases (DDBJ/EMBL/GenBank).

Phylogenetic analysis

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Three steps of the phylogenetic analysis were conducted by use of 1,140 bp sequences of the mtDNA *cytb* gene. First, a preliminary neighbor joining (NJ) tree was

constructed by MEGA3 (Kumar, Tamura & Nei, 2004)– using all sequences

(Appendix). Kimura’s two-parameter (Kimura, 1980) option was chosen and the

bootstrap value was calculated by 10,000 replications. The two mole species were used

as an outgroup. More than two individuals were sequenced from a species or subspecies

5 in the present study, unless only a single sample was available. However, to save

calculation time for maximum likelihood (ML) analysis, 88 sequences were arbitrarily

selected by reviewing the preliminary NJ tree obtained.

Second, a ML tree was constructed for the 88 sequences by the

quartet-puzzling method using TREE-PUZZLE ver. 5.2 (Strimmer & von Haeseler,

10 1996). All codon positions were used for calculations. Confidence of node was

evaluated by quartette supporting values (Strimmer & von Haeseler, 1996). Finally,

ambiguous points in the ML tree obtained from the 88 sequences were reanalyzed

separately by constructing ML trees using individuals of particular interest by

TREE-PUZZLE ver. 5.2, NucML program in MOLPHY ver. 2.3 (Adachi & Hasegawa,

15 1996), or BaseML program in PAML ver. 3.1 (Yang, 1997). The substitution models

were selected by the hierarchical likelihood ratio tests of MODELTEST ver. 3.06

(Posada & Crandall, 1998) with PAUP* ver. 4.0b10 (Swofford, 2002). gamma distribution categories were always eight (Yang, 1996) when gamma distribution for site-heterogeneity was included in the substitution model. Outgroups were carefully selected for each analysis, referring to the remarks by Graham, Olmstead & Barrett (2002) and Van den Bussche & Hooper (2004).

RESULTS

General maximum likelihood tree

10 All samples were successfully sequenced for 1,140 bases of mtDNA *cytb* gene. A preliminary NJ tree (Fig. 1) suggested that sequences revealed herein were authentic. Reviewing the NJ tree (Fig. 1), we selected 86 sequences from 124 sequences in the preliminary analysis. The general time-reversible substitution model (Yang, 1994) with gamma distribution + invariable sites (GTR+G+I) were chosen by the hierarchical
15 likelihood ratio test of MODELTEST. Rate matrix R of the GTR model was as follows:
R [A-C] = 0.2162, R [A-G] = 11.0571, R [A-T] = 0.3886, R [C-G] = 0.7556, R [C-T] = 6.4033, R [G-T] = 1.0000. Using the selected sequences, a ML tree was obtained (Fig.

2) by TREE-PUZZLE. The fraction of invariable sites estimated from the data set was 0.49 (± 0.02 S.E.), and the gamma distribution parameter alpha was 0.80 (± 0.06 S.E.). Unresolved quartets were 5.5%. Total rate heterogeneity was 0.77 (± 0.03 S.E.). Fully resolved quartets were 91.7%, partly resolved quartets were 6.0%, and unresolved quartets were 2.3%.

Specimens of Soricidae except for *Anourosorex* were monophyletic (Fig. 2) with a high supporting value of quartet puzzling (93%). Crocidurinae and Soricinae excluding *Anourosorex* (= main group of Soricinae) composed a monophyletic group (93%) but shrews of the main group of Soricinae did not show monophyly within the group (Fig. 2). Four tribes (Soricini, Blarinini, Notiosoricini, and Neomyini) of Soricinae showed polychotomy and branching orders among the tribes were completely unsolved. *Anourosorex* fell out of the Crocidurinae-main Soricinae cluster associating with neither Crocidurinae nor the main group of Soricinae (Fig. 2). *Blarinella griselda* was included in Blarinini although the supporting value was low (54%). *Megasorex gigas* and *Notiosorex crawfordi* formed the monophyletic Notiosoricini but they were rather differentiated (Fig. 2). *Notiosorex crawfordi* from Arizona and Texas (USA) and

N. crawfordi from Baja California (Mexico) genetically were rather differentiated (Fig. 2; maximum likelihood distance was 0.224-0.224). Within Crocidurinae, *Suncus* and *Crocidura* each were monophyletic (Fig. 2).

The ML tree (Fig. 2) showed four ambiguous points. (1) Within Neomyini relationships among genera were unclear. Especially, it is equivocal whether or not the semi-aquatic shrews (*Chimarrogale*, *Nectogale*, and *Neomys*) are monophyletic. (2) Relationships within *Sorex* were unclear. (3) Relationships among species within Blarinini were obscure. (4) Relationships of within *Crocidura* were partly unclear. Thus, these four specific problems were reanalyzed separately.

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Phylogeny within Neomyini

All species of Neomyini were included in the reanalyses with seven species of Blarinini serving as an outgroup. First, a ML tree was constructed by the quartet puzzling method using TREE-PUZZLE under the substitution model of Hasegawa, Kishino & Yano (1985) with gamma distribution (HKY+G) (Fig. 3A). Statistics of the ML analysis was as follows: transition/transversion parameter = 5.65 (± 0.36 S.E.),

gamma distribution parameter $\alpha = 0.19 (\pm 0.01 \text{ S.E.})$, and unresolved quartets = 10.7%.

Episoriculus c. caudatus, *Ep. c. soluensis*, and *Ep. leucops* were included in a monophyletic group (Fig. 3) although *Ep. caudatus* is paraphyletic. In addition, 5 monophyly of *Episoriculus* was not confirmed. Monophyly of three species of *Chodsigoa* (*Cg. caovansunga*, *Cg. parca*, and *Cg. sodalis*) was strongly supported (96%). *Chimarrogale himalayica* was polyphyletic, as *Cm. himalayica* from Taiwan formed a monophyletic group with *Cm. platycephala*, not with *Cm. himalayica* from Vietnam and Nepal. *Nectogale elegans* was most closely related to *Chimarrogale* sp. 10 *Neomys fodiens* and *Nm. anomalus* were monophyletic but genetically rather distinct. The Eurasian semi-aquatic shrews, *Neomys*, *Chimarrogale*, and *Nectogale*, were monophyletic (Fig. 3A) but the supporting value was low (51%).

Further analysis was conducted to examine the monophyly of the semi-aquatic shrews. Because of high supporting values in the previous ML analysis 15 (Fig. 3A), within-group topology for the following five clusters were fixed in reanalysis: (*Ep. leucops*, *Ep. c. caudatus*, *Ep. c. soluensis*-1); (*Nm. anomalus*, *Nm.*

fodiens); ((*Cg. parca*-1, *Cg. sodalis*-1), *Cg. caovansunga*); (*Cm. himalayica*-TW, (*Cm. platycephala*-HN, *Cm. platycephala*-KS)); and (*Cm. himalayica*-VN, *Cm. himalayica*-NP). Topology of the outgroup also was fixed. Approximate likelihoods (Adachi & Hasegawa, 1996) were calculated for the possible 2,027,025 topologies among the 10 groups (*Ep. macrurus*-1, *Ep. fumidus*-1, *Nc. elegans*, *Sc. nigrescens*, the fixed five groups and outgroup), and then exact likelihoods were calculated for the best 10,000 trees of the approximate likelihood criterion by NucML with HKY model. From the 10,000 trees, 3,663 trees that had exact log likelihood scores greater than the maximum log likelihood minus 2 S.E. were chosen for the more exact analysis. Finally, likelihoods and bootstrap values were calculated for the 3,663 trees by BaseML with HKY+G model. The maximum likelihood tree was obtained from the 3,663 trees (Fig. 3B). The confidence of the node was evaluated by the RELL bootstrap value with 10,000 replications (Kishino, Miyata & Hasegawa, 1990; Hasegawa & Kishino, 1994). For further information regarding this analytical procedure, refer to Kawai *et al.* (2002), where the same analysis was applied.

Monophyly of *Chimarrogale* sp. and *Nectogale elegans* were strongly

supported (94% bootstrap value) but the relationships among the other groups were rather obscure (Fig. 3B). Probability for the monophyly of the semi-aquatic shrews was 24%, calculated by adding bootstrap values of the trees that showed the monophyly of the semi-aquatic shrews in the 3,663 trees. Thus, monophyly of the semi-aquatic shrews of Neomyini was not strongly supported in the present data set although it was not completely rejected. In addition, monophyly of *Episoriculus macrurus* and *E. fumidus* was not supported in the final analysis (Fig. 3B), although they were monophyletic in the previous analysis with 60% supporting value (Fig. 3A).

