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**Molecular phylogeny of Crocidura shrews in northeastern Asia: A special reference to specimens on Cheju Island, South Korea**

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20 Running head: Crocidura shrews on Cheju Island

## Abstract

Molecular phylogeny of crocidurine shrews (Insectivora, Soricidae) in northeastern Asia was investigated to confirm the taxonomic status of unidentified specimens of Crocidura from Cheju Island, South Korea. Phylogenetic trees were constructed by neighbor-joining (NJ) and maximum likelihood (ML) methods, based on the mitochondrial cytochrome b gene sequences (402 base pairs) of 40 individuals of seven crocidurine species and three unidentified specimens from 31 localities mainly in northeastern Asia. Phylogenetic positions of the three unidentified specimens from Cheju Island were compared with those of Suncus murinus, C. attenuata, C. dsinezumi, C. lasiura, C. sibirica, C. suaveolens and C. watasei. Both in NJ and ML trees, the three unidentified specimens were included in the cluster of C. dsinezumi and obviously different from C. suaveolens on Cheju Island. Thus, the present investigation demonstrated that both of C. suaveolens and C. dsinezumi exist on Cheju Island.

Key words: Crocidura dsinezumi, C. suaveolens, Cheju Island, mtDNA sequence, species identification

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## Introduction

Crocidurine shrews (Insectivora, Soricidae) occur in various regions of the Old World (the African and Eurasian Continents), consisting of about 150 - 170 species (Corbet 1978, Corbet and Hill 1991, Hutterer 1993, Wolsan and Hutterer 1998), but there are many problems in their taxonomy (e.g., Vogel et al. 1990, Motokawa 1998, 1999). In northeastern Asiatic region (Russian Far East, Japan, Korea and their neighboring small islands), seven species of the subfamily Crocidurinae are known: Suncus murinus (Linnaeus), C. dsinezumi (Temminck), C. watasei Kuroda, C. orii Kuroda, C. suaveolens (Pallas), C. lasiura Dobson, and C. sibirica Dukelsky (Corbet 1978, Corbet and Hill 1991, Hutterer 1993, Abe 1994, Hoffmann 1996; Motokawa 1998, Wolsan and Hutterer 1998, Motokawa et al. 2000). We tentatively used the taxonomic rank of C. suaveolens in the present study. In this region, particularly, crocidurine fauna on Cheju Island of South Korea is still uncertain since the first description of a crocidurine species (Kuroda 1934, Jones and Johnson 1960, Motokawa 1998, 1999, Iwasa et al. 2001)

Cheju Island is located between the Korean Peninsula and the Japanese Islands (Fig. 1). Therefore, species list on Cheju Island is important to elucidate biogeographical history of crocidurine species in northeastern Asia (Han 1997, Motokawa 1998, Abe 2000, Iwasa et al. 2001). Kuroda (1934) recorded a species of Crocidura at first from the island and described it as a new subspecies, C. dsinezumi quelpartis, based on a single skin and incomplete skull specimen (see also Jones 1959). Kuroda (1934) showed that the holotype specimen had an intermediate morphotype between C. dsinezumi and C. suaveolens. Motokawa (1998) doubted the occurrence of C. dsinezumi on Cheju Island from a biogeographic standpoint, and suspected that C. dsinezumi quelpartis might be C. suaveolens. Iwasa et al. (2001) recently explicated the presence of C. suaveolens on Cheju Island by cytogenetic and molecular analyses. However, we found Crocidura specimens whose DNA sequences obviously differed from C. suaveolens in a preliminary study. Thus, it is desirable to determine the phylogenetic and taxonomic status of these unidentified Crocidura specimens to complete the species list of small mammals on Cheju Island.

Species identification of the genus Crocidura is sometimes difficult if only depending on morphology, because there are great intraspecific variations in morphotype and size (e.g., Vogel et al. 1990). In fact, the specimens of C. suaveolens from Cheju Island in Iwasa et al. (2001) did not show a typical morphotype of C. suaveolens which has been described in literature (e.g., Imaizumi 1970, Abe 1967, 2000), indicating an intermediate type between C. dsinezumi and C. suaveolens (unpublished data). Likewise, the unidentified specimens examined here showed the intermediate morphotypes (e.g., body size and shape of the upper premolar) between C. dsinezumi and C. suaveolens (unpublished data). Even when it is difficult to distinguish species only using morphological data, genetic and phylogenetic information can often solve taxonomic problems (e.g., Vogel et al. 1990; Vogel and Sofianidou 1996; Ohdachi et al 1997; Iwasa and Tsuchiya 2000; Iwasa et al. 2001). In particular, Iwasa et al. (2001) successfully identified two specimens from Cheju Island as C. suaveolens investigating karyotype and restriction fragment length

polymorphism (RFLP) for the nuclear 28S rRNA gene. Thus, we should try again to identify another unidentified specimens from Cheju Island by using genetic markers.

