

A preliminary study on genetic divergence of the Asian lesser white-toothed shrew *Crocidura shantungensis* (Mammalia: Soricomorpha) in mainland Korea, adjacent islands and continental East Asia: cytochrome *b* sequence analysis

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ABSTRACT. To examine genetic divergence of *Crocidura shantungensis* in mainland Korea, adjacent islands and continental East Asia, we obtained 17 complete cytochrome *b* sequences (1140 bp) from mainland Korea and Far Eastern Russia, and these sequences were compared to sequences of *C. shantungensis*, obtained from the GenBank. Jeju *C. shantungensis* was divergent from the mainland Korean population, with average genetic distance of 1.94%. Moreover, Tsushima *C. shantungensis* was distinct from the mainland Korean population, with average distance of 2.08%, but the latter was not divergent from other *C. shantungensis* in Taiwan and Ullung islands, Mongolia, southeastern Siberia, and Far Eastern Russia, indicating that three clades (Jeju, Tsushima, and Taiwan-Ullung-continental East Asia) are recognized within *C. shantungensis*. Our results do not support the current subspecies classification of *C. shantungensis* (i.e., *C. s. quelpartis* from Jeju and Taiwan and *C. s. shantungensis* from other distribution regions), and do support the former classification of Jeju population as *C. s. quelpartis*. Thus, we proposed further systematic analysis with additional specimens from East Asia to clarify the taxonomy and phylogeography in *C. shantungensis*.

KEY WORDS: Soricomorpha, *Crocidura shantungensis*, East Asia, Korea, phylogeography, DNA systematics, cytochrome *b*.

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Предварительное исследование генетической дивергенции азиатской малой белозубки *Crocidura shantungensis* (Soricomorpha: Mammalia) материковой части Кореи, близлежащих островов и континентальной Восточной Азии: анализ цитохрома *b*

Хан Сан Ко, И.В. Картавецва, Бэ Кеунт Ли, Гу Хи Квон, Биёнг Гуг Янг, Сеон Вук Хео, Сеёнг Тэк Ин

РЕЗЮМЕ. В результате предварительного исследования генетической дивергенции азиатской малой белозубки *Crocidura shantungensis* материковой части Кореи, близлежащих островов и континента Восточной Азии, мы получили 17 полных сиквенсов гена цитохрома *b* (1140 пн) из материковой части Кореи и Российского Дальнего Востока и сравнили их с сиквенсами *C. shantungensis*, полученными из ГенБанка. Особи *C. shantungensis* из корейского острова Чеджу дивергировали от материковых популяций с величиной средних расстояний, равных 1,94%. Кроме того, *C. shantungensis* с Цусимы отличаются от материковых популяций Кореи (средние дистанции 2,08%), которые сходны с *C. shantungensis* островов Тайвань и Уллуны и из материковых популяций Монголии, юго-восточной Сибири и Дальнего Востока России, что свидетельствует о существовании внутри вида

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C. shantungensis трех клад (Чеджу, Цусима и острова Тайвань–Уллуь–материк Восточной Азии). Наши результаты не подтвердили подвидовую классификацию, принятую в настоящее время для *C. shantungensis* (т.е., *C. s. quelpartis* из Чеджу и Тайваня и *C. s. shantungensis* из других регионов), но подтвердили ранее предложенное выделение популяции острова Чеджу в отдельный подвид *C. s. quelpartis*. Необходим дальнейший систематический анализ с исследованием дополнительных экземпляров из Восточной Азии для уточнения таксономии и филогеографии *C. shantungensis*.

КЛЮЧЕВЫЕ СЛОВА: Soricomorpha, *Crocidura shantungensis*, Восточная Азия, Корея, филогеография, ДНК систематика, цитохром *b*.