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Phylogeny within *Sorex*

The genus *Sorex* was unambiguously divided into two subgenera *Sorex* and *Otisorex* in the general ML tree (Fig. 2). Thus, the two groups were individually analyzed. *Sorex saussurei* and *S. cinereus* from the subgenus *Otisorex* served as an outgroup for the analysis of the subgenus *Sorex*, whereas *S. caecutiens* and *S. araneus* from the subgenus *Sorex* formed the outgroup for the subgenus *Otisorex*. For the

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subgenus *Sorex*, the substitution model by Tamura & Nei (1993) with gamma distribution + invariable sites (TrN+G+I) were chosen by the hierarchical likelihood ratio test of MODELTEST, whereas GTR+G+I model was the best model for the subgenus *Otisoorex*. Rate matrix R of the GTR model for *Otisoorex* was as follows: R [A-C] = 2.5104, R [A-G] = 12.7828, R [A-T] = 2.8445, R [C-G] = 0.4568, R [C-T] = 36.8306, R [G-T] = 1.0000. Maximum likelihood trees were calculated under these models by TREE-PUZZLE (Fig. 4).

In the analysis for the subgenus *Sorex*, statistics of ML analysis were as follows: transition/transversion parameter = 6.34 (± 0.47 S.E.), Y/R transition parameter = 1.72 (± 0.15 S.E.), fraction of invariable sites (estimated from data set) = 0.63 (± 0.01 S.E.), number of invariable sites = 714, and unresolved quartets = 4.1%.

Sorex alpinus branched first in the subgenus (Fig. 4A). The other species formed a monophyletic group with a marginal supporting value (57%). *Sorex cylindricauda*-*S. excelsus*, *S. minutissimus*-*S. hosonoi*, *S. unguiculatus*-*S. isodon*, and *S. caecutiens*-*S. shinto* formed monophyletic groups, respectively (Fig. 3A). *Sorex araneus*-*arcticus* group (*S. araneus*, *S. antinorii*, *S. granarius*, *S. coronatus*, *S.*

tundrensis, and *S. daphaenodon*) and *S. samniticus* were included in a monophyletic group with a high supporting value (88%). Branching orders among these four monophyletic groups were unclear, although the species, *S. roboratus*, *S. minutus*, and *S. gracillimus* were monophyletic (supporting value = 64%, Fig. 4A). In addition, *S. cylindricauda* was most closely related to *S. excelsus*.

In the analysis of the subgenus *Otisorex*, statistics of ML analysis were as follows: Fraction of invariable sites = 0.57 (± 0.02 S.E.), number of invariable sites = 655, gamma distribution parameter alpha = 1.29 (± 0.24 S.E.), total rate heterogeneity = 0.76 (± 0.06 S.E.), and unresolved quartets = 4.4%.

10 Although *Sorex saussurei* and *S. trowbridgii* formed a monophyletic group, they were genetically rather differentiated from each other (Fig. 4B). The *S. saussurei-trowbridgii* group was branched first within *Otisorex*. Monophyly of the *S. cinereus* group was strongly supported (98%), but there were few genetic differences among *S. ugyunak*, *S. portenkoi*, *S. pribilofensis*, *S. jacksoni*, and *S. camtschatica*. *Sorex*
15 *palustris* was most closely related to *S. monticolus* and they formed a monophyletic group with *S. vagrans* (*S. vagrans* group). *Sorex fumeus* and *S. tenellus* also formed a

monophyletic cluster. Further, the *S. cinereus* group, *S. vagrans* group, *S. fumeus*-*S. tenellus* group, and *S. hoyi* formed a monophyletic group, but branching orders among them were unsolved (Fig. 4B).

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Phylogeny within Blarinini

Two *Sorex* species served as an outgroup in the reanalysis of the tribe Blarinini and the GTR+G+I model was selected by MODELTEST. Rate matrix R of GTR model was as follows: R [A-C] = 3.3397, R [A-G] = 12.3682, R [A-T] = 3.5024, R [C-G] = 0.5779, R [C-T] = 31.023, R [G-T] = 1.0000. A ML tree was constructed by TREE-PUZZLE (Fig. 5). Statistics of ML analysis were as follows: fraction of invariable sites = 0.56 (± 0.02 S.E.), number of invariable sites = 639, gamma distribution parameter alpha = 1.90 (± 0.45 S.E.), total rate heterogeneity = 0.71 (± 0.07 S.E.), and unresolved quartets = 2.4%.

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Blarinella griselda fell out of the group of *Blarina* and *Cryptotis* within Blarinini (Fig. 5). Monophyly of *Cryptotis* and *Blarina* was confirmed with high supporting value (100%). Within *Cryptotis*, three Mexican species (*C. mexicana*, *C.*

magna, and *C. goldmani*) formed a monophyletic group and North American *C. parva* was located outside this group (Fig. 5).

Phylogeny within *Crocidura*

For the closer examination of species within *Crocidura*, MODELTEST selected the TrN+G+I model as the best substitution model, and two *Suncus* species served as an outgroup in the reanalysis. A ML tree was constructed under this condition (Fig. 6) by TREE-PUZZLE. Statistics of ML analysis were as follows: transition/transversion parameter = 8.60 (± 0.80 S.E.), Y/R transition parameter = 3.31 (± 0.42 S.E.), fraction of invariable sites = 0.55 (0.02 S.E.), number of invariable sites = 625, gamma distribution parameter alpha = 1.97 (± 0.40 S.E.), total rate heterogeneity = 0.70 (± 0.06 S.E.), and unresolved quartets = 5.4%.

Within *Crocidura*, three well-supported monophyletic groups were recognized (Fig. 6): (1) *C. suaveolens*, *C. sibirica*, and *C. shantungensis* (= *C. suaveolens* group); (2) *C. dsinezumi*, *C. lasiura*, and *C. t. kurodai* (= *C. dsinezumi* group); and (3) *C. horsfieldii* and *C. watasei* (= *C. horsfieldii-watasei* group). Further, the *C. dsinezumi* group, the *C. horsfieldii-watasei* group, *C. wuchihensis*, *C. attenuata*, *C. a. tanakae*, and *C. fuliginosa* formed a monophyletic group (= Group A) with 80% supporting value

although branching orders among them were unsolved (Fig. 6). *Crocidura attenuata* from Vietnam and *C. a. tanakae* from Taiwan were genetically rather differentiated (Fig. 6); maximum likelihood distance between them was 0.11072, whereas average distance in this reanalysis was 0.12790.

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DISCUSSION

Higher level taxonomy of Soricidae

Extant Soricidae currently are divided into two subfamilies, Soricinae and
10 Crocidurinae (e.g., Reumer, 1987; Hutterer, 1993; Wolsan & Hutterer, 1998). Based primarily on morphology, Reumer (1984) and Wolsan & Hutterer (1998) suggested *Anourosorex* belonged to Soricinae whereas Simpson (1945) and Imaizumi & Obara (1966) suggested it belonged to Crocidurinae. The discrepancy mainly was caused by the difference in interpreting morphological data, such as dental characters. We applied
15 molecular data to resolve this taxonomic problem. The phylogeny of the mtDNA *cytb* sequences indicated that *Anourosorex* could not be included in either Soricinae or Crocidurinae (Fig. 2). A new subfamily rank might be created for *Anourosorex*.

However, to conclude its subfamily position, we need more phylogenetical information from both mitochondrial and nuclear genomes. The subfamily status for *Anourosorex* still is pending although we revealed its phylogenetic position based on the mtDNA *cytb* gene.

5 Although we analyzed only 16 subspecies of *Crocidura* and *Suncus* mainly from eastern Eurasia, monophyly of Crocidurinae (white-toothed shrews) in Eurasia was supported (Fig. 2). In contrast, monophyly of Soricinae (red-toothed shrews) was not confirmed (Fig. 2). Tribal relationships within Soricinae also were unresolved (Fig. 2). Thus, information obtained from the mtDNA *cytb* gene was not sufficient to
10 determine the monophyly of Soricinae and to fully resolve generic and tribal relationships within the subfamily.

 Repenning (1967) placed *Anourosorex* in Neomyini whereas Reumer (1984) placed it in Amblyoptini, based mainly on dental and cranial morphology. Hutterer (1993) noted that Amblyoptini was antedated by Anourosoricini, and treated
15 *Anourosorex* as “Anourosoricini or Neomyini” (Table 1). Our result (Fig. 2) based on mtDNA sequence data, suggested that *Anourosorex* should belong in Anourosoricini.

Thomas (1911) proposed that *Blarinella* in East Asia was more closely related to *Blarina* in North America, whereas Allen (1938) suggested it was more closely related to *Sorex*. Many prominent authors followed Allen's (1938) opinion and placed *Blarinella* in the tribe Soricini (Table 1). However, our result showed *Blarinella* was included in Blarinini (Fig. 2), supporting Thomas's (1911) opinion. However, the supporting value of the monophyly of Blarinini was rather low (Fig. 2) and we could not completely deny the hypothesis that *Blarinella* belongs to Soricini.