In the present study, to determine the taxonomic rank of unidentified *Crocidura* specimens from Cheju Island, we applied a molecular phylogenetic approach based on the cytochrome *b* gene (*Cytb*) of mitochondrial DNA, since result is more straightforward than cytogenetic and RFLP analyses and methodology is more simple. We compared the phylogenetic positions of the unidentified shrews with those of *C. dsinezumi*, *C. suaveolens*, *C. lasiura*, *C. sibirica*, *C. watasei*, *C. attenuata* Milne-Edwards, and *Suncus murinus* collected from northeastern Asia (including Taiwan), and elucidated the taxonomic rank of the target specimens. In addition, several phylogenetic problems among northeastern Asiatic crocidurine shrews were discussed.

## Materials and methods

### Samples

Recently, Hoffmann (1996) and Motokawa (1999) treated northeastern Asiatic "*C. suaveolens*" as a valid species, "*C. shantungensis*", based on morphological characteristics. However, taxonomic relationship between *C. suaveolens* and *C. shantungensis* has not been completely revealed yet. Therefore, we tentatively used the traditional specific rank *C. suaveolens* for the northeastern Asiatic shrew according to Wolsan and Hutterer (1998).

We examined 40 samples of *Suncus murinus*, *C. watasei*, *C. lasiura*, *C. suaveolens*, *C. attenuata*, *C. dsinezumi*, *C. sibirica*, and unidentified *Crocidura* species from 31 localities in northeastern Asia (Table 1 and Fig. 1). Of them, the data of 17 samples were obtained from Ohdachi et al. (1997) and Motokawa et al. (2000) (Table 1) in GenBank/EMBL/DDBJ DNA databases. The three unidentified specimens obtained from Cheju Island (specimen Nos. HSO960925-1, -2 and HSO960926-1), were tentatively treated as an unidentified species, *Crocidura* sp.

### DNA sequencing

The total DNA was extracted from liver tissues by the conventional phenol chloroform method. Fragments of the mitochondrial *Cytb* gene were amplified with universal primers (L14724 and H15915; Irwin et al. 1991) for primary PCR (polymerase chain reaction). Subsequently, a specific secondary PCR amplifications for the first 402-bp (base pair) regions were conducted using nested (N-) primers (N-L14724 and N-H15155; Iwasa et al. 2000). Both reactions of PCR amplifications were carried out according to Iwasa et al. (2000). The second PCR products were directly sequenced for both DNA strands by an automated method using the Dye Primer Cycle Sequencing Kit (ABI) and an automated sequencer (model 373A, ABI).

The *Cytb* gene sequence data, which are determined in this study, were placed in the GenBank/EMBL/DDBJ nucleotide sequence databases (see Appendix).

### Phylogenetic trees

To estimate phylogenetic relationships, neighbor-joining (Saitou and Nei

1987) and maximum likelihood (Felsenstein 1981) methods were applied using all substitutions at all codon positions of the mitochondrial *Cytb* gene sequences (402-bp). *Sorex shinto* was used as a referring taxon. Neighbor-joining (NJ) tree was constructed by Kimura's two parameter method (Kimura 1980) using PAUP 4.0b\* program (Swofford 2000). Maximum likelihood (ML) tree was constructed by quartet-puzzling method (10,000 puzzling steps) using Tree-Puzzle ver. 5.0 program (Strimmer and von Haeseler 1996). The ML analysis was performed using HKY algorithm (Hasegawa et al., 1985) with unequal base frequencies and a discrete approximation to the gamma-distribution. The transition/transversion ratio, the fractions of invariable sites ( ) and the shape parameter ( ) were estimated according to the model. The mixed model of heterogeneity rate (one invariable rate + eight gamma-distribution rate) was executed for the program (Strimmer and von Haeseler 1996). To assess the confidence we used the support values (Strimmer and von Haeseler 1996; Strimmer et al. 1997) for the ML tree.

