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MOLECULAR PHYLOGENETICS OF *CROCIDURA* SHREWS (INSECTIVORA) IN EAST AND CENTRAL ASIA

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Phylogenetic relationships among 8 species of white-toothed shrews (Crocidurinae, Mammalia) in East and Central Asia were evaluated based on mitochondrial cytochrome-*b* gene sequences. The taxon formerly regarded as *Crocidura suaveolens* in East Asia phylogenetically is distinct from that of "true" *C. suaveolens* in Europe, suggesting that specimens in East Asia should be considered a distinct species, *C. shantungensis*. All shrews from Central Asia were regarded as *C. sibirica*, although phylogenetic comparison with the unsampled *C. gmelini* is needed to confirm its taxonomic status. *C. shantungensis*, *C. suaveolens*, and *C. sibirica* formed a well-supported monophyletic group. *C. dsinezumi*, *C. lasiura*, *C. kurodai*, and *C. watasei* also formed a well-supported monophyletic group. Within *C. dsinezumi*, there were 2 clusters, referable to western and eastern Japan. Based on low genetic divergence, *C. dsinezumi* on Cheju and Hokkaido islands appear to be the result of a recent introduction from western Japan and northern-eastern Honshu, respectively.

Key words: *Crocidura dsinezumi*, *Crocidura shantungensis,* cytochrome-*b*, human introduction, white-toothed shrew

RUNNING HEAD: Phylogeny of *Crocidura* shrews in Asia

Approximately 340 species are assigned to the family Soricidae (Wolsan and Hutterer 1998), although an extensive taxonomic revision is necessary. The Soricidae is divided into 2 subfamilies, Crocidurinae (220 species) and Soricinae (120 species). The former is distributed mainly in tropical-temperate area in the Old World (Africa and Eurasia), whereas the latter occurs in the Holarctic region.

Approximately 8-10 species of crocidurine shrews (white-toothed shrews) are known from East Asia (Russian Far East, northeastern China, Korea, Japan, Taiwan, and neighboring small islands). These include *Suncus murinus*, *Crocidura attenuata*, *C. dsinezumi*, *C. kurodai*, *C. lasiura*, *C. orii*, *C. suaveolens*, and *C. watasei* (Abe et al. 1994; Corbet and Hill 1991; Motokawa 1998; Motokawa et al. 2000; Wolsan and Hutterer 1998), whereas Jiang and Hoffmann (2001) included *C. horsfieldii* and *C. dsinezumi* from Taiwan and Fang and Lee (2002) treated *C. kurodai* as a subspecies of *C. tadae*. Some researchers (e.g., Hoffmann 1996; Jiang and Hoffmann 2001; Motokawa 1999) recognized specimens of *C. suaveolens* from East Asia as *C. shantungensis*, a species distinct from *C. suaveolens* in central-western Eurasia.

From Central Asia (western China, central Siberia and Mongolia), Yudin (1971), Wolsan and Hutterer (1998), and Zhang et al. (1997) listed *Crocidura sibirica* (= *C. leucodon sibirica*) and *C. suaveolens*. However, Hoffmann (1996) and Jiang and

Hoffmann (2001) recognized *C. gmelini* in addition to *C. sibirica* and excluded *C. suaveolens* from Central Asia.

Motokawa et al. (2000, 2001) and Han et al. (2002) investigated phylogenetic relationships of East Asian crocidurine shrews, using 402-base pair (bp) sequences of the mitochondrial cytochrome-*b* gene (mtDNA *Cytb*); however, they could not clarify the phylogenetic position of *C. shantungensis*, which they regarded as *C. suaveolens*, because no samples of *C. suaveolens* from western Eurasia were analyzed. Therefore, it is necessary to investigate the phylogenetic relationship of *C. shantungensis* ("*C. suaveolens*" in East Asia) and *C. suaveolens* in western Eurasia to address this taxonomic problem. To clarify the problem, a molecular phylogenetic investigation is needed, specifically a comparison of samples from the eastern and western ranges of "*C. suaveolens*" and related species.

Crocidurine species also are found on some islands in East Asia. Han et al. (2002) suggested, based on degree of divergence of mtDNA *Cytb* sequences, that *C. dsinezumi* on Cheju Island near the Korean Peninsula was accidentally introduced from western Japan as a result of human activity. Therefore, to investigate the biogeography of crocidurine shrews in Asia, it is necessary to compare genetic divergence in order to determine whether shrews naturally inhabit islands or were recently introduced by humans.

In this paper, we analyzed the molecular phylogenetic relationships of crocidurine shrews in East and Central Asia using complete (1,140 bp) and partial (402 bp) sequences of the mtDNA *Cytb* gene. In addition, we discuss several taxonomic and phylogeographic problems.