Repenning (1967) and Hutterer (1993) classified *Megasorex* and *Notiosorex* into Neomyini with *Chimarrogale*, *Neomys*, *Nectogale*, *Soriculus* (= *Soriculus*, *Episoriculus*, and *Chodsigoa*), whereas Reumer (1984) placed *Megasorex* and *Notiosorex* in Notiosoricini and *Chimarrogale*, *Neomys*, *Nectogale*, *Soriculus*, *Episoriculus*, and *Chodsigoa* in Soriculini (Table 1). Our tribal treatment for these genera was different from both of these opinions (Table 1). We classified *Megasorex* and *Notiosorex* into Notiosoricini as in Reumer (1987) but placed *Chimarrogale*, *Neomys*, *Nectogale*, *Soriculus*, *Episoriculus*, and *Chodsigoa* in Neomyini as in Reppening (1967) and Hutterer (1993).

Neomyini

Repenning (1967) recognized *Soriculus*, *Episoriculus*, and *Chodsigoa* as separate genera whereas Hoffmann (1985) and Hutterer (1993) treated them as subgenera of the genus *Soriculus*. We followed the taxonomic scheme of the former author (Table 1). Monophyly of each of *Chodsigoa*, *Neomys*, and *Chimarroga* was supported by the mtDNA *cytb* phylogeny (Fig. 3). However, monophyly of *Episoriculus* was not confirmed (Fig. 3), although *E. leucops*, *E. c. caudatus* and *E. c. soluensis* formed a monophyletic group (Fig. 3A). This finding suggests that *Episoriculus* may be polyphyletic.

Abe (1977) regarded *Episoriculus caudatus caudatus* and *E. c. soluensis* as subspecies of *E. caudatus* although he used the genus name *Soriculus* instead of *Episoriculus*. The molecular phylogenetic trees of the *cytb* gene (Figs. 1-3), however, indicated a large genetic difference between the two “subspecies” and paraphyly of *E. caudatus*. Further investigations including morphological analysis should be conducted to determine taxonomic ranks for *E. c. caudatus* and *E. c. soluensis*.

Hutterer (1993) regarded *Chodsigoa sodalis* in Taiwan as a synonym of

Episoriculus fumidus (he originally used *Soriculus* instead of *Chodsigoa* and *Episoriculus*). However, *C. sodalis* and *E. fumidus* obviously are distinct taxa (Figs. 2 and 3), which has been recognized by researchers in East Asia (e.g., Motokawa *et al.*, 1997, 1998; Cheng *et al.*, 2000; Ci, 1998). Hence, *C. sodalis* is a valid species.

5 Jones & Mumford (1971) reported a species of water shrew from Taiwan for the first time and assigned it to *Chimarrogale himalayica*. In the molecular phylogenetic trees (Figs. 2 and 3), *C. himalayica* from Nepal and Vietnam were monophyletic with high supporting values, whereas *C. himalayica* from Taiwan formed a monophyletic group with *C. platycephala* from Japan. However, the Taiwanese water shrew was
10 genetically rather different from *C. platycephala* from Japan (Figs. 2 and 3). Thus, either a new species name or a new subspecies name of *C. platycephala* should be given to the *Chimarrogale* species in Taiwan.

 Water shrews (*Neomys*, *Chimarrogale*, and *Nectogale*) of Neomyini were monophyletic with low supporting values in a ML tree (Fig. 3A). According to the
15 reanalyzed tree, monophyly among them was not supported (Fig. 3B) and the probability of a monophyletic relationship among them was only 24%. Non-monophyly

among the water shrews may have been caused by a lack of phylogenetic information within the mtDNA *cytb* gene. If water shrews actually are polyphyletic, they acquired semi-aquatic adaptations independently. Thus, further examination using other gene regions should be conducted to examine the evolution of the semi-aquatic mode of life in the Neomyini.

Notiosorex and Megasorex

We analyzed *Notiosorex crawfordi* from Arizona, Texas, Baja California, and Baja California Sur (Appendix). There were almost no genetic difference between shrews from Arizona and Texas (Fig. 2; maximum likelihood distance was 0.011). However, *N. crawfordi* from the Baja California Peninsula was genetically rather different from those in Arizona and Texas (Fig. 2; distance ranged from 0.224 to 0.227) and the genetic distance was small between shrews from Baja California and Baja California Sur (maximum likelihood distance = 0.016; also see Fig. 1).

Carraway & Timm (2000) described three species of *Notiosorex* mainly based on morphology and Baker, O'Neil & MacAliley (2003) recently described a new species

of *Notiosorex*. Thus, *Notiosorex* is composed of four species. According to Carraway & Timm (2000), the shrew from the Baja California Peninsula is classified as *N. crawfordi*.

However, herein we showed (Figs. 1 and 2) that *Notiosorex* on the Baja California Peninsula are phylogenetically distinct from *N. crawfordi* in the U.S.A. Baker *et al.*

5 (2003) also found that a sequence of the *cytb* gene of *Notiosorex* from Baja California was different from those of *N. crawfordi* in Texas and Arizona. *Notiosorex* in the Baja California Peninsula may be a species different from *Notiosorex* in Texas and Arizona.

Megasorex gigas originally was placed in the genus *Notiosorex* (Merriam, 1897) and Hall (1981) regarded *Megasorex* as a synonym of *Notiosorex*. However, other
10 authors have treated it as a separate genus (Table 1). George (1986), based on an allozyme study, suggested a closer relationship between *Megasorex* and *Neomys* than with *Notiosorex*. In contrast, Ducommun, Jeanmaire-Besancon & Vogel (1994) found greater similarity of hair morphology between *Megasorex* and *Notiosorex* than with *Neomys*, and suggested they should be treated as related genera. However, Ducommun
15 *et al.* (1994) treated *Megasorex* and *Notiosorex* as separate genera because there are some morphological differences between them. The phylogenetic tree of the *cytb* gene

(Fig. 2) also showed a close relationship between *Megasorex* and *Notiosorex* and supported the opinion that they should be treated as different genera as they were genetically rather differentiated.

5

Anourosorex

Hutterer (1993) treated *Anourosorex yamashinai* as a synonym of *A.*

squamipes, whereas Motokawa *et al.* (2004) insisted *A. yamashinai* was a distinct

species because their karyotypes were quite different. *Anourosorex yamashinai* occurs

10 only at higher elevations of Taiwan whereas *A. squamipes* occurs in higher regions of

Southeast Asia including Yunnan, China and Assam, India (Motokawa & Lin, 2002).

Considering the large genetic differences of the *cytb* gene sequence between *A.*

squamipes and *A. yamashinai* (Fig. 1), we support the taxonomic treatment by

Motokawa *et al.* (2004) and consider *A. yamashinai* as a valid species.

15

Sorex

Sorex was clearly divided into two subgenera, *Sorex* and *Otisorex* (Fig. 2).

Molecular phylogeny based on mtDNA *cytb* sequences for the subgenus *Sorex* has been examined by Ohdachi *et al.* (1997) and Fumagalli *et al.* (1999); however, branching
5 orders among the species could not be clarified. We also could not determine some branching orders (Fig. 4A). This means that species of the subgenus *Sorex* diverged so rapidly that nucleotide substitutions of the *cytb* gene have not accumulated sufficiently, thus contain limited phylogenetic information.

The *Sorex araneus-arcticus* group is defined as having XY₁Y₂ sex
10 chromosome (Dannelid, 1991). The monophyly of this group was well supported in the present mtDNA analysis (Fig. 4A). Br nner *et al.* (2002) suggested, based on the *cytb* gene sequence that *S. antinorii*, that was regarded as a chromosomal race of *S. araneus*, should be treated as a separate species. The present result (Fig. 4A) also supported their taxonomic treatment. Further, Dannelid (1991) stated that *S. samniticus* was
15 morphologically closest to the *S. araneus-arcticus* group although it does not have the XYY system. Their close relationship was confirmed in the present molecular

phylogenetic analysis (Fig. 4A).

The chromosome number (2N) of *Sorex caecutiens*, *S. shinto*, *S. unguiculatus*,
S. isodon, and *S. minutissimus*, (and probably *S. hosonoi*) is 42 (Dannelid, 1991; Zima
et al., 1998); we refer to this species group as the “true” 2N = 42 group as *S. minutus*
5 independently obtained 42 chromosome numbers (Dannelid, 1991). Further, *S.*
caecutiens, *S. shinto*, *S. unguiculatus*, and *S. isodon* also have very similar karyotypes
(Tsuchiya, 1985; Tada & Obara, 1988; Dannelid, 1991). There is no contradiction
between these karyological features and the phylogenetic relationships determined
herein although monophyly of these karyological groups was not verified in the
10 phylogenetic tree (Fig. 4A). Finally, *S. mirabilis* and *S. alpinus* have a tripartite penis
(Dannelid, 1991), but their monophyly was not confirmed herein (Fig. 4A).