Introduction

The Asian lesser white-toothed shrew (*Crocidura shantungensis* Miller, 1901) is distributed in southeastern Siberia, eastern China, and mainland Korea, including Taiwan, Jeju (Korea), Ullung (Korea), and Tsushima (Japan) islands (Hutterer, 2005). Six subspecies had been reported: *C. s. utsuryoensis* from Ullung, *C. s. quelpartis* from Jeju, and *C. s. coreae* from mainland Korea (Jones & Johnson, 1960), *C. s. coreae* from Tsushima (Thomas, 1908), *C. s. orientis* from Ussuri region and eastern Siberia (Corbet, 1978), and *C. s. hosletti* from Taiwan (Jameson & Jones, 1977). Recently, two subspecies of *C. shantungensis* (*C. s. quelpartis* from Jeju and Taiwan and *C. s. shantungensis* from other distribution regions) were recognized (Hutterer, 2005), indicating that subspecies classification of *C. shantungensis* is still in confusion.

Nuclear genes with a slower rate of evolution are useful for the phylogeny reconstruction at a higher level, whereas mitochondrial DNA is more suitable for classification at the species level and for the examination of population structures within a species (Sunucks, 2000). Molecular phylogeny within six species of *Crocidura* shrews in northeastern Asia was investigated from partial (402 bp) cytochrome *b* sequences (Han *et al.*, 2002), and phylogenetic relationships among eight *Crocidura* shrews in East and Central Asia were analyzed on the basis of complete or partial cytochrome *b* sequences (Ohdachi *et al.*, 2004), whereas phylogeographic structure within six species of *C. suaveolens* group was examined based on nuclear Breast Cancer Susceptibility 1 (BRAC1) and mitochondrial cytochrome *b* sequences (Dubey *et al.*, 2006).

From a molecular study with six partial (402 bp) cytochrome *b* haplotypes of *C. shantungensis*, Jeju population (one haplotype) made a unique local cluster and diverged slightly from other *C. shantungensis* (five haplotypes) in mainland Korea, Ullung and Kagu Islands (Korea), Tsushima, and Taiwan (Han *et al.*, 2002). In a sequencing study of *C. shantungensis* on the basis of complete or partial (402 bp) cytochrome *b* sequences, one Jeju haplotype was divergent from other six haplotypes of *C. shantungensis* from Primorye (Far Eastern Russia), mainland Korea, and Ullung (Ohdachi *et al.*, 2004), whereas based on one partial (998 bp) cytochrome *b* sequence from Tsushima and complete eight sequences from other regions, three subgroups of

C. shantungensis were shown (Jeju; Tsushima; and Primorye, mainland Korea, and Ullung) (Dubey *et al.*, 2006). Recently, on the basis of 15 complete cytochrome *b* haplotypes with different sequences three subgroups of *C. shantungensis* were reported (Jeju and Tsushima; Buryatia, southeastern Siberia; and other regions of Mongolia, Primorye, mainland Korea, and Ullung) (Bannikova *et al.*, 2009), indicating that the previous researches on *C. shantungensis* utilized two partial (402 bp) haplotypes or one complete haplotype from mainland Korea, and their results on population subdivisions are not comparable with one another.

In addition, peripheral populations are often genetically and morphologically divergent from central populations, and natural barriers to dispersal, which limit species distribution, include mountain range and rivers (Goldberg & Land, 2007). The Yellow Sea separating Korea and eastern China was retreated during the last glacial maximum (Chung, 2007), whereas large areas of continental shelf were dry land at the end of the last glacial period, allowing easy exchange of plant and animal species by land bridge connections to what are now isolated islands (Lomolino *et al.*, 2010). Northern boundary of the Korean Peninsula is formed naturally by Yalu River, Baitou Mountain (the main peak of the Changbai Mountains in northeastern China, 2744 m above sea level), and Tumen River. Five major populations of *C. shantungensis* (four insular isolates from Jeju, Tsushima, Taiwan, and Ullung and one large continental population from eastern China, eastern Russia, and Mongolia) are surrounding a mainland Korean population.

Moreover, the ancestors of Jeju and Tsushima *C. shantungensis* moved from continental East Asia through mainland Korea to Jeju and Tsushima Islands, respectively (Motokawa *et al.*, 2003). Thus, it appeared that a cytochrome *b* sequencing analysis using several *C. shantungensis* specimens from mainland Korea is necessary to examine genetic diversity of mainland Korean population and investigate the genetic divergence of a peripheral mainland Korean isolates within the Korean Peninsula from other populations in adjacent islands and continent of East Asia. However, the genetic analysis with more *C. shantungensis* specimens from mainland Korea has not been performed yet.

