

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

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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 mitochondrial cytochrome *b* gene sequences (402 base pairs) of 37 individuals of seven crocidurine species and three unidentified specimens from 31 localities mainly in northeastern Asia. Phylogenetic position 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 were obviously different from *C. suaveolens* on Cheju Island. Thus, the present investigation demonstrated that both *C. suaveolens* and *C. dsinezumi* exist on Cheju Island.

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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

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there are many problems in their taxonomy (eg 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, 1758), *Crocidura dsinezumi* (Temminck, 1843), *C. watasei* Kuroda, 1924, *C. orii* Kuroda, 1924, *C. suaveolens* (Pallas, 1811), *C. lasiura* Dobson, 1890, and *C. sibirica* Dukelsky, 1930 (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, crocidurine fauna on Cheju Island of South Korea remains 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 that of *C. suaveolens*. 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 (eg Vogel *et al.* 1990). In fact, the specimens of *C. suaveolens* from Cheju Island in Iwasa *et al.* (2001) did not show the typical morphotype of *C. suaveolens* which has been described in the literature (eg Imaizumi 1970, Abe 1967, 2000), indicating an intermediate position between *C. dsinezumi* and *C. suaveolens* (M. A. Iwasa, unpubl.). Likewise, the unidentified specimens examined here showed the intermediate morphotypes (eg body size and shape of the upper premolar) between *C. dsinezumi* and *C. suaveolens* (M. A. Iwasa, unpubl.). Even when it is difficult to distinguish species only using morphological data, genetic and phylogenetic information can often solve taxonomic problems (eg Vogel *et al.* 1990, Vogel and Sofianidou 1996, Ohdachi *et al.* 1997, Iwasa and Tsuchiya 2000, Iwasa *et al.* 2001). 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 such results are more straightforward than cytogenetic, and RFLP analyses and methodology, are 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, 1872, 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.

### Material and methods

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, 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 *Crocidura* sp.

### DNA sequencing

The total DNA was extracted from liver tissues by the conventional phenol chloroform method. The whole mitochondrial *Cytb* gene was amplified with universal primers (L14724 and H15915; Irwin *et al.* 1991) for primary polymerase chain reaction (PCR). Subsequently, a secondary PCR amplifications for the first 402-bp (base pair) regions was conducted using nested (N-) primers (N-L14724 and N-H15155; Iwasa *et al.* 2000). Both 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, determined in this study were placed in the GenBank/EMBL/DDBJ database (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 ( $\theta$ ) and the shape parameter ( $\alpha$ ) 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.

Table 1. *Crocidura* and *Suncus* specimens examined in this study (including outgroup, *Sorex shinto*). Numbers in parenthesis represent collecting localities (see Fig. 1). \* Collecting localities are not shown in Fig. 1.