Materials and Methods

DNA analysis.--Sequences from 60 individuals, including 8 species from East and Central Asia, were examined (Appendix I; Fig. 1). Total DNA was extracted from liver or muscle tissues preserved in 70--95% ethanol by the conventional phenol-chloroform-proteinase *K* method (Maniatis et al. 1982). Fragments (approximately 1,250 bp) of the mtDNA *Cytb* gene were amplified by the polymerase chain reaction (PCR) method using the following primer set: L14724 andH15915 (Irwin et al. 1991) or L14734/H15985 (Ohdachi et al. 2001). When needed, nested PCR was conducted using primer sets L14734/H15392 (Ohdachi et al. 2001), and L15330 (5'-GTGCCGCACTCGCAGGAGT-3')/H15985. Conditions for PCR followed Iwasa et al. (2000) or Ohdachi et al. (2001). Among 44 samples examined, 38 individuals were sequenced for 1,140 bp and the remaining 6 specimens were examined for 402 bp (Appendix I). In addition, we included 16 mtDNA *Cytb* sequences from the DNA databases GenBank/EMBL/DDBJ (Appendix I).

Phylogenetic analysis.--Complete sequences (1,140 bp) were used in the 1st phylogenetic analysis. *Sorex caecutiens* was used as an outgroup. When ≥2 identical haplotypes were found, a single representative haplotype was used to reduce computation time. Second, to increase the number of individuals and locations available for analyses, 402-bp data sets were used for samples of *C. dsinezumi* and a group of *C. shantungensis*, *C. suaveolens*, and *C. sibirica*. In these analyses, all haplotypes were used to generate trees even when ≥2 identical haplotypes were found. *C. watasei* and *C. lasiura* were used as outgroups for *C. dsinezumi* and the *C. shantungensis-C. suaveolens-C. sibirica* group, respectively.

To infer phylogenetic relationships among shrews, maximum likelihood trees were estimated by the quartet-puzzling method using TREE-PUZZLE ver.4.0.2 software (Strimmer and von Haeseler 1996). According to the hierarchical likelihood ratio tests estimated by MODELTEST ver. 3.06 (Posada and Crandall 1998) with PAUP* ver. 4.0b10 (Swofford 2002), the substitution model by Tamura and Nei (1993) with gamma distribution + invariable sites (TrN+G+I model) was selected for the 1,140-bp data set. For the 402-bp data sets of *C. shantungensis-C. suaveolens-C. sibirica* and *C. dsinezumi*, the model by Tamura and Nei (1993) with gamma distribution (TrN+G model) and the model by Hasegawa et al. (1985) with gamma distribution (HKY+G model) were selected, respectively. The number of gamma categories was 8 (Yang 1996)

and puzzling steps was 10,000. Confidence of nodes was assigned by a support value from the quartet-puzzling analysis (Strimmer and von Haeseler 1996).

Results

The transition/transversion ratio estimated from complete sequences (1,140 bp) was 8.19 (±1.19 *SEI*), pyrimidine/purine (Y/R) transition parameter was 2.72 (±0.48 *SE*), and fraction of invariable site was 0.60 (±0.02 *SE*). The gamma distribution parameter (alpha) was 2.44 (±0.77 *SE*), total rate heterogeneity 0.71 (±0.08 *SE*), percentage of unresolved quartets 3.5%, and -ln L 4886.81 (without a molecular clock). In the analysis of the 402-bp data set for *C. shantungensis*/*C. suaveolens*/*C. sibirica*, the transition/transversion ratio was 9.13 (±2.30 *SE*), Y/R transition parameter 3.26 (±1.13 *SE*), gamma distribution parameter 0.49 (±0.18 *SE*), percentage of unresolved quartets 22.3%, and -ln L 1057.05. In the analysis of the 402-bp data set for *C. dsinezumi*, the transition/transversion parameter was 9.94 (±3.80 *SE*), gamma distribution parameter alpha 0.10 (±0.04 *SE*), unresolved quartets 40.1%, and -ln L 781.92.

In the maximum likelihood analysis (Fig. 2), *C. dsinezumi*, *C. lasiura*, *C. kurodai*, and *C. watasei* were monophyletic with a high degree of confidence (support value 96%). *C. shantungensis*, *C. suaveolens*, and *C. sibirica* also formed a strong (100%) monophyletic group (Fig. 2), but *C. shantungensis*

in East Asia was phylogenetically distinct from *C. suaveolens* in Europe and *C. sibirica* in Central Asia (Figs. 2 and 3). In addition, all samples from Central Asia examined were included in a single cluster, recognized as *C. sibirica* (Figs. 2 and 3).

Within the *C. shantungensis* cluster, shrews from Cheju Island or Taiwan (Taichung) demonstrated some genetic divergence (1.5-2.4%) from those from other locations (Fig. 3). Within *C. dsinezumi*, there were 2 unambiguous clusters (Figs. 2 and 4). The geographic distributions of the clusters coincided with samples of locations 1—12 in eastern Japan and locations 13—19 in western Japan including Cheju Island (Figs. 1 and 4). Demarcation of range between the populations of the 2 clusters exists in central Honshu (Figs. 1 and 4). Three specimens of *C. dsinezumi* from Cheju Island showed little or no genetic divergence (< 0.3%) from those from Kyushu or western Honshu (Figs. 2 and 4). Four individuals of *C. dsinezumi* from Hokkaido shared an identical sequence, and showed little or no genetic difference (< 0.2%) from shrews from northern or eastern Honshu (Figs. 2 and 4).