In this paper we obtained 17 complete cytochrome *b* sequences (1140 bp) of *C. shantungensis* from four locations in mainland Korea and one location in Far Eastern Russia (Vladivostok), and these sequences were

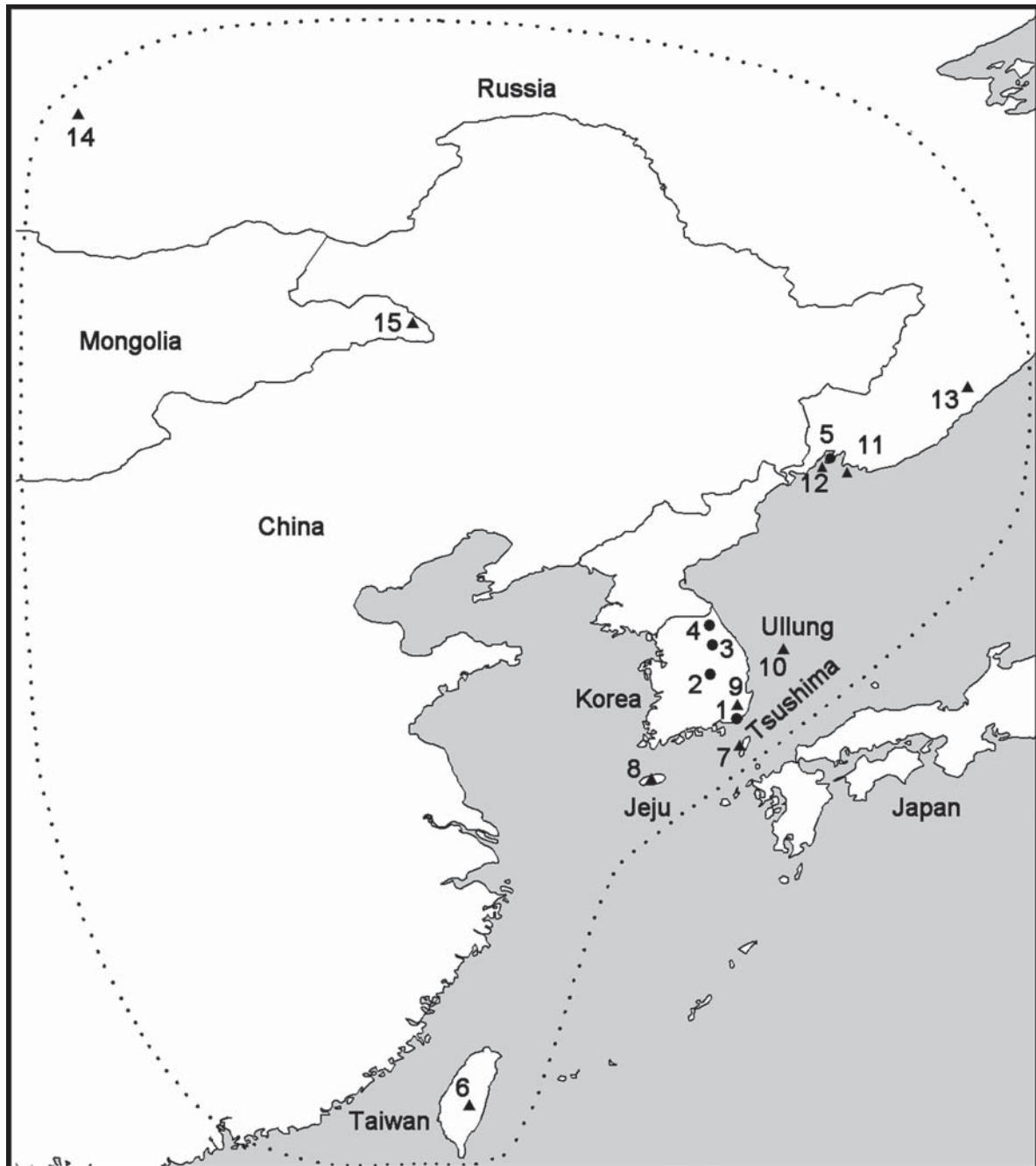


Figure 1. Map showing the distribution range (indicated in dotted line) of *Crocidura shantungensis* and the localities of studied specimens. The specimens collected during this study are indicated in circles, the specimens from GenBank are indicated in triangles. See locality numbers and additional information in Table.