Species	Acronym of species	Specimen number or reference	Collecting locality	Specimen Code in Figs 2 and 3
<i>C. watasei</i>	Cwt	Motokawa <i>et al.</i> 2000	Okinawajima Is., Japan (1)	Okinawajima
		HS600	Tokunoshima Is., Japan (2)	Tokunoshima
		Motokawa <i>et al.</i> 2000	Amamioshima Is., Japan (3)	Amamioshima
<i>C. lasiura</i>	Cls	<i>ditto</i>	Changchun, Jilin Province, China*	Changchun
		HS1252	Ussuriski Reservation, Russia (4)	Ussurisk
		SHH931106-1	Mt. Naejang, Korea (5)	Naejang
		Motokawa <i>et al.</i> 2000	Hapcheon Gun, Kyungsangnam Do, Korea (6)	Hapcheon
		SHH97004	Seokmo Is., Korea (7)	Seokmo
<i>C. suaveolens</i>	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 <i>et al.</i> 2000	Tsushima Is., Japan (12)	Tsushima
		<i>ditto</i>	Taichung, Taiwan (13)	Taichung-1
		<i>ditto</i>	Taichung, Taiwan (13)	Taichung-2
<i>C. attenuata</i>	Cat	<i>ditto</i>	Taichung, Taiwan (13)	Taichung
		<i>ditto</i>	Pingtung Hsein, Taiwan (14)	Pingtung
<i>C. dsinezumi</i>	Cds	HS1491	Iwate, Honshu, Japan (15)	Iwate
		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 <i>et al.</i> 1997	Sado Is., Japan (20)	Sado
		Motokawa <i>et al.</i> 2000	Toyama, Honshu, Japan (21)	Toyama
		<i>ditto</i>	Gifu, Honshu, Japan (22)	Gifu
		<i>ditto</i>	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		
Motokawa <i>et al.</i> 2000	Yakushima Is., Japan (26)	Yakushima		
<i>C. sibirica</i>	Csb	KT-Mon95-07	Sharga, Govi-Altay, Mongolia*	Mongolia
		Motokawa <i>et al.</i> 2000	Academgorodok, Novosibirsk, Russia*	Novosibirsk
<i>Crocidura</i> sp.	Csp	HSO960925-1	Songsan, Cheju Is., Korea (27)	Cheju-1
		HSO960926-1	Songsan, Cheju Is., Korea (27)	Cheju-2
		HSO960926-2	Songsan, Cheju Is., Korea (27)	Cheju-3
<i>Suncus murinus</i>	Smn	Ohdachi <i>et al.</i> 1997	Okinawajima Is., Japan (1)	Okinawa
		Motokawa <i>et al.</i> 2000	Taramajima Is., Japan (28)	Taramajima
<i>Sorex shinto</i>	–	Ohdachi <i>et al.</i> 1997	Shikoku, Japan	<i>Sorex shinto</i>

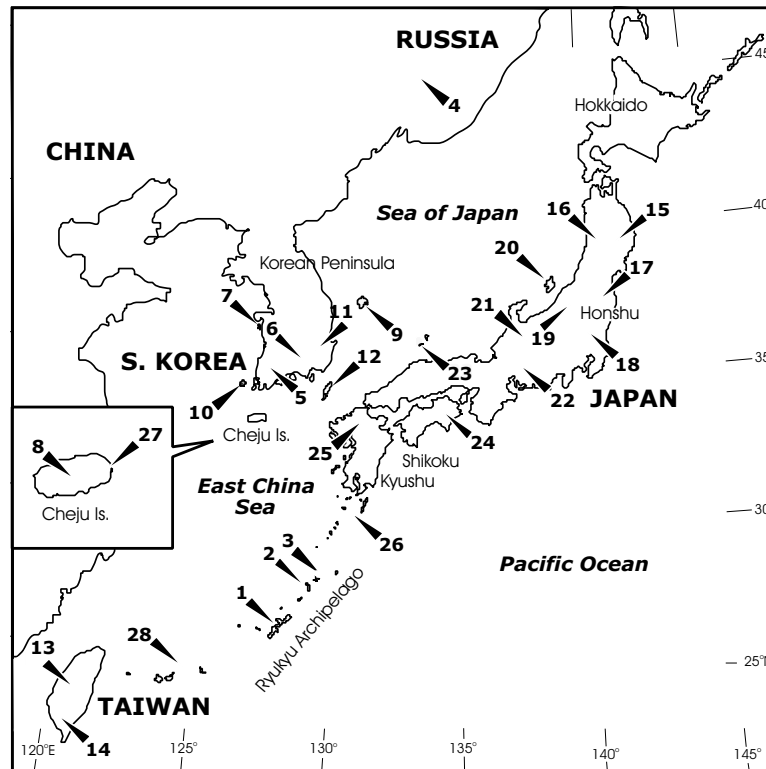


Fig. 1. Collection localities of crocidurine samples in northeastern Asia. Numbers indicate localities marked in Table 1.

## 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 with previously published by Ohdachi *et al.* (1997) and Motokawa *et al.* (2000).

