Article type: Full Length

A phylogeographic study of the Japanese earthworm, *Metaphire sieboldi* (Horst, 1883) (Oligochaeta: Megascolecidae): inferences from mitochondrial DNA sequences

Yukio MINAMIYA [1]*, Jun YOKOYAMA [2], Tatsuya FUKUDA[3]

[1]: The United Graduate School of Agricultural Science, Ehime University, Monobe, Nankoku 783-8502, JAPAN

[2]: Faculty of Science, Yamagata University, Koshirakawa-machi 1-4-12, Yamagata 990-8560, JAPAN

[3]: Faculty of Agriculture, Kochi University, Monobe Nankoku 783-8502, JAPAN

*: Author for correspondence:

Yukio MINAMIYA

The United Graduate School of Agricultural Science, Ehime University, Monobe, Nankoku 783-8502, JAPAN

Tel: 088-864-2124

e-mail: b0mf025@s.kochi-u.ac.jp

Running title: Phylogeography of *Metaphire sieboldi*

1. Introduction

Phylogeography, the combined analysis of genealogical and geographic data, has become a powerful tool for inferring historical biogeographic events. Patterns of variation in DNA markers have allowed inferences of past biogeographic events on every geographic scale from continental to local [1, 2]. In addition, recent development of molecular techniques has allowed phylogenies to be reconstructed and systematic relationships to be evaluated among species or populations within a given species.

Modern fauna and flora species assemblages have been affected by eradications by glaciers and migration associated with connections and disjunctions of islands resulting from climate changes. After the Japanese archipelago was formed during the early Miocene (approx. 15-20 mya), glaciers did not form during the Quaternary except in alpine areas of northern Japan, thus, across most of Japan, glaciers have not affected the biota [20]. However, climatic changes did dramatically affect Japanese biota by changing the flora; evergreen broad-leaved forests present in warm periods were replaced with deciduous broad-leaved forests or coniferous forests during cold periods [36]. Moreover, land bridges, formed by lowering of the sea level after global cooling, affected the fauna of Japan, as animal populations that were initially divided by

the sea could then cross the land bridges. Honshu, Shikoku, and Kyushu islands of Japan were repeatedly connected and divided by the raising and lowering of the sea level during the glacial period; therefore, the fauna and flora of Shikoku were affected by those of neighbouring areas, including the Kinki and Chugoku regions of Honshu, and Kyushu. Studies of fauna in western Japan such as asian black bear *Ursus thibetanus* [38], sika deer *Cervus nippon* [37] and Japanese salamander *Hynobius boulengeri* [23], have shown that various taxa of Shikoku are partially composed of elements from neighbouring areas.

Earthworms are one of the most important components of the soil biota, and make up the largest contribution to the biomass in the temperate zone. They cannot readily cross seas, rivers, or mountains; therefore, the modes by which they expand their distribution are restricted, and it is uncertain how their present distribution pattern has been formed. The phylogeographic history of earthworms can be inferred from a phylogenetic analysis. Molecular phylogenetic studies of some earthworm groups have greatly increased in number (review in Dupont [11]); most of these studies are concerned with closely related species or variations within species [7-10, 13, 14, 18, 26] but none yet based on primary type specimens nor considering species' synonyms [5]. Recently, some studies have been conducted on the phylogeography of certain earthworm species in relation to palaeogeography [9, 18].

Metaphire sieboldi (Horst, 1883) was the first earthworms described from Japan (its types are in Leiden Museum), it is a striking metallic blue only when adult and is one of the largest of Japanese species at ca. 30 cm [3,4]. This species occurs in extreme southwestern Chubu, Kinki, and the Chugoku (Honshu), as well as on the islands of Shikoku and Kyushu [19, 21]. Given this distribution, it is intriguing to consider how such a large earthworm colonized Shikoku despite the apparent barrier to expansion presented by the sea. It is probably that *M. sieboldi* colonized from Honshu into Shikoku though the connected islands because earthworms could not survive in seawater. However, phylogeographic studies of Japanese earthworms have not been conducted to date.