## Results

The 402-bp upstream region of the *Cytb* gene sequences was successfully sequenced for 23 *Crocidura* specimens newly obtained in the present study. No insertion and deletion were found for all the specimens sequenced here. Thus, we compared our raw sequence data and previously published data by Ohdachi et al. (1997) and Motokawa et al. (2000).

The Kimura's distances (*d*) between *C. suaveolens* and the *Crocidura* sp. on Cheju Island were ranged from 0.1023 to 0.1297. On the contrary, the *d* values between *C. dsinezumi* and the *Crocidura* sp. were ranged from 0.0000 to 0.0469. In addition, the extent of *d* within *C. suaveolens* and *C. dsinezumi* haplotypes, were ranged from 0.0000 to 0.0361 and from 0.0000 to 0.0495, respectively. Considering the species groups based on the distances, the extent of substitutions between *C. suaveolens* and the *Crocidura* sp./*C. dsinezumi* groups, were also ranged from 0.1023 to 0.1453. Thus, the substitution states between the two species groups showed certain extents as interspecific levels among the other species considering all *d* values in the crocidurine species of our data. On the other hand, only one specimen (Csp: Cheju-2) from Cheju Island showed 0.0050 in *d* within the *Crocidura* sp./*C. dsinezumi* group from southwestern Japan (Tokushima and Fukuoka) and Cheju Island. However, it was considered that the *Crocidura* sp. specimens from Cheju Island carried fundamentally same sequences with those of *C. dsinezumi* from southwestern Japan (Fig. 1). In contrast, the specimens of *C. suaveolens* from Cheju Island had peculiar sequences with relatively larger differentiation (*d*: max. 0.0361) in comparison with those from the Korean Peninsula (Naejang and Kyungju; Figs. 1 and 2). Therefore, in the Cheju Island specimens of *C. suaveolens* and the *Crocidura* sp., only the former had the Island-specific divergence genetically based on the present calculations of the *Cytb* gene (Fig. 1). Pairwise ML distances of the specimens showed similar divergences mentioned above as the Kimura's distances (data not shown).

In the NJ tree, the three specimens of *C. suaveolens* from Cheju Island were clustered into a unique cluster and diverged from the cluster of *C.*

suaveolens from the Korean Peninsula (Naejang and Kyungju), Ullung Island, Kagu Island and Tsushima Islands and that from Taiwan (Fig. 2). In C. dsinezumi, specimens from northeastern Japan (Iwate, Akita, Fukushima, Niigata, Sado, Gunma, Toyama, and Gifu) and those from southwestern Japan (Chiburijima, Tokushima, Fukuoka, and Yakushima) were clearly separated (Fig. 2). Furthermore, two clear monophyletic species groups, C. suaveolens/C. sibirica and the Crocidura sp./C. dsinezumi/C. lasiura were recognized (Fig. 2). Suncus murinus was located inside the cluster of the genus Crocidura (Fig. 2).

The ML tree was not completely resolved (unresolved quartet = 10.1%), but the topology represented in Fig. 3 was fundamentally the same as that of the NJ tree (Fig. 2). The most striking difference is that branching orders among species were obscure in the ML tree (Fig. 3), although C. suaveolens/C. sibirica and the Crocidura sp./C. dsinezumi/C. lasiura formed monophyletic groups, respectively, as in the NJ tree.

The three specimens of the Crocidura sp. from Cheju Island were clearly included in the cluster of C. dsinezumi from the Japanese Islands both in the NJ and ML trees (Figs. 2 and 3). Furthermore, the three specimens formed a sub-cluster with C. dsinezumi from southwestern Japan (Fukuoka and Tokushima) in both trees.

## Discussion

The present analysis about phylogenetic relationship of northeastern Asiatic Crocidura shrews was fundamentally identical to that of Motokawa et al. (2000), although no samples from Cheju Island were analyzed in the latter. Motokawa et al. (2000) demonstrated monophyly of C. dsinezumi/C. lasiura and of C. suaveolens/C. sibirica in their NJ tree, although C. dsinezumi/C. lasiura did not form a monophyletic group in a maximum parsimony tree in their data. The present study also supported the monophyly of C. dsinezumi/C. lasiura and of C. suaveolens/C. sibirica (Figs. 2 and 3). Thus, C. dsinezumi and C. suaveolens can be definitely separated from each other by the Cytb gene sequences.