compared to the corresponding sequences of *C. shantungensis*, obtained from GenBank, in order to examine genetic divergence of mainland Korean population from other East Asian *C. shantungensis* populations.

Material and methods

Specimens consisted of 17 *C. shantungensis* individuals from four locations in mainland Korea and one

location in Far Eastern Russia (see Table) were studied. Collection sites are shown in Figure 1. Muscle biopsies were taken and preserved in a deep freezer.

From the muscle samples, total cellular DNA was extracted using a genomic DNA extraction kit (Bioneer Co., Korea). The cytochrome *b* gene was PCR-amplified, using primers L14724 and H15915, designed by Irwin *et al.* (1991), and PCR thermal cycle for cytochrome *b* gene was as follows: 94°C for 5 min; 94°C for

Table. List of the specimens analysed for the complete cytochrome *b* gene sequences (1140 bp). Locality numbers correspond to those in Fig. 1. Haplotype codes correspond to those in Fig. 2.

Specimen No.	Haplotype code	Sampling locality	Locality number	Accession No.	Source
2560	Bu01Korea	Korea, Busan, 35°09'N, 129°07'E	1	KF144152	This study
2471	SoOd01Korea	Korea, Mt. Sobaek, 36°54'N, 128°27'E	2	KF144159	This study
2472	So02Korea			KF144154	This study
2473	So03Korea			KF144155	This study
2474	So04Korea			KF144156	This study
2475, 2477, 2479, 2480	So05Korea			KF144157	This study
				KF144157	
				KF144157	
2476, 2478	So06Korea			KF144158	This study
		KF144158			
2109	SoOd01Korea	Korea, Mt. Odae, 37°47'N, 128°32'E	3	KF144159	This study
2481	Se01Korea	Korea, Mt. Seolak, 38°06'N, 128°28'E	4	KF144153	This study
2556	V101FERussia	Russia, Far East, Vladivostok, 43°08'N, 131°54'E	5	KF144160	This study
2557	V102FERussia			KF144161	This study
2558	V103FERussia			KF144162	This study
2559	V104FERussia			KF144163	This study
	FJ814020Taiwan	Taiwan, Taichung, 23°46'N, 121°02'E	6	FJ814020	GenBank
	DQ630395Tsushima	Japan, Tsushima Island, 34°25'N 129°20'E	7	DQ630395*	GenBank
	AY843447Tsushima			AY843447**	
	AB077078Jeju	Korea, Jeju Island, 33°30'N, 126°32'E	8	AB077078	GenBank
	HQ993055Jeju			HQ993055	
	HQ709234Jeju			HQ709234	
	AB077079Korea	Korea, Kyengju, 35°50'N, 129°13'E	9	AB077079	GenBank
	AB077075Ullung	Korea, Ullung Island, 37°29'N, 130°54'E	10	AB077075	GenBank
	AB077076Ullung			AB077076	
	AB077081FERussia	Russia, Far East, Putyatin Island, 42°50'N, 132°25'E	11	AB077081	GenBank
	AB077278FERussia	Russia, Far East, Popov Island, 42°57'N, 131°43'E	12	AB077278	GenBank
	EU742594FERussia	Russia, Far East, Lazo Nature Reserve, 45°20'N, 136°10'E	13	EU742594	GenBank
	EU742593FERussia			EU742593	
	EU742584ERussia	Russia, Buryatia Republic, 53°48'N, 109°20'E	14	EU742584	GenBank
	EU742585ERussia			EU742585***	
	EU742590Mongolia	Mongolia, West Khingan, 47°15'N, 119°32'E	15	EU742590	GenBank
	EU742589Mongolia			EU742589	