Discussion

Systematics of C. suaveolens/C. shantungensis/C. sibirica.--Jiang and Hoffmann (2001) demonstrated that *C. shantungensis* and *C. suaveolens* were morphologically distinct and should be treated as independent species. In the present phylogenetical analyses, *C. shantungensis* in East Asia also was

phylogenetically distinct from *C. suaveolens* in Europe (Figs. 2 and 3). Both morphological and phylogenetical analyses indicated that these 2 taxa should be treated as separate species. Thus, we recommend that the shrews in East Asia be considered as *C. shantungensis*, as suggested by Hoffmann (1996), Motokawa (1999), and Jiang and Hoffmann (2001).

In Xinjiang, China, we obtained 3 morphologically different types of *Crocidura* in 1999 and 2000. Based on Yudin's (1971) univariate morphological criteria, larger specimens from Mosuowan (location 35; Fig. 1) were regarded as *C. sibirica* and the smaller ones from Qarqan (location 33) as *C. suaveolens*. A specimen from Korla (location 34) showed the intermediate morphotype between the 2 "species" by Yudin (1971). The other specimens from Central Asia were classified as *C. sibirica*. However, in our analyses (Figs. 2 and 3), all samples from Central Asia (locations 33-37) belonged to a single cluster and should be recognized as *C. sibirica*. Thus, Yudin (1971) probably recognized small specimens of *C. sibirica* as *C. suaveolens*, and his morphological criteria may be inappropriate.

Jiang and Hoffmann (2001) concluded from a multivariate morphological analysis that *C. suaveolens* did not occur in Xinjiang and the eastern end of its range was Kazakhstan, northeastern Pakistan, and northeastern Afghanistan (it was described as northwestern Afghanistan but their figure indicated northeastern Afghanistan). Further, Jiang and

Hoffmann (2001) listed *C. gmelini* from Central Asia including Xinjiang in addition to *C. sibirica* without comparison. Therefore, it is possible that *C. sibirica* might be a synonym of *C. gmelini*. It is necessary to clarify the phylogenetical and morphological relationships between the specimens of *C. gmelini* they used and those of *C. sibirica* examined in the present study to determine how many species exist in Central Asia, especially in Xinjiang.

Intraspecific variation of C. dsinezumi.--Crocidura dsinezumi clearly was divided into 2 clusters: western and eastern Japanese clusters (Figs. 2 and 4). A separation in the distributions between populations comprising the 2 clusters occurs in central Japan (Fig. 1), possibly with a narrow or even no transitional zone. However, there is no obvious geographic or climatic barrier between the ranges of shrews from western and eastern Japan. Similar phenomena of genetic structure between the western and eastern parts of Honshu are known for some vertebrates (within species or between closely related species): chromosome races of the greater Japanese wood mouse *Apodemus speciosus* (Saitoh and Obara 1988; Tsuchiya 1974; Tsuchiya et al. 1973), Y chromosomal variation of Smith's red-backed voles *Eothenomys smithii* (Ando et al. 1988; Iwasa et al. 1999), autosomal variation (Harada et al. 2001) and mtDNA haplotype (Shinohara 2001) of the greater Japanese shrew mole *Urotrichus talpoides*, and mtDNA haplotype of the Japanese

minnows *Pseudorasbora* (Watanabe et al. 2000). Common biogeographic events that might underlie these distribution patterns remains unknown.

It also is interesting that shrews from 2 offshore islands (Chiburi and Yaku islands, locations 14 and 18) formed a monophyletic group (Fig. 4), although these 2 islands are distantly located (Fig. 1). An ancient haplotype might have been retained on those offshore islands whereas new haplotypes might have replaced old ones on Kyushu and Honshu islands.

Human introduction of shrews.--*Crocidura* shrews are sometimes unintentionally introduced onto islets by ships (Vogel et al. 1986; Vogel and Sofianidou 1996). Cheju Island geologically was separated from Kyushu about 150,000 years ago, whereas its separation from the Korean Peninsula was 12,000--16,000 years ago (Ohshima 1990). Therefore, it is expected that there should be more genetic divergence between populations of shrews from Cheju and western Japan than between those from Cheju and the Korean Peninsula, if shrews are naturally distributed. In reality, *C. shantungensis* on Cheju Island genetically are divergent from the shrews from the Korean Peninsula and adjacent islands (about 1.0-1.5% difference in *Cytb* gene--Figs. 2 and 3; unique restriction site pattern of the nuclear ribosomal DNA, Iwasa et al. 2001), whereas there is little or no genetic divergence in *C. dsinezumi* between Cheju Island and Kyushu or western Japan (< 0.3% in *Cytb* gene, Figs.