Within the subgenus *Otisorex*, Demboski & Cook (2001) revealed
phylogenetic relationships among “*S. monticolus*” which may contain several distinct
species and 8 other related species. Then, Demboski & Cook (2003) reported on the
15 molecular phylogeny of the *S. cinereus* group using mtDNA *cytb* gene sequences. The
data set of *Otisorex* used herein basically was the pruned set of Demboski & Cook

(2003); (Appendix); we obtained almost the same result of ML tree topology (Fig. 4A).

Additional species in the present analysis were *S. fumeus* and *S. saussurei*. Semi-aquatic *S. palustris* was most closely related to terrestrial *S. monticolus* that formed a

monophyletic group with *S. vagrans* (= *S. vagrans* group) as in the ML tree by

5 Demboski & Cook (2003). However, unlike their result, the *S. vagrans* group did not

form a monophyletic group with *S. hoyi* in the present analysis (Fig. 4B). We analyzed

the present data set deleting sequences of *S. fumeus* and used *S. saussurei* and *S.*

trowbridgii as an outgroup under the GTR+G+I model, as did Demboski & Cook (2003),

and under the GTR+G model, which was the best model for our data set. However, we

10 did not obtain a ML tree wherein the *S. vagrans* group and *S. hoyi* were monophyletic,

as in the maximum parsimony tree by Demboski & Cook (2003). Thus, the

monophyletic relationship of the *S. vagrans* group and *S. hoyi* is delicate, depending on

minor difference in parameters of the substitution model and on the method of tree

reconstruction. A further examination is needed to conclude the monophyly of the *S.*

15 *vagrans* group and *S. hoyi*.

Sorex trowbridgii and *S. saussurei* formed a monophyletic group although

they were distantly related (Fig. 3B). *Sorex trowbridgii* is distributed along the West Coast of U.S.A., whereas *S. saussurei* occurs in higher elevations of Mexico and is one of the southernmost species of *Sorex*. Hutterer (1993) did not apply subgeneric designations to *S. trowbridgii* or *S. saussurei*. We tentatively placed them in the subgenus *Otisorex* as they were phylogenetically closest to it (Fig. 4).

Crocidura* and *Suncus

Monophyly of *Crocidura* and *Suncus* examined herein was supported (Fig. 2); however, we did not examine any crocidurine species from Africa. Han *et al.* (2002), based on 402 bp *cytb* sequences, showed that *Crocidura* in Asia might be paraphyletic because *Suncus murinus* was located within the *Crocidura* species. However, the present analysis, using 1,140 bp, clearly demonstrated that the Asian *Crocidura* species we examined are monophyletic (Fig. 2).

Crocidura fuliginosa, *C. attenuata*, *C. a. tanakae*, *C. wuchihensis*, the *C. horsfieldii-watasei* group, and the *C. dsinezumi* group formed a monophyletic group (=

Group A) but their branching orders were unsolved (Fig. 6).

Hutterer (1993) treated *Crocidura watasei* as a synonym of *C. horsfieldii* and

Abe *et al.* (1994) treated it as a subspecies of *C. horsfieldii*, *C. h. watasei*. However,

Motokawa *et al.* (1996) and Motokawa (1999, 2000) regarded it as a valid species, and

5 we tentatively followed this taxonomic scheme. The phylogeny of the *cytb* gene showed

that *C. horsfieldii* and *C. watasei* are phylogenetically closest to each other, but

genetically are rather different (Fig. 6). In addition, the distribution of *C. watasei* is

limited to the northern Ryukyu Islands, Japan (Motokawa, 1999), whereas *C. horsfieldii*

occurs widely in Southeast Asia and southern East Asia (Hutterer, 1993). Thus, *C.*

10 *watasei* certainly speciated after the Ryukyu Islands were separated from the Asian

Continent.

In the general phylogenetic tree (Fig. 2), *C. orii* made a monophyletic group

with the *C. dsinezumi* group. However, in the reanalyzed tree (Fig. 6), branching orders

among *C. orii*, the *C. suaveolens* group, and Group A were unclear. Thus, the *cytb* gene

15 region did not contain enough information to estimate the phylogenetic positions of *C.*

orii.

Crocidura attenuata tanakae has been treated as a subspecies of *C. attenuata* in Taiwan (e.g., Cheng *et al.*, 2000). However, *C. a. tanakae* had a different phylogenetic position from *C. attenuata* in Vietnam (Figs. 2 and 6). Motokawa *et al.* (2001) also showed that *C. a. tanakae* in Taiwan had a karyotype different from *C. attenuata* in southern mainland China. Therefore, *tanakae* should be considered as a distinct species, although there may be “true” *C. attenuata* in Taiwan in addition to “*C. a. tanakae*”. Extensive sampling of *Crocidura* in Taiwan is needed to resolve the *tanakae* problem.

10 Acknowledgments

First of all, we would like to express our deep gratitude to the following persons and institutes arranging tissue samples of their institutes: D. Lunde, American Museum of Natural History, New York, USA; J. Dines, Natural History Museum of Los Angeles County, California, USA; R. C. Dowler, Angelo State Natural History Collections, Dept. of Biology, Angelo State Univ., Texas, USA; T. Theimer, Museum of

Vertebrate, Northern Arizona Univ., USA; C. Denys, Museum National d'Histoire Naturelle, Paris, France; S. -W. Chang, Taiwan Endemic Species Research Institute, Nantou, Taiwan; and M. P. Moshkin, Institute of Animal Systematics and Ecology, Novosibirsk, Russia. F. A. Cervantes, N. E. Dokuchaev, V. Haukialmi, M. Kita, and R. Masuda offered us samples and/or assisted sampling by ourselves. K. Kawai assisted in the laboratory and phylogeny reconstruction. L. N. Carraway and M. Motokawa reviewed an earlier draft of this manuscript. U. Arnason, J. Demboski, L. Fumagalli, and J. Moribe gave us information of sequences cited. We also thank J. Cuisin and R. Cornette.

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Table 1. Generic and tribal designations for the extant subfamily Soricinae previously published and presented herein.

Repenning (1967)		Reumer (1984)		Hutterer (1993)		Present study	
genus	tribe	genus	tribe	genus (subgenus)	tribe	genus (subgenus)	tribe
<i>Anourosorex</i>	Neomyini	<i>Anourosorex</i>	Amblycoptini	<i>Anourosorex</i>	Neomyini or Anourosoricini	<i>Anourosorex</i>	Anourosoricini
<i>Chimarrogale</i>	Neomyini	<i>Chimarrogale</i>	Soriculini	<i>Chimarrogale</i>	Neomyini	<i>Chimarrogale</i>	Neomyini
<i>Neomys</i>	Neomyini	<i>Neomys</i>	Soriculini	<i>Neomys</i>	Neomyini	<i>Neomys</i>	Neomyini
<i>Nectogale</i>	Neomyini	<i>Nectogale</i>	Soriculini	<i>Nectogale</i>	Neomyini	<i>Nectogale</i>	Neomyini
<i>Soriculus</i>	Neomyini	<i>Soriculus</i>	Soriculini	<i>Soriculus</i> (<i>Soriculus</i>)	Neomyini	<i>Soriculus</i>	Neomyini
<i>Episoriculus</i>	Neomyini	<i>Episoriculus</i>	Soriculini	<i>Soriculus</i> (<i>Episoriculus</i> ^a)	Neomyini	<i>Episoriculus</i> ^a	Neomyini
<i>Chodsigoa</i>	Neomyini	<i>Chodsigoa</i>	Soriculini	<i>Soriculus</i> (<i>Chodsigoa</i> ^a)	Neomyini	<i>Chodsigoa</i> ^a	Neomyini
<i>Notiosorex</i>	Neomyini	<i>Notiosorex</i>	Notiosoricini	<i>Notiosorex</i>	Neomyini	<i>Notiosorex</i>	Notiosoricini
<i>Megasorex</i> ^b	Neomyini	<i>Megasorex</i>	Notiosoricini	<i>Megasorex</i> ^b	Neomyini	<i>Megasorex</i> ^b	Notiosoricini
<i>Sorex</i>	Soricini	<i>Sorex</i>	Soricini	<i>Sorex</i> (<i>Sorex</i> , <i>Otisorex</i> ^d , & <i>Stroganovia</i> ^e)	Soricini	<i>Sorex</i> (<i>Sorex</i> & <i>Otisorex</i> ^e)	Soricini
<i>Microsorex</i> ^c	Soricini	<i>Sorex</i>	Soricini	<i>Sorex</i> ^c (<i>Otisorex</i>)	Soricini	<i>Sorex</i> ^c (<i>Otisorex</i>)	Soricini
<i>Blarinella</i>	Soricini	<i>Blarinella</i>	Soricini	<i>Blarinella</i>	Soricini	<i>Blarinella</i>	Blarinini
<i>Blarina</i>	Blarinini	<i>Blarina</i>	Blarinini	<i>Blarina</i>	Blarinini	<i>Blarina</i>	Blarinini
<i>Cryptotis</i>	Blarinini	<i>Cryptotis</i>	Blarinini	<i>Cryptotis</i>	Blarinini	<i>Cryptotis</i>	Blarinini

^aHutterer (1993) regarded *Soriculus sodalis* as a synonym of *S. (Episoriculus) fumidus*, whereas it is considered a valid species belonging to the genus *Chodsigoa*, *C. sodalis*, in the present study.