Iwasa et al. (2001) identified two Crocidura specimens from Cheju Island as C. suaveolens, judging from karyotype and RFLP for nuclear 28S rRNA gene. In the present study, the identical specimens (Csv: Cheju-2 and -3) as Iwasa et al. (2001) were included in the analysis. The sequence data of the Cytb gene supported the taxonomic conclusion by Iwasa et al. (2001), including the two specimens in the cluster of C. suaveolens (Figs. 2 and 3). Iwasa et al. (2001) also showed some differentiation of the RFLP pattern of C. suaveolens between the individuals from Cheju Island and the Korean Peninsula. In the phylogenetic trees of the present study (Figs. 2 and 3), C. suaveolens from Cheju Island made a unique local cluster and diverged slightly from three clusters of the Korea Peninsula (including Ullung and Kagu Islands), Tsushima Islands, and Taiwan.

According to both NJ and ML trees (Figs. 2 and 3) and genetic divergences, we identified the three specimens of the Crocidura sp. from Cheju Island as C. dsinezumi. Furthermore, these specimens of the Crocidura sp. (= C. dsinezumi) from Cheju Island had fundamentally identical sequences as C. dsinezumi from the southwestern parts of Japan (Fukuoka and Tokushima, see

Table 1, Figs. 1 and 2).

Three specimens of *C. dsinezumi* from Cheju Island had identical or very similar sequences to those from the southwestern parts of Japan, whereas *C. suaveolens* from Cheju Island formed a unique cluster and was diversified slightly from other conspecific local clusters (Figs. 2 and 3). There are two possible reasons for the genetic closeness between Cheju Island and the southwestern parts of Japan (Kyushu/Shikoku: Figs. 1, 2 and 3) in *C. dsinezumi*.

One is that it has not passed sufficient time enough for genetic diversification since *C. dsinezumi* population of Cheju Island was separated from that of southwestern Japan after geological or population vicariance events (e.g., Futuyma 1993). Separation period between Kyushu and Cheju islands is estimated to be ca. 150,000 years ago, while separation of Cheju Island from the Korean Peninsula is 12,000-16,000 years ago (Ohshima 1990). Thus, if *C. dsinezumi* showed a little genetic divergence between Kyushu and Cheju, *C. suaveolens* should have also shown a little genetic variation between the Korean Peninsula and Cheju Island. In reality, *C. suaveolens* on Cheju Island had a unique genetic structure different from that from peninsular Korea (Iwasa et al. 2001, Figs. 2 and 3). Thus, this hypothesis might be less plausible.

The other reason of the genetic closeness between Cheju Island and southwestern Japan is the shrews on Cheju Island was recently introduced from southwestern Japan through human activity, such as trades by ships. The crocidurine species are sometimes moved by humans. For example, *Suncus murinus* in southeastern Asia are thought to be introduced into other regions by humans (Hutterer and Tranier 1990, Motokawa 1998), and several species of the *Crocidura* on the Mediterranean Islands also seems to have been accidentally introduced by humans (Vogel et al. 1986, Vogel and Sofianidou 1996). It is thought that people interchanged frequently between Kyushu and Cheju islands by ships since the 14th century (Takahashi 1987) until now, although the oldest official record of interchange of people between Cheju Island and ancient Japan dates back to the 7th century (Amino 2000). Hence, *C. dsinezumi* might have been accidentally introduced from Kyushu to Cheju Island by ships. However, investigations of population genetics (e.g., using microsatellite DNA polymorphism) with more collection localities have to be conducted to answer the question of whether *C. dsinezumi* on Cheju Island is a natural population or it was recently introduced from southwestern Japan.

In conclusion, it has been revealed from molecular phylogeny based on the mitochondrial *Cytb* gene that both *C. dsinezumi* and *C. suaveolens* inhabit Cheju Island. Co-occurrence of these two species on Cheju Island has probably caused the confusion of taxonomy of the *Crocidura* on Cheju Island (e.g., Han 1997, Iwasa et al. 2001). Now, it is possible to identify the *Crocidura* species on Cheju Island using molecular cues. However, more extensive investigations for the relationships of morphology and distribution between the two species on Cheju Island are necessary to solve the taxonomic problem completely.