The Kimura's distances ( $d$ ) between *C. suaveolens* and *Crocidura* sp. on Cheju Island ranged from 0.1023 to 0.1297. On the contrary, the  $d$  values between *C. dsinezumi* and the *Crocidura* sp. ranged from 0.0000 to 0.0469. In addition, the extent of  $d$  within *C. suaveolens* and *C. dsinezumi* haplotypes, ranged from 0.0000 to 0.0361 and from 0.0000 to 0.0495, respectively. Considering the species groups based on distances, the extent of substitutions between *C. suaveolens* and the *Crocidura* sp./*C. dsinezumi* groups, 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 similar sequences with those of *C. dsinezumi* from southwestern Japan (Fig. 1). In contrast, the specimens of *C. suaveolens* from Cheju Island had distinct sequences which diverged considerably ( $d$ : maximum 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

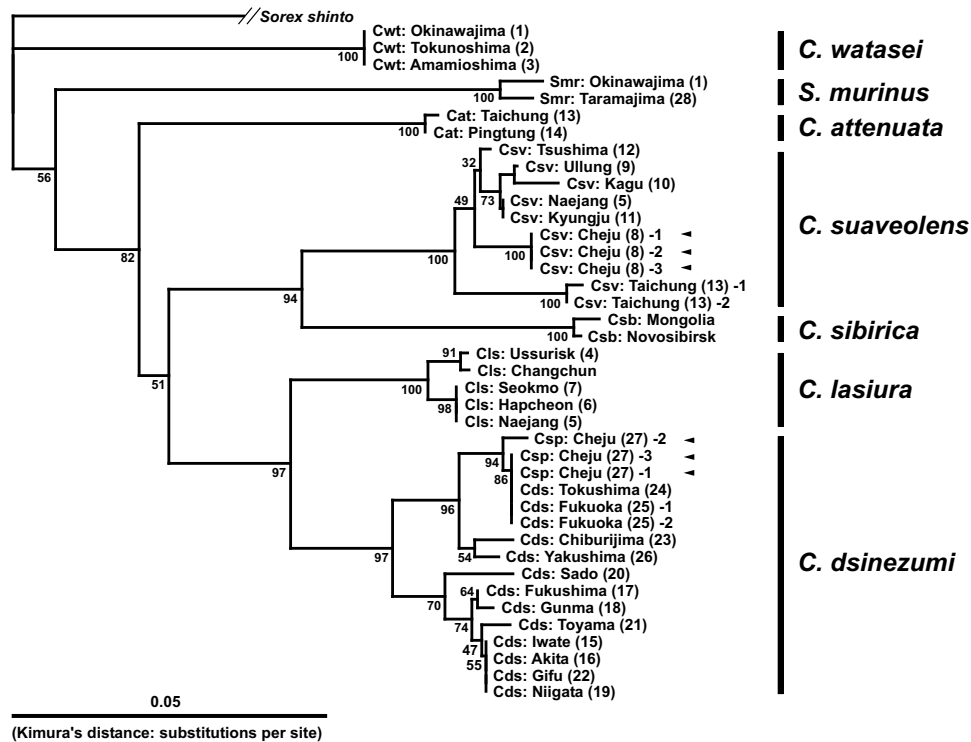


Fig. 2. Neighbor-joining tree of the mitochondrial cytochrome *b* gene sequences (402-bp) by the Kimura's two parameter method applied to crocidurine shrews in northeastern Asia. Bootstrap values of percentage (1,000 replicates) are indicated to assign the confidence of nodes. Specimen codes and locality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

the Korean Peninsula (Naejang and Kyungju), Ullung Island, Kagu Island and Tsushima Islands, and those 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