^bIncluding only one species *Megasorex gigas* = *Notiosorex gigas*.

^cIncluding only one species, *Microsorex hoyi* = *Sorex hoyi*.

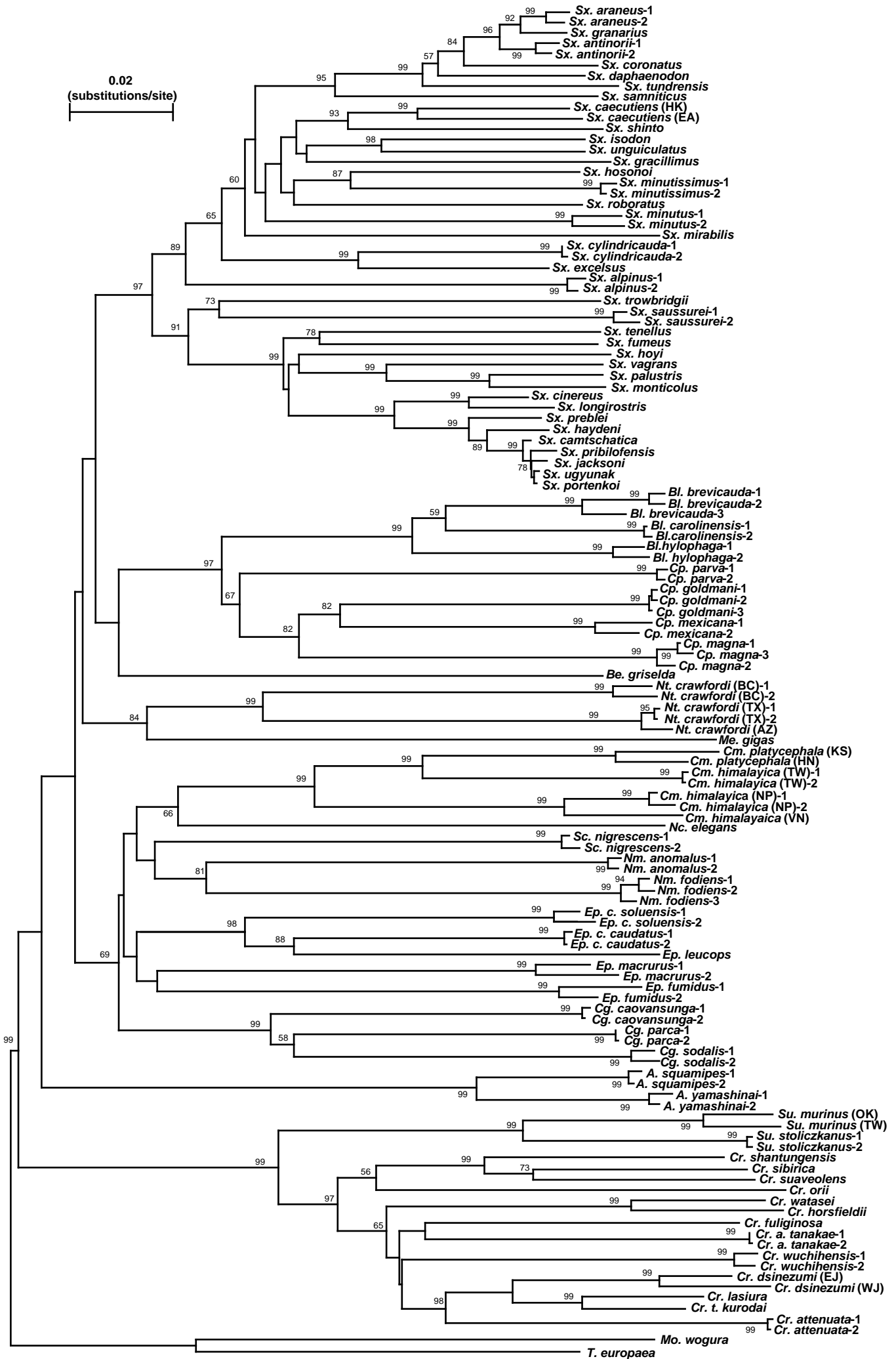
^dNo subgeneric name was designated to *S. trowbridgii* and *S. saussurei* according to Hutterer (1993); whereas, herein they are designated as belonging to the subgenus *Otisorex*.

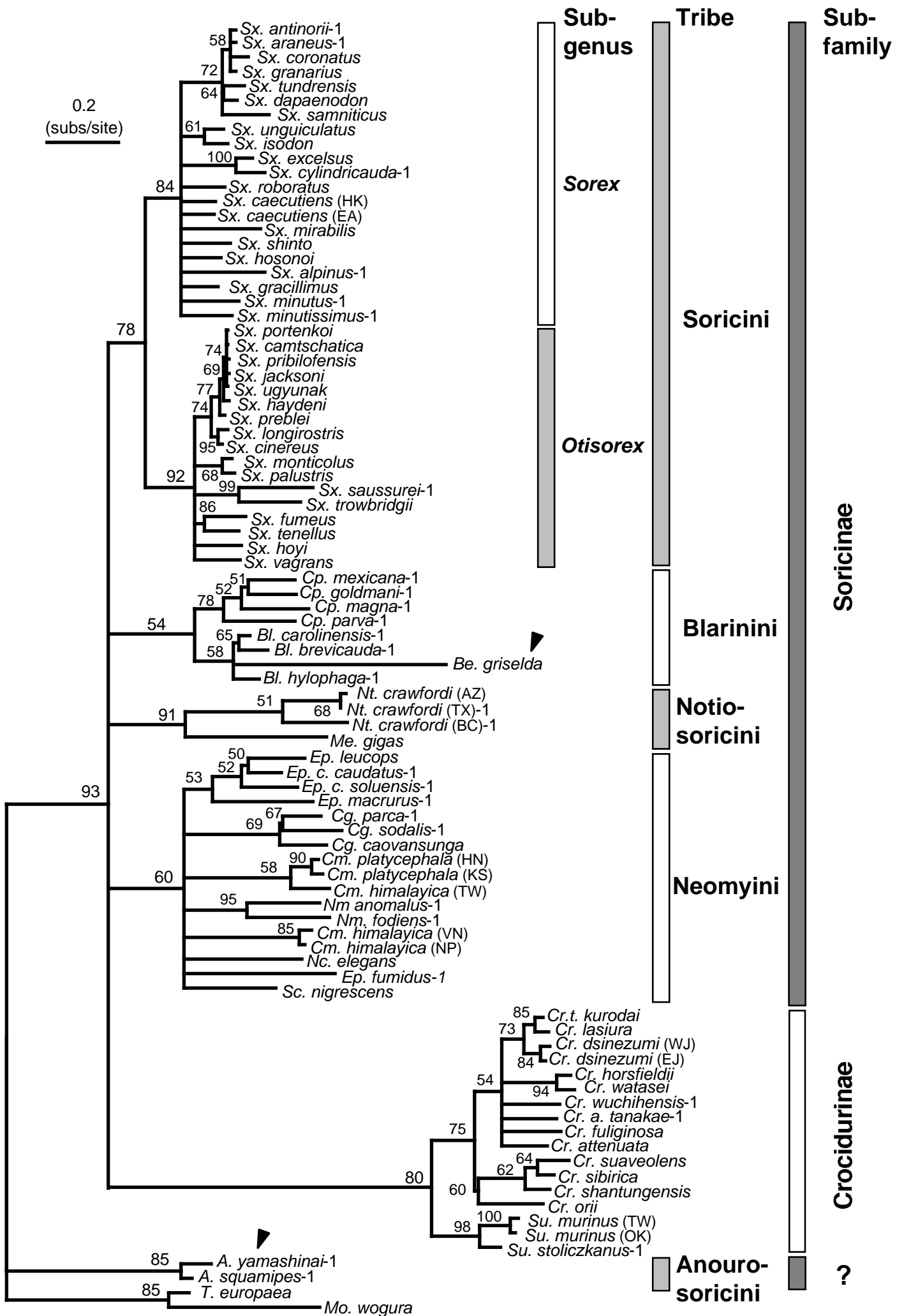
^eThe subgenus *Stroganovia*, which contains only *S. daphaenodon*, was included in the subgenus *Sorex* in the present study.