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## **Appendix**

Accession numbers of our DNA sequence data in DNA databases as follows (acronyms and specimen codes as shown in Table 1): C. watasei - Cwt:

Tokunoshima, AB077074; C. sibirica - Csb: Mongolia, AB077088; C. suaveolens

- 5 - Csv: Cheju-1, AB077151; Cheju-2, AB077077; Cheju-3, AB077078; Naejang, AB077152; Kyungju, AB077079; Ullung, AB077149; Kagu, AB077150; C. lasiura  
- Cls: Ussurisk, AB077071; Naejang, AB077321; Seokmo, AB077148; C. dsinezumi - Cds (= Csp): Akita, AB077061; Iwate, AB077146; Fukushima, AB077147; Gunma, AB077063; Niigata, AB077274; Tokushima, AB077066;  
10 Fukuoka-1, AB077069; Fukuoka-2, AB077275; Cheju-1, AB077276; Cheju-2, AB077070; Cheju-3, AB077277.

## Figure legends

**Fig. 1.** Collection localities of crocidrine samples in northeastern Asia examined.

5

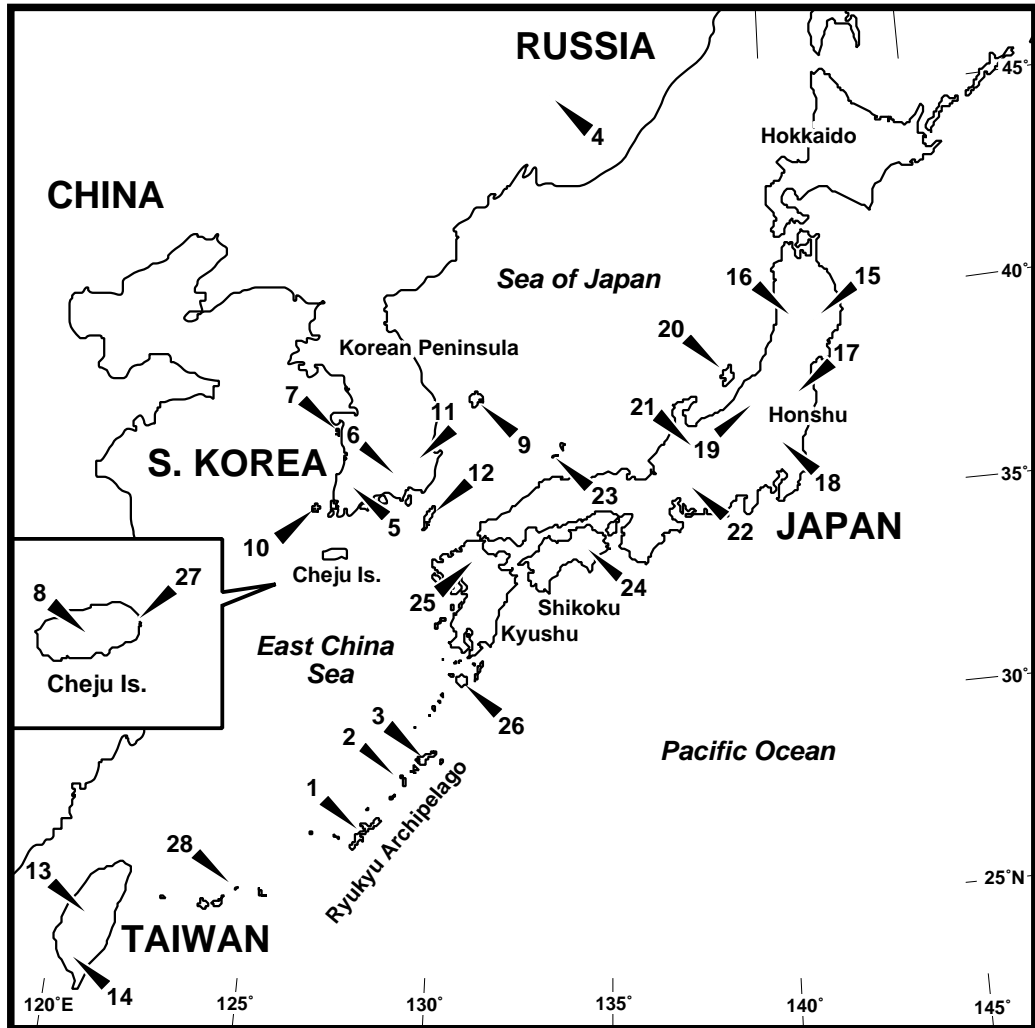
**Fig. 2.** Neighbor-joining tree of the mitochondrial cytochrome *b* gene sequences (402-bp) by the Kimura's two parameter method using in crocidrine shrews in eastern Asia. Bootstrap values of percentage (1,000 replicates) are indicated to assign the confidence of nodes. Specimen codes and locality numbers correspond with those in Table 1 and Fig. 1.