* partial sequences (1117 bp),

** partial sequences (998 bp),

*** partial sequences (1132 bp).

1 min, 57°C for 1 min, and 72°C for 1 min (30 cycles); and 72°C for 5 min. The amplified products were purified using a DNA PrepMate kit with a silica-based matrix at Bioneer Co. (Cheongju, Korea) for the removal of primers and the unincorporated nucleotides. Sequencing of the purified PCR products was carried out using an automated DNA Sequencer (Perkin Elmer 377) at Macrogen Co. (Seoul, Korea).

The complete 17 cytochrome *b* sequences from mainland Korea and Far Eastern Russia were obtained and compared to the corresponding 17 sequences of *C. shantungensis* from GenBank, as listed in Table.

Sequence alignments, model selections, and tree constructions with 1000 bootstrapped replications were conducted using MEGA5 (Tamura *et al.*, 2011): the Jukes-Cantor (JC) model, which showed the lowest Bayesian information criterion score, was chosen as the best model for our data by the program, and maximum likelihood tree was constructed using the JC model. *Crocidura dsinezumi* (AB077276) and *Sorex caecutiens* (JX192950) were used as outgroups.

Results

From 17 complete cytochrome *b* sequences (1140 bp) of mainland Korea and Far Eastern Russia *C. shantungensis*, 12 haplotypes were identified, as listed in the Table. Within the 29 cytochrome *b* haplotypes of *C. shantungensis* (12 haplotypes from this study and 17 haplotypes from GenBank), 66 sites (5.78%) were variable and 42 sites (3.68%) were parsimony informative.

On the maximum likelihood tree with the 29 cytochrome *b* haplotypes from *C. shantungensis* (Fig. 2) three subgroups were recognized: Gp 1 (Jeju clade) with three Jeju haplotypes; Gp 2 (Tsushima clade) with two Tsushima haplotypes; and Gp 3 (Taiwan-Ullung-continental East Asian clade) with 24 haplotypes from Mongolia, Buryatia, Primorye (Far Eastern Russia), mainland Korea, Taiwan, and Ullung. Average JC distances within the Gp 1 and the Gp 3 were 0.53 and 0.64%, respectively.

In addition, the Jeju clade (Gp 1) was divergent from neighboring mainland Korean population (Gp 3, in part), with average JC distance of 1.94% and 15 fixed site differences (site nos. 67, 126, 127, 151, 321, 411, 516, 561, 618, 642, 732, 771, 945, 1083, and 1122), and the Tsushima clade (Gp 2) was divergent from the latter (Gp 3, in part), with average distance of 2.08% and 14 fixed site differences (site nos. 126, 243, 411, 468, 498, 618, 732, 771, 822, 924, 1041, 1087, 1107, and 1122): all of the 15 fixed site differences between Jeju and mainland Korean populations were resulted from transition, and only one of the 14 fixed site differences between Tsushima and mainland Korean populations was from transversion.

Additionally, the Jeju clade (Gp 1) was divergent from the Taiwan-Ullung-continental East Asian clade (Gp 3), with average JC distance of 1.82%, and the Tsushima clade (Gp 2) was divergent from Taiwan-

Ullung-continental East Asian clade (Gp 3), with average JC distance of 1.78%.

Discussion

Insular populations should diverge over time (morphologically and genetically) from populations of respective mainland species (Johnson *et al.*, 2000). Insular Jeju haplotypes of *C. shantungensis* was distinct in the present study with nine complete cytochrome *b* haplotypes from mainland Korea (Fig. 2) and previous researches with one complete cytochrome *b* haplotype from mainland Korea (Ohdachi *et al.*, 2004; Dubey *et al.*, 2006; Bannikova *et al.*, 2009). Additionally, one Jeju haplotype was divergent from other haplotypes of *C. shantungensis* in Far Eastern Russia, mainland Korea, and Ullung, with about 1.0-1.5% differences by complete or partial (402 bp) sequence analyses (Ohdachi *et al.*, 2004). We found that the Jeju clade (Gp 1) was one of three distinct clades, and that it was divergent from neighboring mainland Korean population (Gp 3, in part), with average JC distance of 1.94% and 15 fixed site differences, indicating that distinctiveness of the Jeju population was recognized more evidently from this study.