Table 2. Primer list for the PCR of the mitochondrial cytochrome *b* gene. *L and H are light and heavy strands and numeral is the 3' end position of the primer in the human mitochondrial DNA sequence (Anderson et al., 1981).

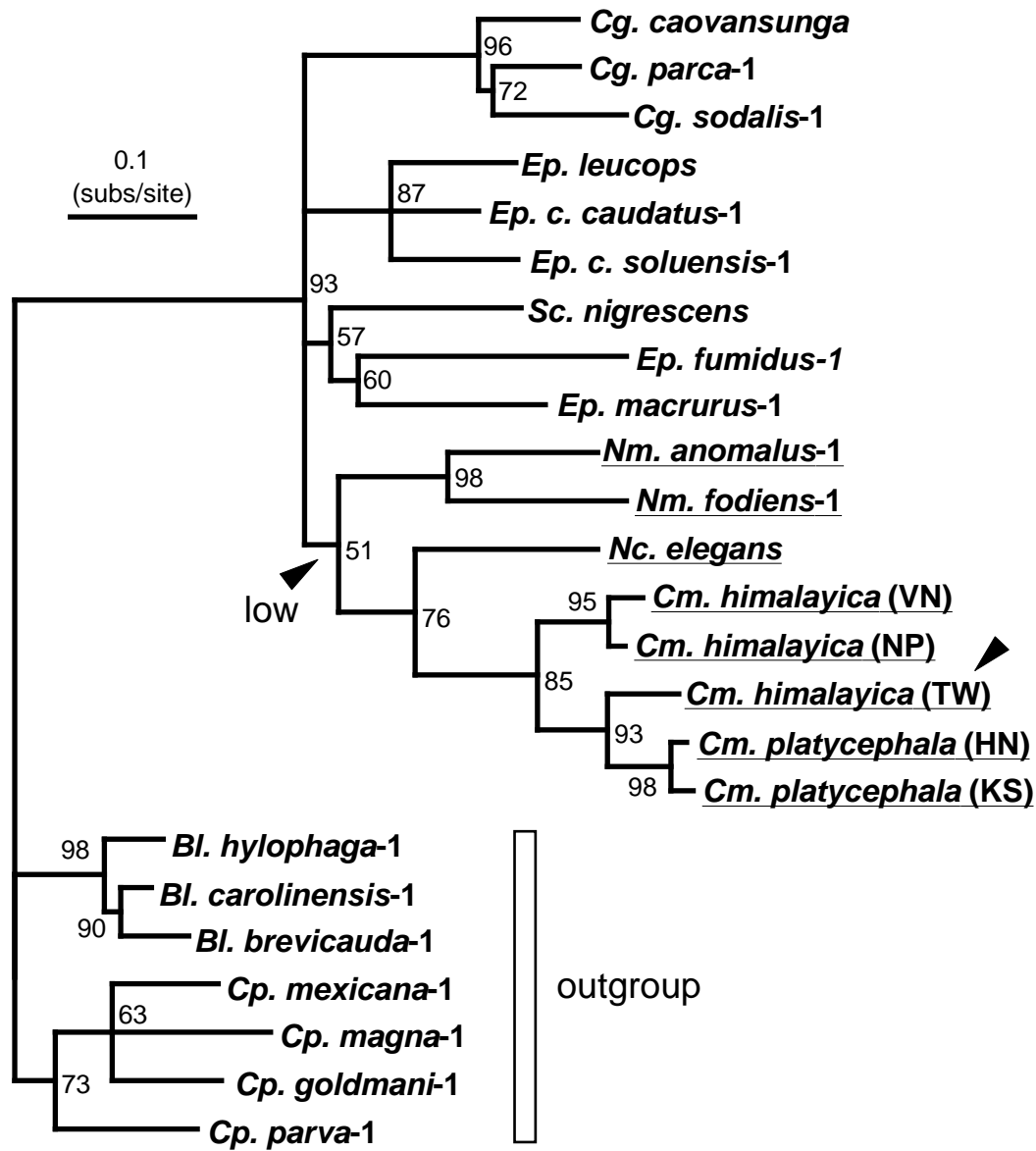
Primer Code	position*	sequence (5'-3')	Reference
Forward			
F1	L14721	GACCAATGATATGAAAAACCATCG	1
F2	L14727	TGACATGAAAAATCATCGTTG	2
F3	L14734	AAAAACCATCGTTGTTATTCAACT	1
F4	L14986	ATTATGGCTGACTAATCCGT	3
F5	L14988	ACTACGGCTGACTAATCCGATA	3
F6	L15129	GCAGTCATAGCCACAGCATT	3
F7	L15131	GCAGTAATAGCCACAGCCTTTA	3
F8	L15131	GCTGTAATAGCAACTGCCTTCA	3
F9	L15345	CTGGAGTTCACCTACTATTTCT	3
F10	L15346	GAGTCCATCTCTTATTTCTT	3
F11	L15347	GGAGTACACCTCCTATTTCTCC	3
F12	L15347	GGAGTCCACCTCCTTTTTTCTCC	3
F13	L15347	GGAGTCCACCTCCTATTTCTCC	3
F14	L15366	CATGAAACAGGCTCAAACAA	3
F15	L15366	CATGAAACCGGCTCAAATAA	3
F16	L15379	AACCCAACAGGACTACAATC	3
F17	L15505	TCCAGACCTTCTTGGAGATCCG	3
F18	L15507	CAGACTTACTTGGAGACCCAGA	3
F19	L15507	CAGATCTGCTTGGAGACCCAGA	3
F20	L15507	CAGACCTATTAGGAGACCCAGA	3
F21	L15525	GACAATTATATCCCCGCAA	3
F22	L15526	ATAACTATACACCTGCCAAC	3
F23	L15561	CCACATATTAAACCAGAATG	4
F24	L15738	TTCTGAATCCTAGTGGCAGA	3
Reverse			
R1	H14995	AATATTGATGCTCCGTTTGCG	3
R2	H15142	ACATTTGTCCCTCATGGTAAT	3
R3	H15149	CTCAGAATGATATTTGTCCTCA	1
R4	H15155	TGCCCTCAAAGGATATTTG	4
R5	H15392	GGGTGGAAGGGATTTTGTC	5
R6	H15392	GGGTGGAATGGGATTTTATC	3
R7	H15548	AAAATATCATTCAGGTTTAAT	3
R8	H15548	GAAATATCATTCAGGTTTATC	3
R9	H15548	GAAGTATCATTCAGGTTTAAT	3
R10	H15707	ATTCAGAATAAGCATTGGCT	3
R11	H15752	GGTTGACCTCCGATTCATGT	3
R12	H15985	TAGAATGTCAGCTTTGGGTGCT	5

¹Ohdachi et al. (1997), ²Nikaido et al. (2000), ³present study, ⁴Iwasa et al. (2000), ⁵Ohdachi et al. (2001).