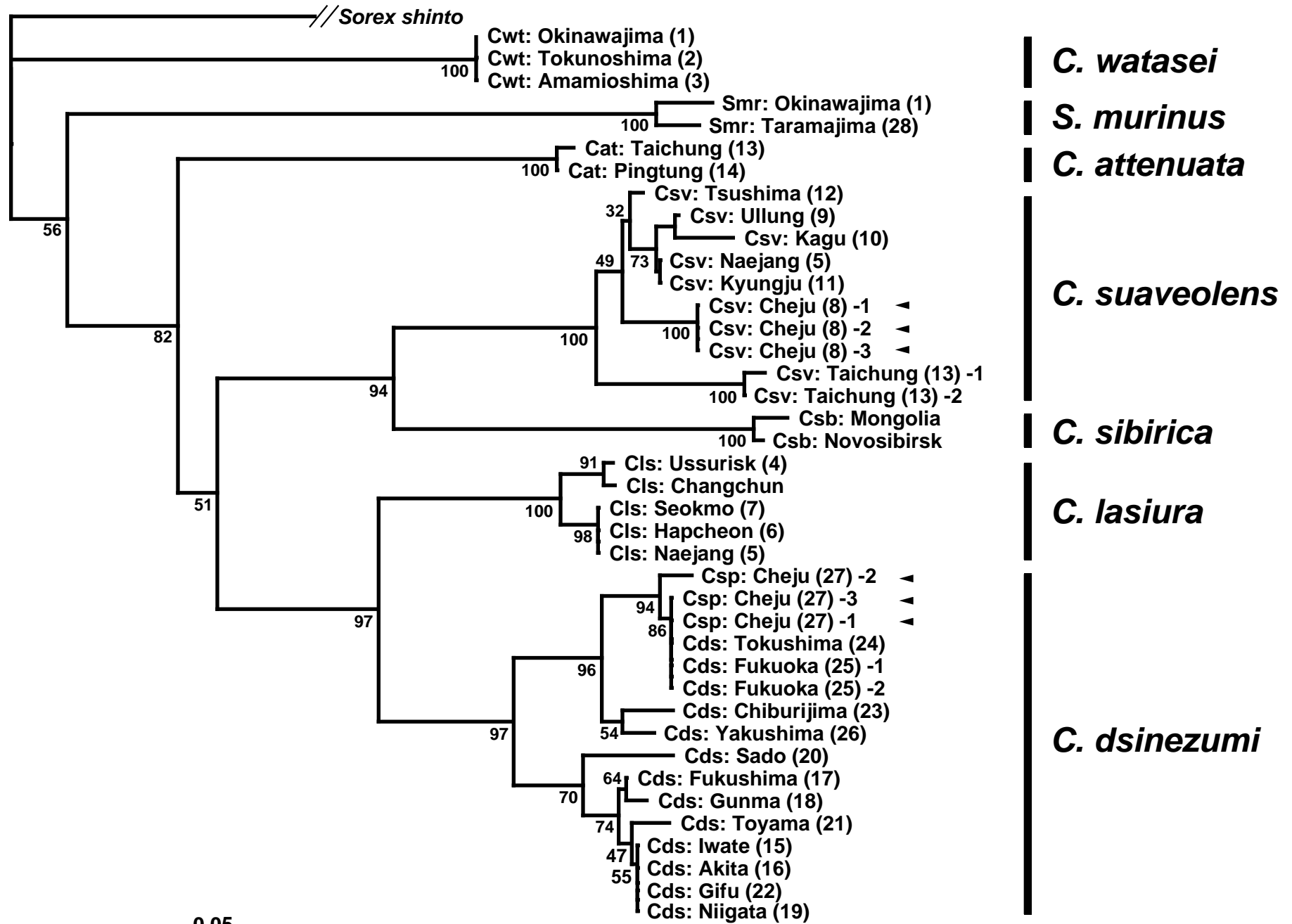
10

**Fig. 3.** Maximum likelihood tree of the mitochondrial cytochrome *b* gene sequences (402-bp) in crocidrine shrews in eastern Asia. Percentage of support values (10,000 steps) are indicated to assign the confidence of nodes. Specimen codes and locality numbers correspond with those in Table 1 and Fig. 1.