2 and 4). In addition, fauna of terrestrial mammals on Cheju Island is generally part of that of the Korean Peninsula, and it is unusual that this island includes a species which is an element of Japanese mammalian fauna, i.e. *C. dsinezumi*. These findings suggest that *C. shantungensis* naturally is distributed on Cheju Island whereas *C. dsinezumi* was recently introduced by humans from Kyushu or western Japan. This argument was 1st proposed by Han et al. (2002) using the 402-bp data set. The present study supports this hypothesis. In contrast, it is difficult to conclude whether or not *C. shantungensis* was distributed naturally on Ullung, Popov, and Putjatin islands, as their level of genetic divergence from the Korean Peninsular individuals is negligible (Figs. 2 and 3). In addition, it would be desirable to compare phylogenetic relationships among specimens from those islands and mainland Russia and China to estimate the biogeographic origin of shrews on these islands.

It is unknown if *C. dsinezumi* naturally occurs in Hokkaido or whether it recently was introduced from trading and/or immigration ships. Dobson and Kawamura (1998) suggested *C. dsinezumi* was introduced into Hokkaido from Honshu by humans whereas Motokawa (1998) and Ohdachi (1999) suggested it was naturally distributed. Hokkaido was separated from Honshu about 140,000 years ago (Ohshima 1990). If the shrews were distributed naturally in Hokkaido, there should be genetic divergence between populations of Hokkaido and Honshu. In addition, the

Tsugaru Strait between Honshu and Hokkaido is a prominent biogeographic line, Blakiston's line, between which fauna, flora or phylogeny of many organisms drastically differentiates (Ohdachi 1999). In the present study, however, the phylogenetic position of Hokkaido *C. dsinezumi* showed little or no divergence from northern or eastern Honshu (Figs. 2 and 4), suggesting that *C. dsinezumi* recently was introduced by humans from northern or eastern Honshu. The extremely rare occurrences of collection records from the southwestern parts of Hokkaido (Abe et al. 1987; Nakata 1981) might support the present conclusion.

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Figure legends

Fig. 1. Collection localities for specimens of *Crocidura* examined in this study. Locality numbers correspond to those in Appendix I. Location of Vienna (Austria) is not shown on this map. The distribution of shrews was obtained from the literature (Abe et al. 1994; Jiang and Hoffmann 2001; Motokawa 1999; Nesterenko 1999; Stroganov 1957; Wolsan and Hutterer 1998; Won 1968; Yudin 1971; Zhang et al. 1997). A "?" denotes uncertain boundaries of distribution.

Fig. 2. Tree generated from maximum likelihood analysis (TrN+G+I model) of *Crocidura* shrews based on 1,140 base pairs of the mitochondrial cytochrome-*b* gene sequences. Numbers at nodes are quartet-puzzling support values (%).

Fig. 3. Tree generated from maximum likelihood analysis (TrN+G model) of *Crocidura shantungensis*, *C. suaveolens*, and *C. sibirica* based on 420 base pairs of the mitochondrial cytochrome-*b* gene sequences. Numbers at nodes are quartet-puzzling support values (%).

Fig. 4. Tree generated from maximum likelihood analysis (HKY+G model) of *Crocidura dsinezumi* based on 420 base pairs of the mitochondrial cytochrome-*b* gene sequences. Numbers at nodes are quartet-puzzling support values (%).

APPENDIX I

Specimens examined—All specimens are listed with specimen codes in phylogenetic trees, localities with locality numbers in parentheses, reference numbers, and accession numbers of GenBank/EMBL/DDBJ databases for each species.

Locality numbers correspond to those in Fig.1. Reference numbers: 1, Present study; 2, Shinohara et al. (unpublished); 3, Motokawa et al. (2000); 4, Ohdachi et al. (unpublished); 5, Ohdachi and Ablimit (unpublished). Full 1,140 base pairs sequences were used except for specimens with asterisks in specimen code. Partial 402 base pairs sequences were available for those with asterisks.

Crocidura

dsinezumi—Dsi (Hokkaido)-1: Shimamaki, Hokkaido (1), ref.

1, AB077059. Dsi (Hokkaido)-2: Shimamaki, Hokkaido (1), ref.

1, AB077270. Dsi (Hokkaido)-3: Sapporo, Hokkaido (2), ref.

1, AB077271. Dsi (Hokkaido)-4: Shizunai, Hokkaido (3), ref.

1, AB077272. Dsi (Aomori): Aomori Pref., Honshu (4), ref.

1. AB077060. Dsi (Iwate)-1: Iwate Pref., Honshu (5), ref.

1, AB077273. Dsi (Iwate)-2*: Iwate Pref., Honshu (5), ref.

1, AB077146. Dsi (Akita)-1: Akita Pref., Honshu (6), ref.

1, AB077061. Dsi (Akita)-2: Akita Pref., Honshu (6), ref.

2, AB076837. Dsi (Sado Is.): Sado Is. (7), ref.

1, AB077062. Dsi (Niigata): Niigata Pref., Honshu (8), ref.

1, AB077274. Dsi (Fukushima)*: Fukushima

Pref., Honshu (9), ref.1, AB077147. Dsi (Gunma): Gunma

Pref., Honshu (10), ref. 1, AB077063. Dsi (Toyama)*: Toyama

Pref., Honshu (11), ref.

3, AB066248. Dsi (Gifu)*: Gifu Pref., Honshu (12), ref.