Other insular Tsushima haplotypes of *C. shantungensis* were distinct with nine complete cytochrome *b* sequences from mainland Korea (this study: Fig. 2) and with one complete cytochrome *b* haplotype from mainland Korea (previous researches: Dubey *et al.*, 2006; Bannikova *et al.*, 2009). However, from this study the Tsushima clade (Gp 2) was one of three distinct clades, and it was divergent from the mainland Korean population (Gp 3, in part), with average JC distance of 2.08% and 14 fixed site differences, indicating that distinctness of the Tsushima *C. shantungensis* was revealed more clearly from this study.

On the other hand, other insular Taiwan and Ullung haplotypes and continental haplotypes from Mongolia, Buryatia, and Primorye formed the Taiwan-Ullung-continental East Asian clade (Gp 3) together with the mainland Korean haplotypes, as shown in Figure 2. The Buryatia haplotype was reported as a distinct subgroup (Bannikova *et al.*, 2009), but they used no Taiwan haplotypes and only one mainland Korean haplotype for the comparison. In addition, Ohdachi *et al.* (2004) noted the distinctiveness of Taiwan or Jeju *C. shantungensis* from other *C. shantungensis* in Ullung, mainland Korea, and Primorye, with genetic distances of 1.5%-2.4%, but they used partial (402 bp) cytochrome *b* sequences. Thus, we considered that the present analysis, including complete cytochrome *b* sequences from Taiwan (one haplotype) and mainland Korea (eight haplotypes), is more informative than the previous researches, and concluded that two insular (Taiwan and Ullung) populations and one continental population (from Mongolia to Far Eastern Russia) were not divergent from adjacent mainland Korean population.