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1.





(Kimura's distance: substitutions per site)



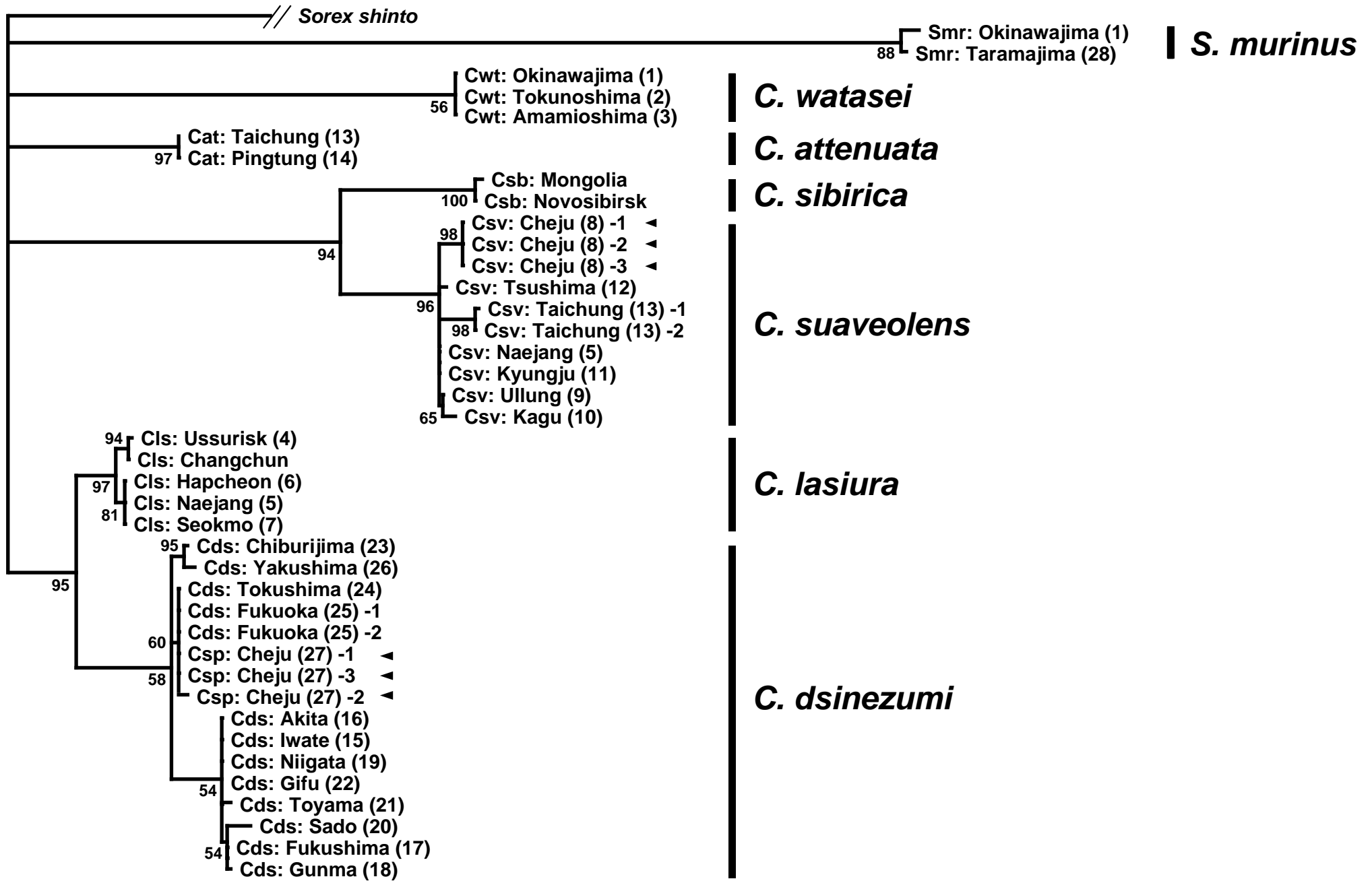


Table 1. Crocudira and Suncus specimens examined in this study (including outgroup, Sorex shinto).