3, AB066249. Dsi (Wakayama)-1: Wakayama Pref., Honshu (13), ref.

1, AB077064. Dsi (Wakayama)-2: Wakayama Pref., Honshu (13), ref.

1, AB077065. Dsi (Chiburi Is.)*: Chiburi Is. (14), ref.

3, AB066250. Dsi (Tokushima): Tokushima

Pref., Shikoku (15), ref.

1, AB077066. Dsi (Ehime)-1: Ehime Pref., Shikoku (16), ref.

1, AB077067. Dsi (Ehime)-2: Ehime Pref., Shikoku (16), ref.

1, AB077068. Dsi (Fukuoka)-1: Fukuoka Pref., Kyushu (17), ref.

1, AB077069. Dsi (Fukuoka)-2: Fukuoka Pref., Kyushu (17), ref.

1, AB077275. Dsi (Yaku Is.)*: Yaku Is. (18), ref.

3, AB066251. Dsi (Cheju Is.)-1: Cheju Is. (19), ref.

1, AB077276. Dsi (Cheju Is.)-2: Cheju Is. (19), ref.

1, AB077070. Dsi (Cheju Is.)-3: Cheju Is. (19), ref.

1, AB077277.

Crocidura lasiura--Las (Ussuriisk): Ussuriisk,

Primorye (20), ref. 1, AB077071. Las (Kraskino): Kraskino Vill.,

Primorye (21), ref. 1, AB077072. Las (Odae): Mt.

Odae, South Korea (22), ref. 1, AB077073

Crocidura kurodai--Kur (Xitou)-1: Xitou, Taiwan (23), ref. 4, AB057420. Kur (Xitou)-2: Xitou, Taiwan (23), ref. 4, AB062686.

Crocidura watasei--Wat: Tokunoshima Is. (24), ref. 1, AB077074.

Crocidura shantungensis (="*suaveolens*") --Sha (Tsushima Is.)*: Tsushima Is. (25), ref. 3, AB066257. Sha (Ullung Is.)-1: Ullung Is. (26), ref. 1, AB077075. Sha (Ullung Is.)-2: Ullung Is. (26), ref. 1, AB077076. Sha (Ullung Is.)-3*: Ullung Is. (26), ref. 1, AB077149. Sha (Kagu Is.)*: Kagu Is. (27), ref. 1, AB077150. Sha (Cheju Is.)-1: Cheju Is. (19), ref.1, AB077077. Sha (Cheju Is.)-2: Cheju Is. (19), ref. 1, AB077078. Sha (Cheju Is.)-3*: Cheju Is. (19) ref. 1, AB077151. Sha (Naejang)*: Mt. Naejang, South Korea, ref. 1, AB077152. Sha

(Kyunju): Kyunju, South Korea (29), ref. 1, AB077079. Sha (Putjatin Is.)-1: Putjatin Is. (30), ref. 1, AB077080. Sha (Putjatin Is.)-2: Putjatin Is. (30), ref. 1, AB077081. Sha (Popov Is.)-1: Popov Is. (31), ref. 1, AB077082. Sha (Popov Is.)-2: Popov Is. (31), ref. 1, AB077278. Sha (Taichung)-1*: Taichung, Taiwan (32), ref. 3, AB066258. Sha (Taichung)-2*: Taichung, Taiwan (32), ref. 3, AB066259.

Crocidura sibirica--Sib (Qarqan)-1: Qarqan (Qiemo), Xinjiang (33), ref. 5, AB077083. Sib (Qarqan)-2: Qarqan (Qiemo), Xinjiang (33), ref. 5, AB077084. Sib (Korla): Korla, Xinjiang (34), ref. 5, AB077085. Sib (Mosuowan)-1: Mosuowan, Xinjiang (35), ref. 5, AB077086. Sib (Mosuowan)-2: Mosuowan, Xinjiang (35), ref. 5, AB077087. Sib (Mongolia): Sharga Vill., Mongolia (36), ref. 1, AB077088. Sib (Novosibirsk)-1: Nobosibirsk, central Siberia (37), ref. 1, AB077089. Sib (Novosibirsk)-2: Nobosibirsk, central Siberia

(37), ref. 1, AB077279. Sib (Novosibirsk)-3*: Nobosibirsk, central Siberia (37), ref. 3, AB066260.

Crocidura suaveolens--Sua (Wien)-1: Vienna (Wien), Austria, ref. 1, AB077090. Sua (Wien)-2: Vienna (Wien), Austria, ref. 1, AB077280.