A. Tree 1



B. Tree 2 (reanalysed)

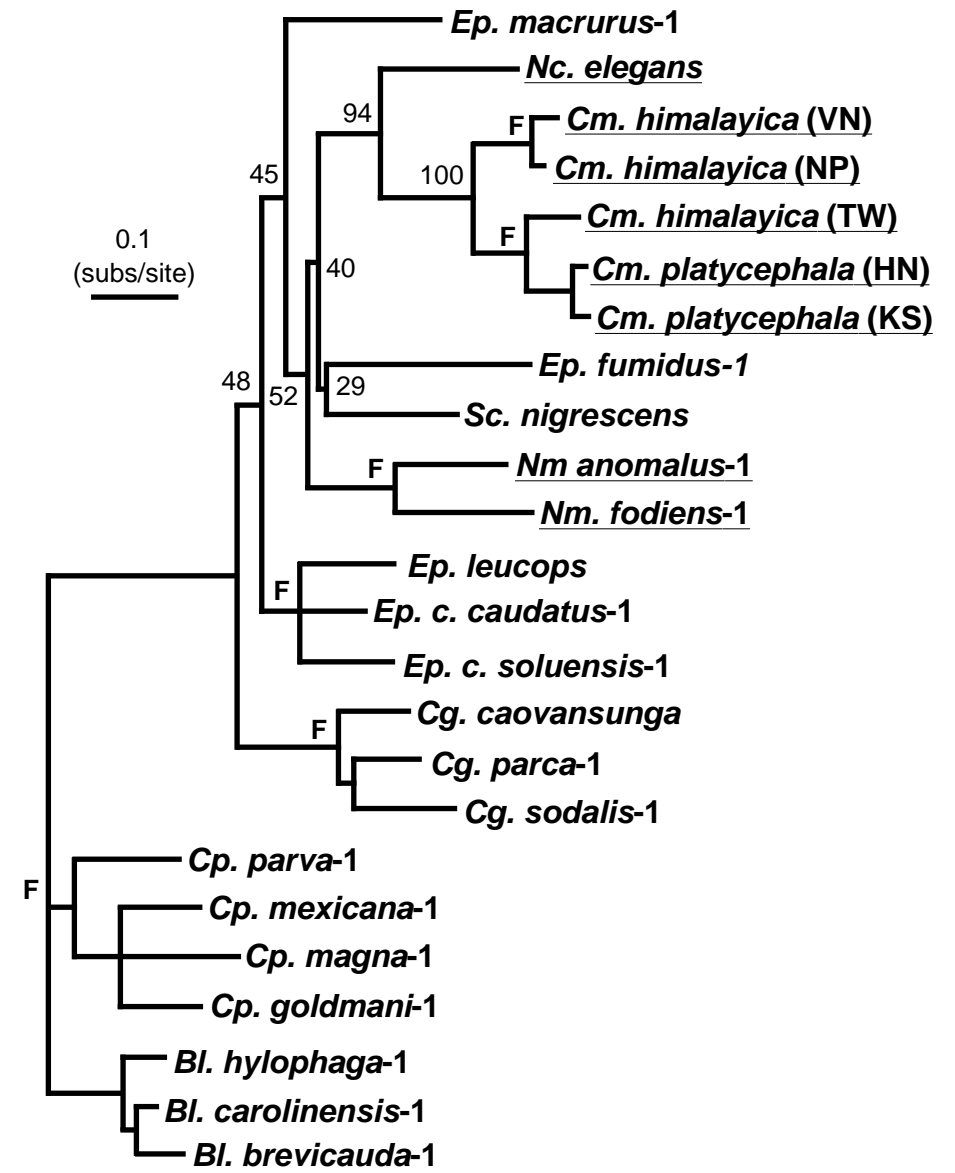
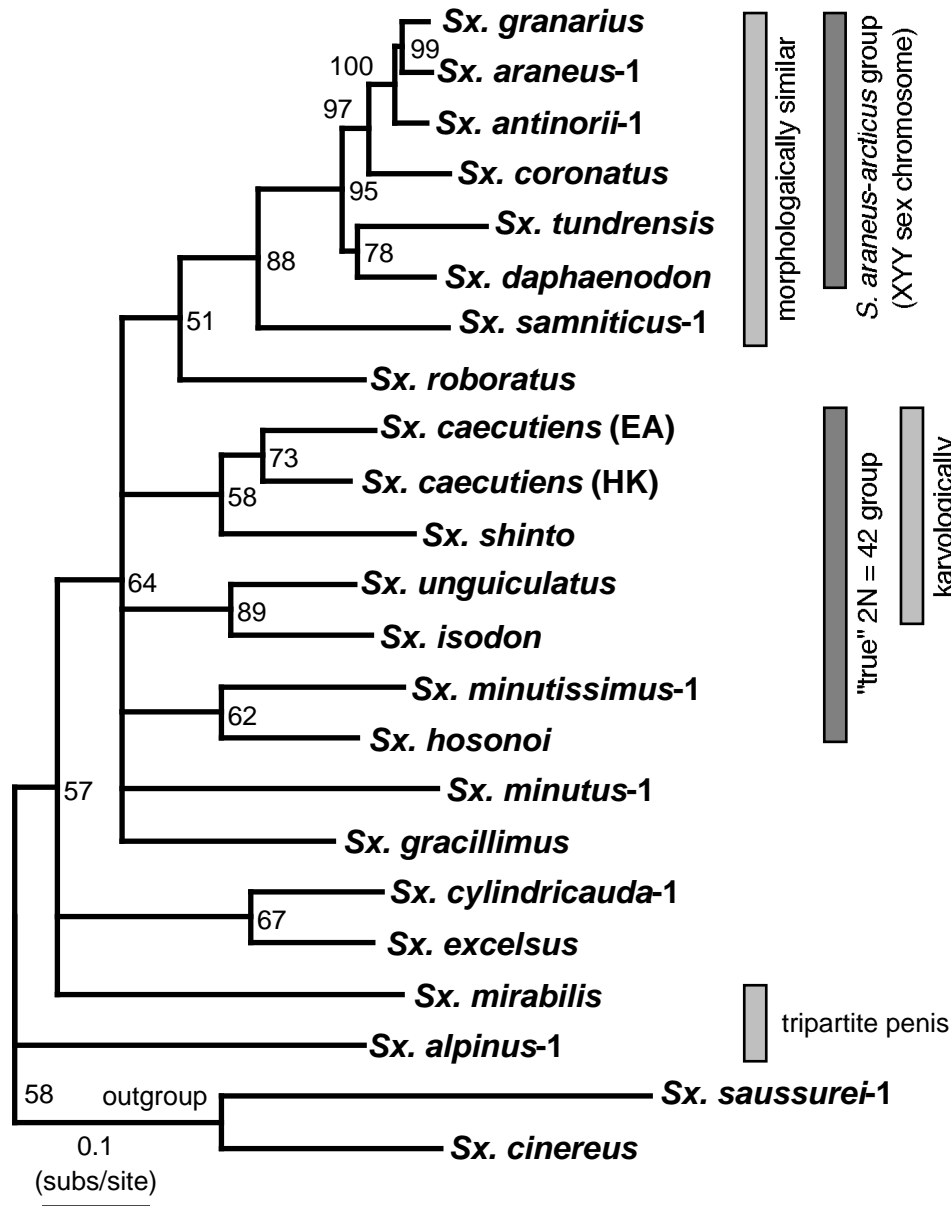


Fig. 3. Ohdachi et al.

A. Subgenus *Sorex*



B. Subgenus *Otisorex*

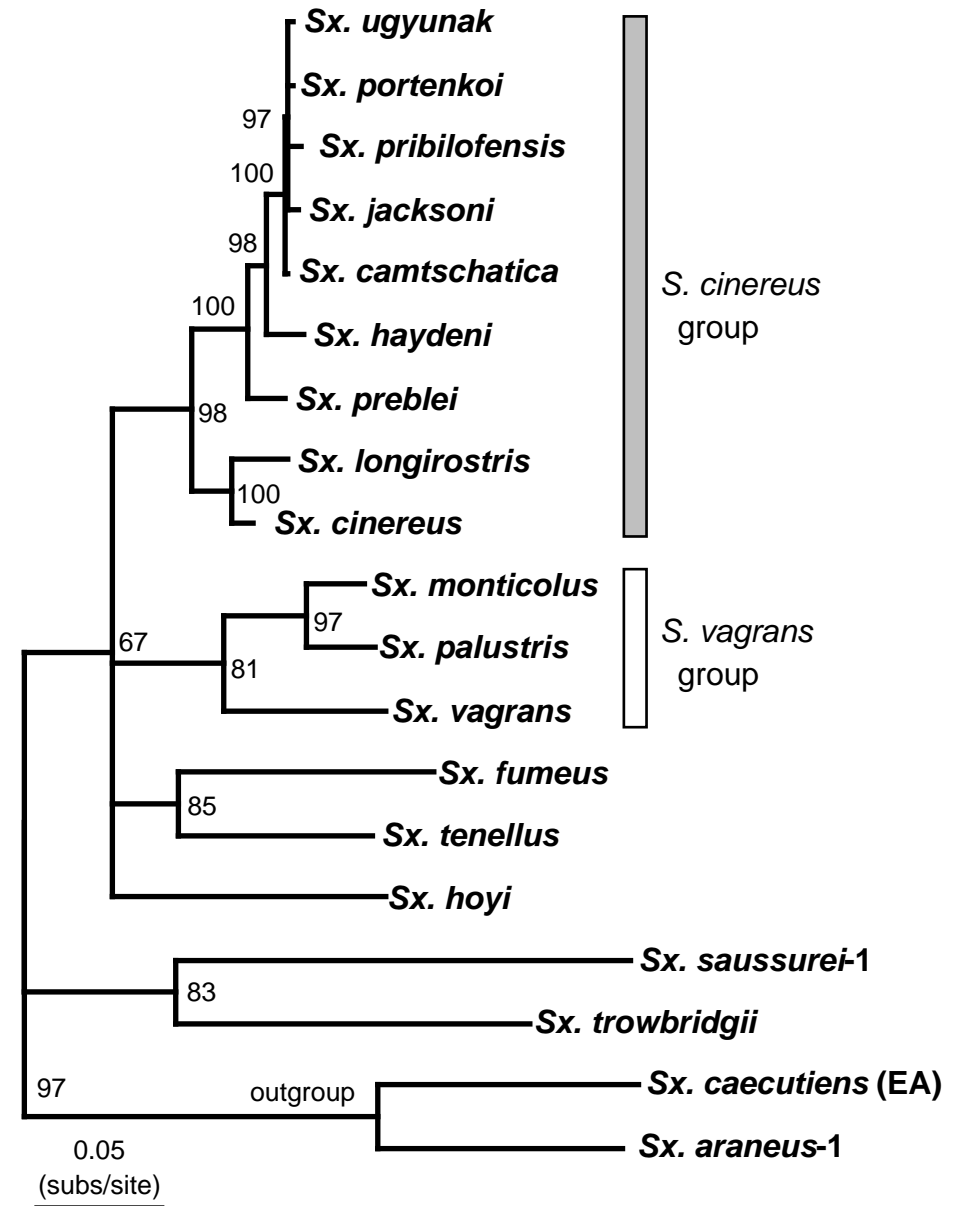


Fig. 4 Ohdachi et al.