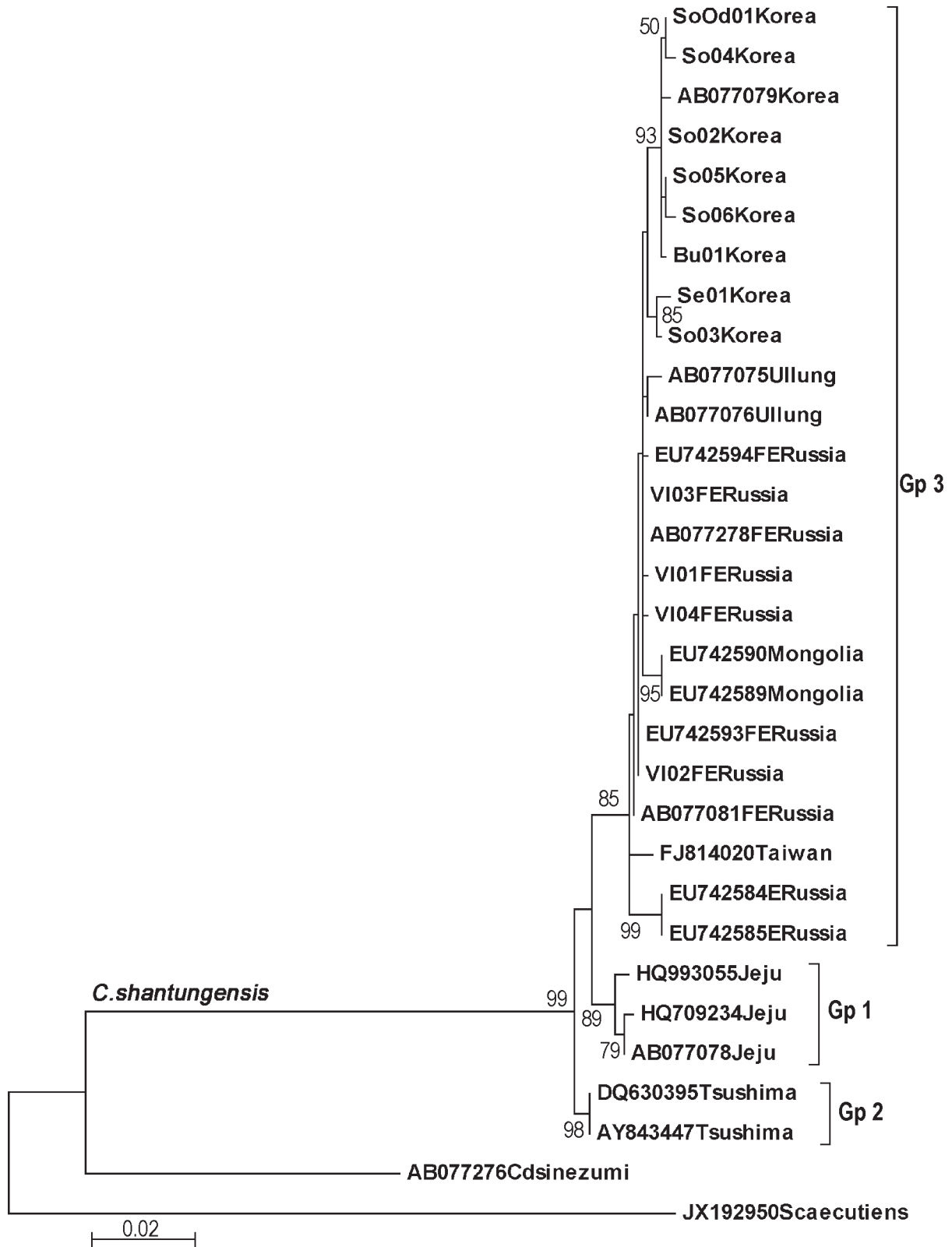


Figure 2. Maximum likelihood tree with 29 cytochrome *b* haplotypes of *Crocidura shantungensis*. Specimen number and haplotype codes are listed in Table. The bootstrap values >50% are reported at the internodes, and *C. dsinezumi* (AB077276) and *Sorex caecutiens* (JX192950) were used as outgroups.

A subspecies is an aggregate of phenetically similar populations of a species differing taxonomically from other populations of that species (Mayr & Ashlock, 1991), although it was advocated that a classification should reflect all available characters distributed as widely and evenly as possible over the organisms studied (Huelsenbeck *et al.*, 1996). From a morphometric analysis Jeju (*C. s. quelpartis*) and Taiwan (*C. s. hosletti*) populations were average larger than mainland Korean and Tsushima populations (Motokawa *et al.*, 2003), and *C. s. hosletti* was considered as a synonym of *C. s. quelpartis* (Hutterer, 2005). However, in this cytochrome *b* sequence analysis (Fig. 2) Jeju *C. shantungensis* forms a distinct Jeju clade (Gp 1), and it was different from Taiwanese haplotype, indicating that Jeju *C. s. quelpartis* is a subspecies with distinct morphology and cytochrome *b* sequences.

In addition, from this study (Fig. 2) Tsushima *C. shantungensis* forms another distinct Tsushima clade (Gp 2), although *C. shantungensis* from Tsushima was considered as *C. s. shantungensis* (Hutterer, 2005) or *C. s. coreae* (Thomas, 1908). Additionally, Taiwan, Ullung, Mongolia, eastern Russia, and mainland Korean populations formed another Taiwan-Ullung-continental East Asian clade (Gp 3) in this study (Fig. 2), although *C. shantungensis* from Mongolia, eastern Russia, mainland Korea, Ullung, and Tsushima were also considered as *C. s. shantungensis* (Hutterer, 2005). Thus, our results appeared not to support the current subspecies classification of *C. shantungensis* (i.e., *C. s. quelpartis* from Jeju and Taiwan and *C. s. shantungensis* from other distribution regions), and do support the former classification of Jeju population as *C. s. quelpartis*. We proposed further systematic analysis with additional specimens from East Asia to confirm our findings in this study.

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