S. caecutiens--S. caecutiens: Yufutsu, Hokkaido, ref. 6, AB028564.

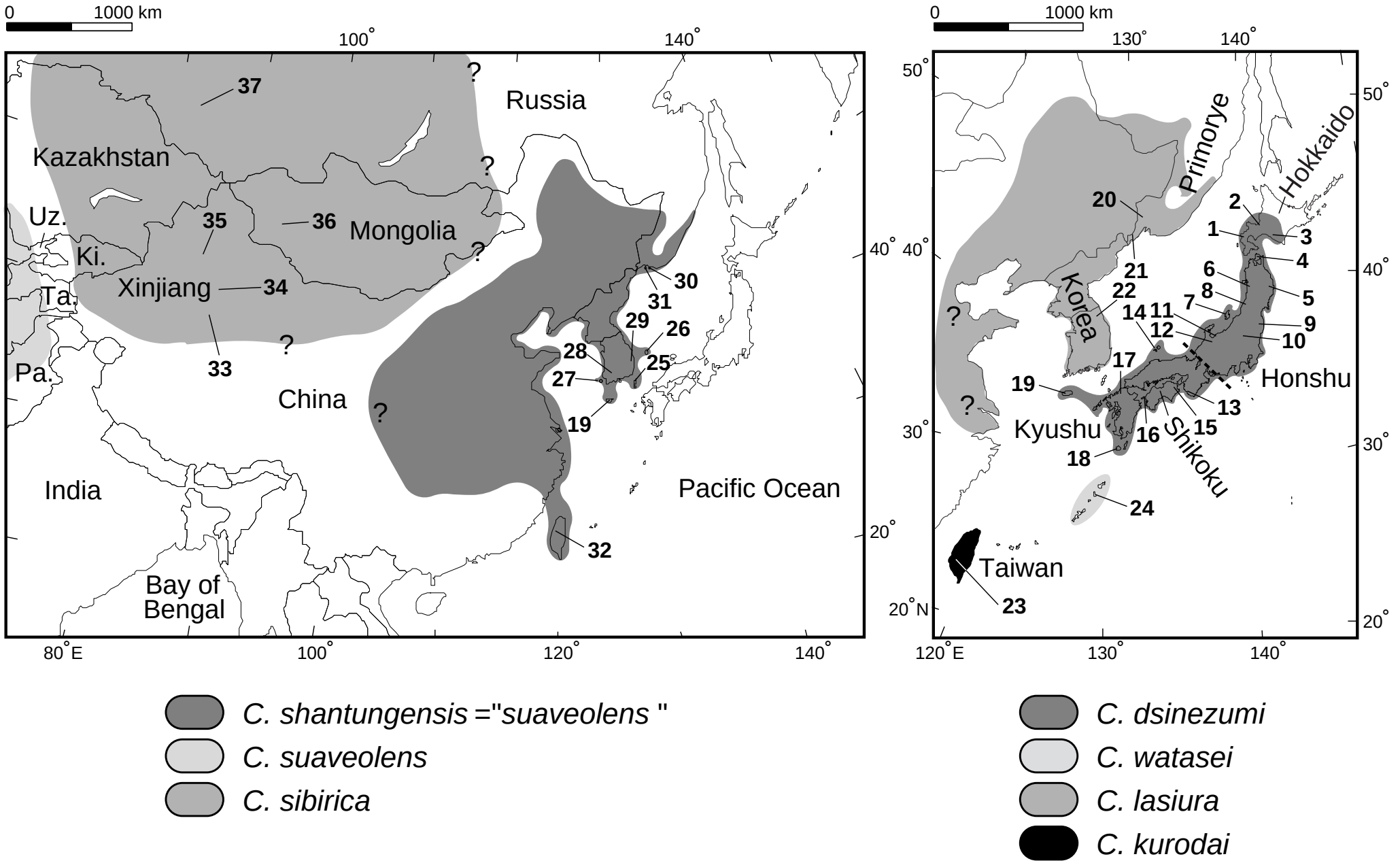