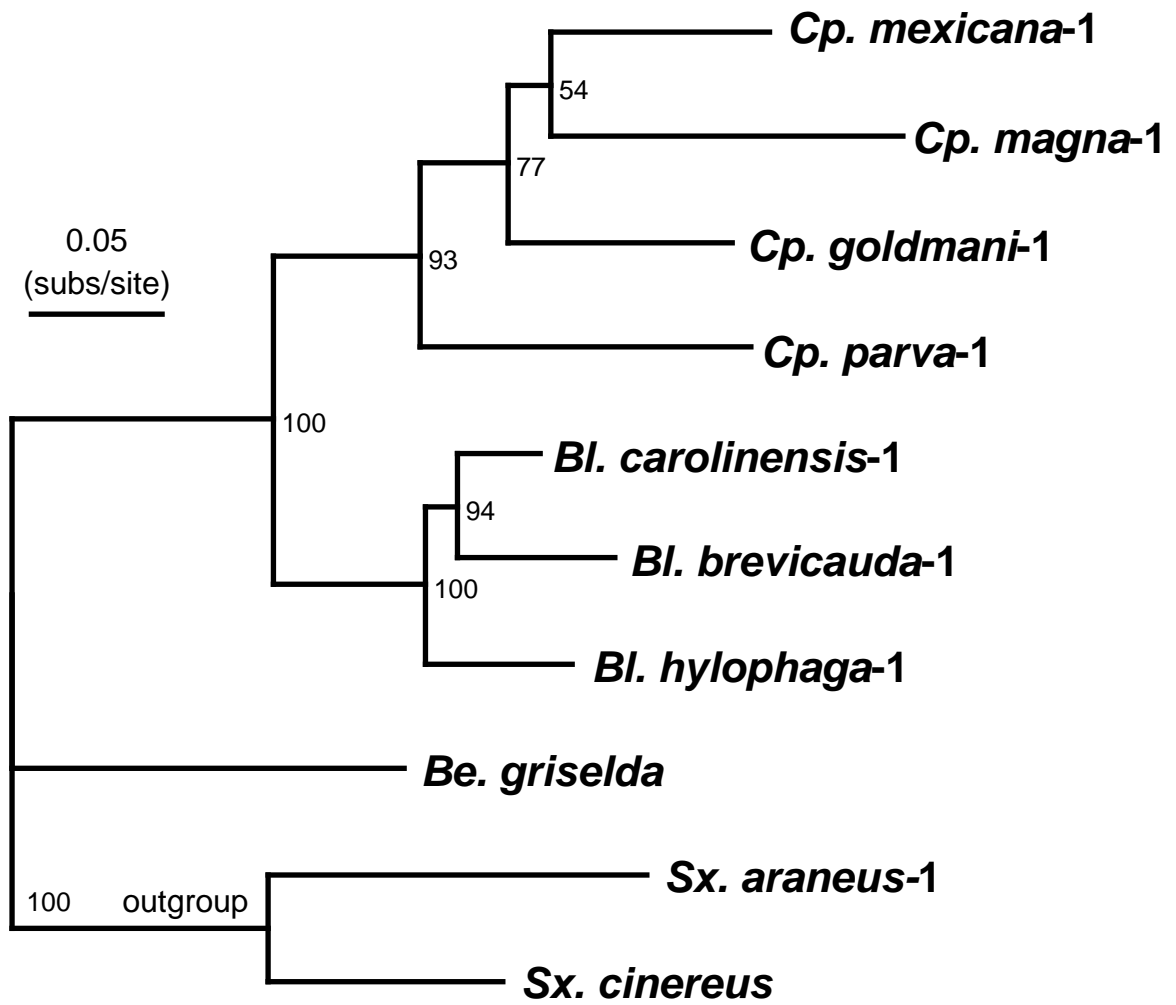


Fig. 5. Ohdachi et al.

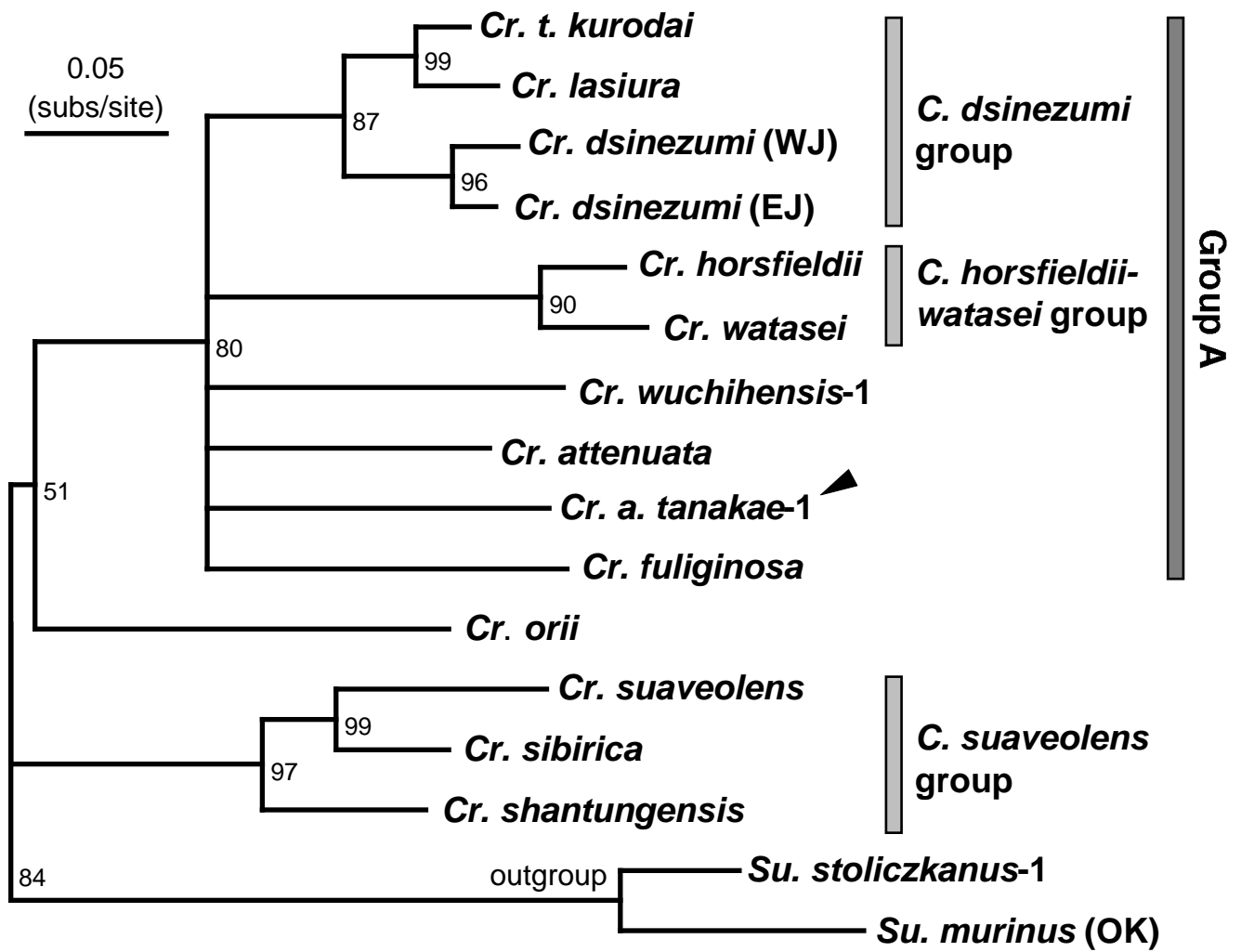


Fig. 6. Ohdachi et al.