Species	Acronym of species	Specimen No. or reference	Collecting locality	Specimen Code in Figs. 2 & 3		
<u>C. watasei</u>	Cwt	Motokawa et al. (2000)	Okinawajima Is., Japan (1)	Okinawajima		
		HS600	Tokunoshima Is., Japan (2)	Tokunoshima		
		Motokawa et al. (2000)	Amamioshima Is. Japan (3)	Amamioshima		
<u>C. lasiura</u>	Cls	ditto	Changchun, Jilin Province, China*	Changchun		
		HS1252	Ussuriskiyi Reservation, Russia (4)	Ussurisk		
		SHH931106-1	Mt. Naejang, Korea (5)	Naejang		
		Motokawa et al. (2000)	Hapcheon Gun, Kyungsangnam Do, Korea (6)	Hapcheon		
		SHH97004	Seokmo Is., Korea (7)	Seokmo		
		<u>C. suaveolens</u>	Csv	HSO980821-4	Mt. Halla, Cheju Is., Korea (8)	Cheju-1
SO99/10/11-1	Mt. Halla, Cheju Is., Korea (8)			Cheju-2		
SO99/10/14-2	Mt. Halla, Cheju Is., Korea (8)			Cheju-3		
SHH921216-1	Ullung Is., Korea (9)			Ullung		
SHH940807-1	Kagu Is., Korea (10)			Kagu		
SHH970624-1	Mt. Naejang, Korea (5)			Naejang		
SHH970426-1	Kyungju, Korea (11)			Kyungju		
Motokawa et al. (2000)	Tsushima Is., Japan (12)			Tsushima		
ditto	Taichung, Taiwan (13)			Taichung-1		
ditto	Taichung, Taiwan (13)			Taichung-2		
<u>C. attenuata</u>	Cat			ditto	Taichung, Taiwan (13)	Taichung
				ditto	Pingtung Hsein, Taiwan (14)	Pingtung
				HS1491	Iwate, Honshu, Japan (15)	Iwate
<u>C. dsinezumi</u>	Cds	HEG246-98	Akita, Honshu, Japan (16)	Akita		
		SHH930805-1	Fukushima, Honshu, Japan (17)	Fukushima		
		HEG232-98	Gunma, Honshu, Japan (18)	Gunma		
		HEG240-98	Niigata, Honshu, Japan (19)	Niigata		
		Ohdachi et al. (1997)	Sado Is., Japan (20)	Sado		
		Motokawa et al. (2000)	Toyama, Honshu, Japan (21)	Toyama		
		ditto	Gifu, Honshu, Japan (22)	Gifu		
		ditto	Chiburijima Is., Japan (23)	Chiburijima		
		HA6134	Tokushima, Shikoku, Japan (24)	Tokushima		
		HS1296	Fukuoka, Kyushu, Japan (25)	Fukuoka-1		
		HS1297	Fukuoka, Kyushu, Japan (25)	Fukuoka-2		
<u>C. sibirica</u>	Csb	Motokawa et al. (2000)	Yakushima Is., Japan (26)	Yakushima		
		KT-Mon95-07	Sharga, Govi-Altay, Mongolia*	Mongolia		
<u>Crocidura</u> sp.	Csp	Motokawa et al. (2000)	Academgorodok, Novosibirsk, Russia*	Novosibirsk		
		HSO960925-1	Songsan, Cheju Is., Korea (27)	Cheju-1		
		HSO960926-1	Songsan, Cheju Is., Korea (27)	Cheju-2		
<u>Suncus murinus</u>	Smr	HSO960926-2	Songsan, Cheju Is., Korea (27)	Cheju-3		
		Ohdachi et al. (1997)	Okinawajima Is., Japan (1)	Okinawa		
<u>Sorex shinto</u>	---	Motokawa et al. (2000)	Taramajima Is., Japan (28)	Taramajima		
		Ohdachi et al. (1997)	Shikoku, Japan	<u>Sorex shinto</u>		

\*Collecting localities are not shown in Fig. 1.