Fig. 1 Ohdachi et al

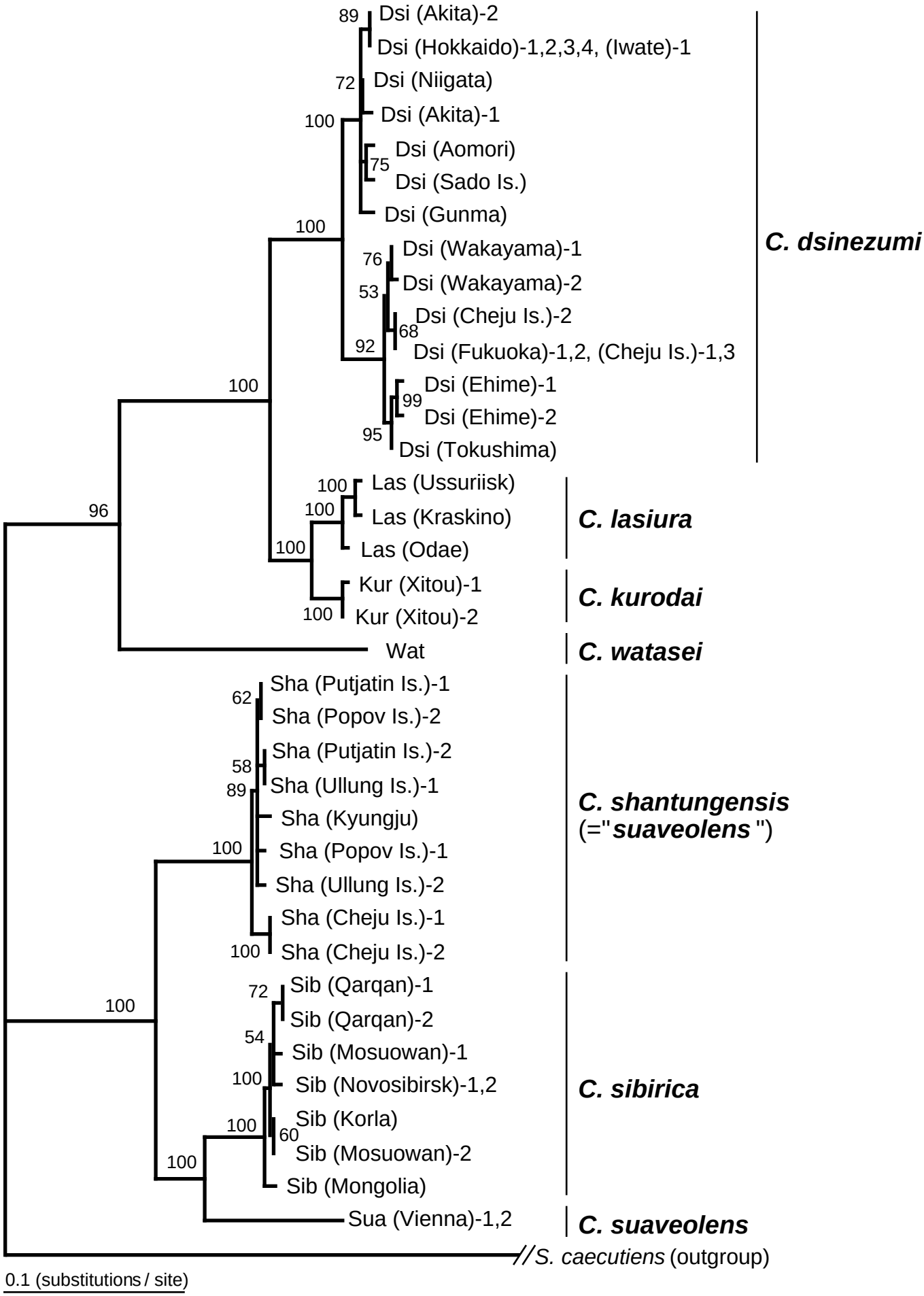

Fig. 2 Ohdachi et al.