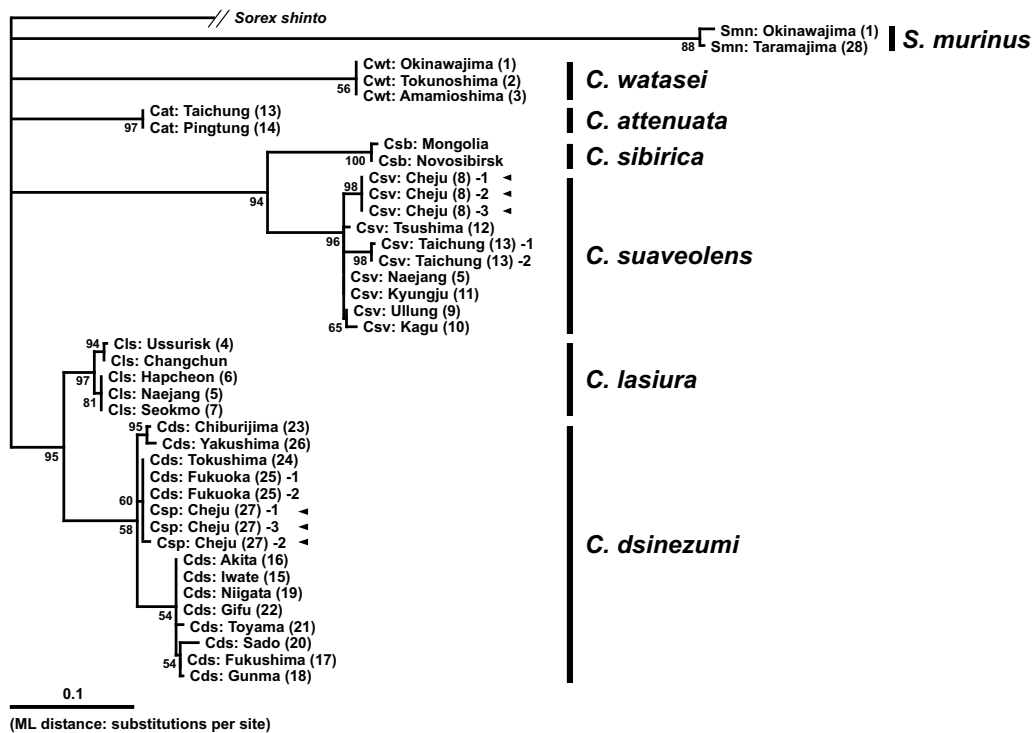


Fig. 3. Maximum likelihood tree of the mitochondrial cytochrome *b* gene sequences (402-bp) in crocidurine shrews in northeastern Asia. Percentage of support values (10,000 steps) are indicated to assign the confidence of nodes. Specimen codes and locality numbers correspond to those in Table 1 and Fig.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

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 *Crociodura* 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 *Crociodura* specimens from Cheju Island as *C. suaveolens*, judging from karyotype and RFLP for nuclear 28S rRNA gene. In the present study, the same 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 *Crociodura* sp. from Cheju Island as *C. dsinezumi*. Furthermore, these specimens of the *Crociodura* 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 time since *C. dsinezumi* population of Cheju Island was separated from that of southwestern Japan after geological or population vicariance events was too short for genetic diversification (eg 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 occurred 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 (Figs 2 and 3; Iwasa *et al.* 2001). Thus, this hypothesis might be less plausible.

The other possible reason of the genetic closeness between Cheju Island and southwestern Japan is that shrews on Cheju Island were recently introduced from southwestern Japan through human activity, such as trade by ships. The crocidurine species are sometimes transferred by humans. For example, *Suncus murinus* in southeastern Asia are thought to have been 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 known 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 (eg using microsatellite DNA polymorphism) with more collection localities have to be conducted to clarify if *C. dsinezumi* on Cheju Island is a natural population or it was recently introduced from southwestern Japan.

In conclusion, we demonstrate the existence of both *C. dsinezumi* and *C. suaveolens* on Cheju Island. Co-occurrence of these two species on Cheju Island has probably caused the confusion of their taxonomy (eg Han 1997, Iwasa *et al.* 2001). Now, it is possible to identify them using molecular cues. However, more extensive investigations of morphology and distribution are necessary.

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Appendix. Accession numbers of our DNA sequence data in DNA databases are as follows (acronyms and specimen codes as shown in Table 1): *C. watasei* – Cwt: Tokunoshima, AB077074; *C. sibirica* – Csb: Mongolia, AB077088; *C. suaveolens* – 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; Fukuoka-1, AB077069; Fukuoka-2, AB077275; Cheju-1, AB077276; Cheju-2, AB077070; Cheju-3, AB077277.