It is clear that both demographic and phylogeographic forces shape the depth and distribution of lineages in a phylogenetic tree. In addition, selecting the correct genes in reconstructing a phylogeny is of importance due to differing evolutionary rates between different DNA regions. The cytochrome oxidase subunit I (COI) gene of mitochondrial DNA (mtDNA) has been extensively used in phylogenetic studies due to ease of primer design and the range of its phylogenetic signal [12]. The rate of evolution of this gene is also sufficiently rapid to allow discrimination at the species level [35], and have been shown to be informative in earthworms [7-9, 13, 14, 18, 25, 26, 29]. However, only COI

gene is not enough to discriminate between individuals of *M. sieboldi*. Additionally, sequences of 16S ribosomal DNA (rDNA) of mtDNA are among the most frequently employed markers for phylogenetic analyses and have also been shown to be informative in earthworms [9, 16, 26]. Substitution rate of 16S rDNA gene were lower than those of COI gene [9, 18]. However, some studies indicated that phylogeographic studies of earthworms were conducted using both COI and 16S genes because of accumulating informative characters. Therefore, the goal of this study was to provide the first broad-scale screening of mtDNA variation in *M. sieboldi*, to infer phylogeographic relationships within this species.

2. **Materials and Methods**

2.1. Sampling of *M. sieboldi* and allied species

To obtain a comprehensive understanding of the underlying historical population structure of *M. sieboldi*, 68 individuals representing 62 populations were sampled from across the geographic range of the species (Table 1). When identical sequences were present in a single locality, one sequence was included and the others were omitted. For use as outgroup taxa, *M. megascolidioides* (Goto and Hatai, 1899), *M.* (now *Duplodicodrilus*) *schmardae* (Horst, 1883), *M. tosaensis* (Ohfuchi, 1938), and *A. vittatus* (Goto and Hatai, 1898) were collected.

2.2. DNA extraction, amplification, and sequencing

The adult, clitellate earthworms were anesthetized in a 30-50% ethanol solution. Muscle tissue of anterior body wall was isolated and either stored in a freezer at -20°C. All DNA extractions were performed using QIAGEN DNeasy™ kits, following the manufacturer's protocol for animal tissue samples. The isolated DNA was resuspended in TE buffer and stored at –20 °C until use. For all specimens, a 690-base fragment in the coding region of the COI and a 377-base fragment of the 16S rDNA

were amplified using the following primers which were designed in this study: Meta-2F (5′ - ATR CCA GTA TTY ATT GGD GG -3′) and Meta-1R (5′- CTR AAT ACT TTR ATT CCT GT - 3′) for the COI region, 16S-Meta-F (5′ – AAC GGC CGC GGT AYM YTA AC -3′) AND 16S-Meta-R (5′ – CYW AAG CCA ACA TSG AGG TG -3′) for 16S rDNA. Double-stranded DNA was amplified by incubating at 94 °C for 10 sec, followed by 45 cycles of incubation at 94 °C for 1.5 min, 48 °C for 2 min, and 72 °C for 3 min, with a final extension at 72 °C for 15 min. DNA was amplified using polymerase chain reaction (PCR) in a 50-μl reaction volume containing approximately 50 ng total DNA, 10 mM Tris-HCl buffer (pH 8.3) with 50 mM KCl and 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 units *Taq* DNA polymerase (TAKARA), and 0.5 μM of each primer. After amplification, reaction mixtures were subjected to electrophoresis in 1% low-melting-temperature agarose gels and purified using QIAGEN QuickSpin™ kits following the manufacturer's specifications. The purified PCR products were sequenced using a Big DYE-terminator Cycle Sequencing Kit (ABI PRISM DNA Sequencing Kit; Perkin-Elmer Applied Biosystems) and an ABI PRISM 3100-*Avant* Genetic Analyzer according to the manufacturers' instructions. For sequencing, the same primers used for amplification were used.