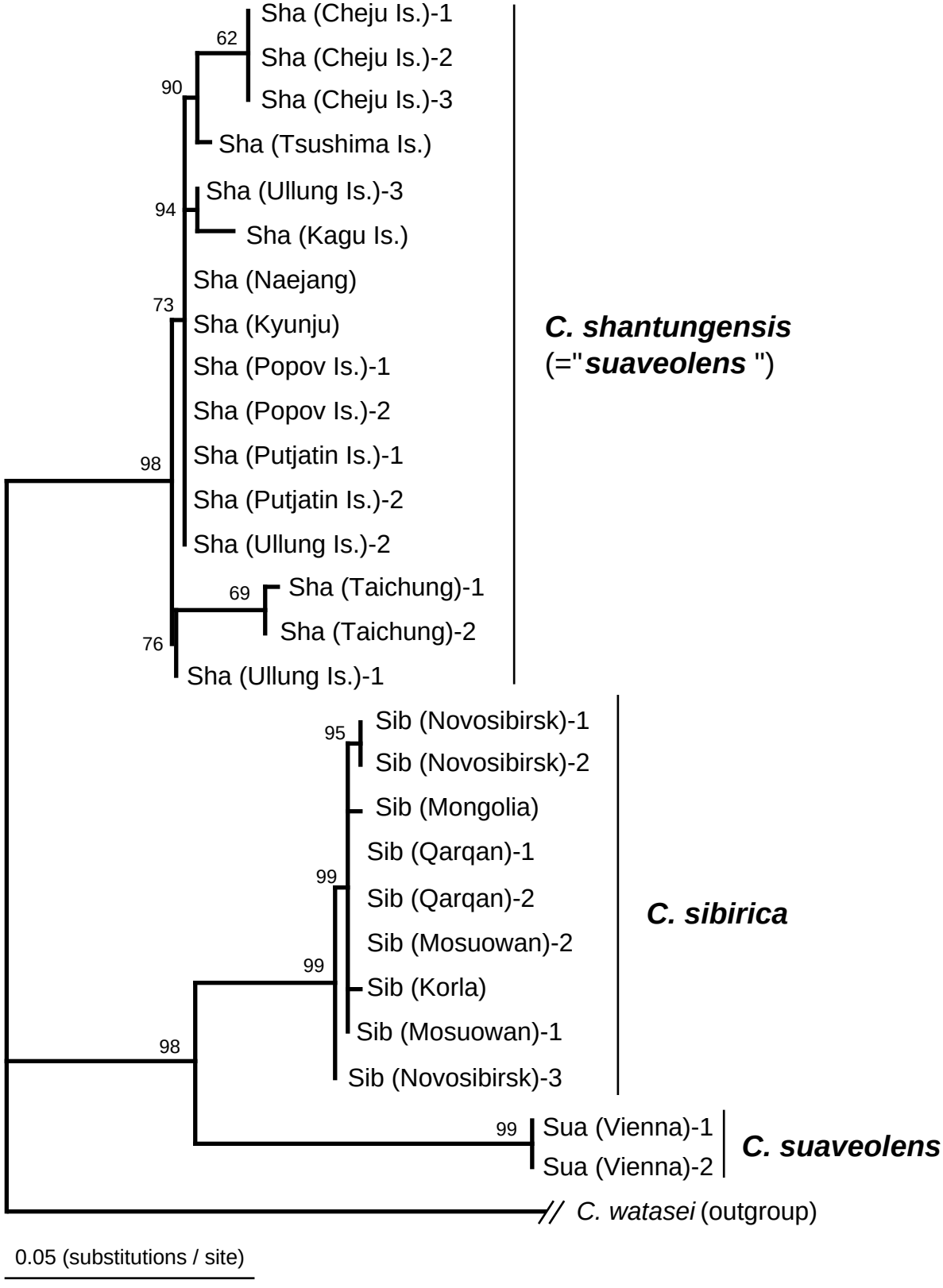

Fig. 3 Ohdachi et al.

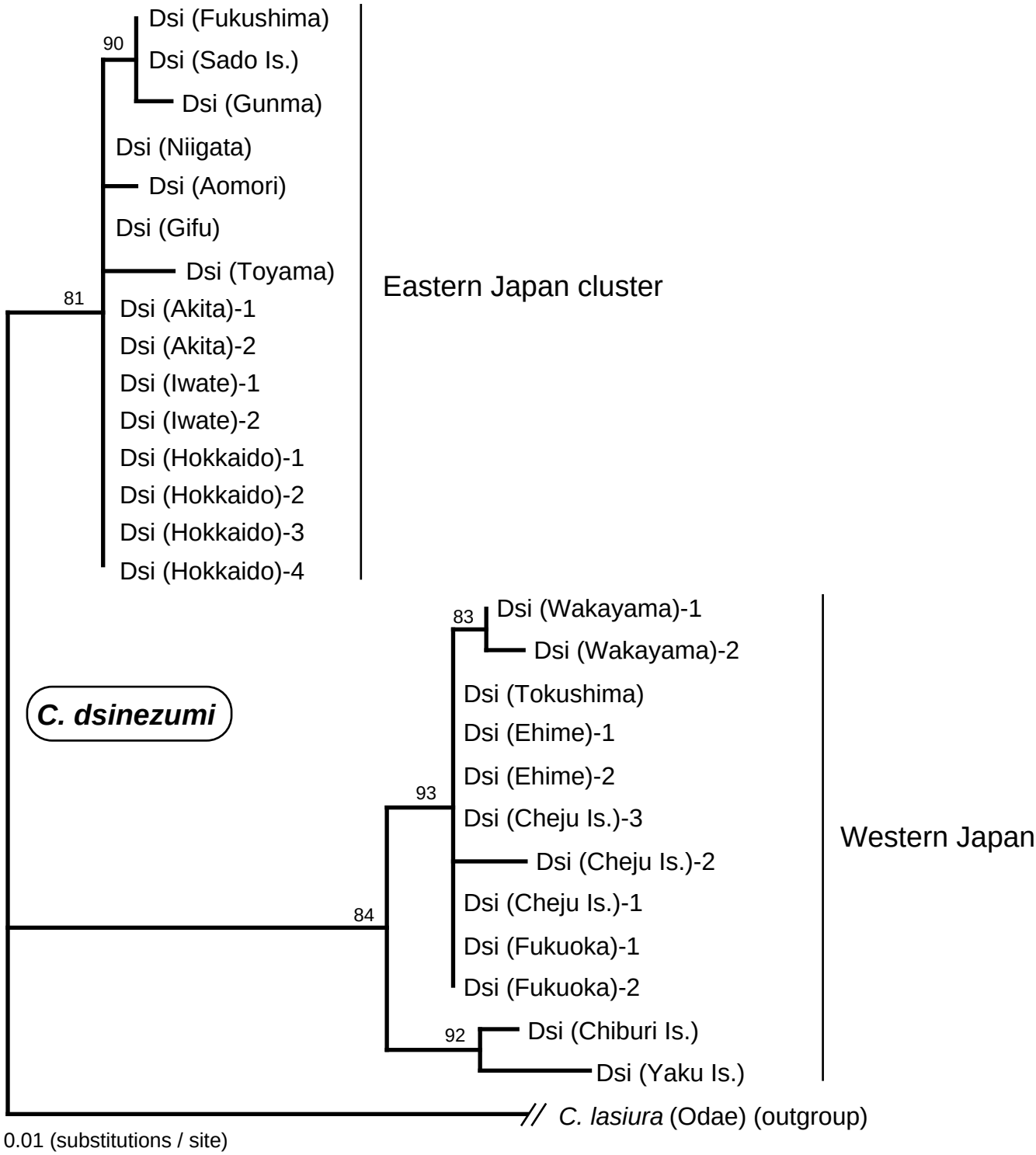

Fig. 4 Ohdachi et al

cluster