2.3. Data analysis

To construct phylogenetic trees, sequences were aligned using Clustal W [34], and were improved manually using MEGA 4 [32]. The positions of deletions or insertions were determined manually. Alignment began at position 240 for COI gene and 11,689 for 16S rDNA of *Lumbricus terrestris* (Linnaeus, 1758), which was retrieved from GenBank (accession no. U24570 [6]).

In the phylogenetic analyses, the most appropriate model of DNA substitution was chosen using hierarchical likelihood ratio tests with PAUP 4.0b10 [30] and Modeltest v3.06 [27]. For the combined dataset of COI and 16S rDNA, Tamura-Nei's model [31] with invariable sites of 0.6385 and a gamma shape parameter of 0.7595 (TrN+I+G) was chosen (Base frequencies: A, 0.3764; C, 0.2241; G, 0.1174 and T, 0.2821. Substitution rates: A–C, 1.0000; A–G, 12.0519; A–T, 1.0000; C–G, 1.0000; C–T, 9.1328; and G–T, 1.0000). For the COI region, the general time-reversible model [33] with invariable sites of 0.4707 and a gamma shape parameter of 0.5544 (GTR+I+G) was chosen (Base frequencies: A, 0.4029; C, 0.1645; G, 0.1672; and T, 0.2654. Substitution rates: A–C, 3.1784; A–G, 6.4862; A–T, 4.1422; C–G, 0.5389; C–T, 19.5915; and G–T, 1.0000).

Neighbour-joining (NJ) analyses were performed using PAUP 4.0b10 on model of TrN+I+G with 1000 bootstrap replicates. Bayesian analysis was applied to generate a posterior probability distribution using

the Metropolis-coupled Markov Chain Monte Carlo (MCMC) with MrBayes 3.0b4 [15, 28]. The search was run for 4.5×10^6 generations, and every 100th tree was sampled. Posterior probabilities for each branch were calculated from the sampled trees.

3. **Results**

3.1. Sequence characteristics

MtDNA sequences from the COI region were determined from a total of 72 samples, including 68 samples of *M. sieboldi* and four outgroup species. The length of the COI region in all samples, including outgroups, was 690 bp, without insertions or deletions. Of the 690-nucleotide positions, 252 (36.5%) were polymorphic and 208 (30.1%) were parsimony informative. Among the *M. sieboldi* samples, the infraspecific sequence divergences of the COI gene ranged from 0.1% to 18.1%, and the mean infraspecific sequence divergences between phylogenetic groups of our study ranged 5.7% to 15.9% (Table 2). The lengths of the 16S rDNA among the samples, including outgroups, ranged from 376 bp to 377 bp. Among the *M. sieboldi* samples, the infraspecific sequence divergences of the 16S rDNA gene ranged from 0.0% to 6.2%, and the mean infraspecific sequence divergences between phylogenetic gourps of our study ranged 4.1% to 10.5% (Table 2).

The lengths of the combined sequences of the COI and 16S rDNA ranged from 1066 to 1067 bp. The total of the aligned sequences was 1068 bp, with insertions or deletions of 1 bp. Gaps and ambiguously aligned regions in the sequence were eliminated for all of the subsequent analyses,

thus only 1065 bp were ultimately used. Of the 1065 nucleotide positions, 333 (31.3%) were polymorphic and 253 (23.8%) were parsimony informative.

3.2. Phylogenetic analyses

Phylogenetic trees were constructed based on the NJ method and Bayesian analysis. Based on NJ tree, each monophyletic group named group I and group III to VII, and remaining individuals named group II, therefore group II formed palaphyletic assemblies (Fig. 1). The two phylogenetic analyses yielded only slightly different topologies, particularly at the bases of the trees and for the topologies of group IV (Figs. 1, 2). In the NJ tree (Fig. 1), a monophyletic group composed of individuals from southwestern Chubu and western Kinki (Group I) occupies the basal position (99% support). However, in the Bayesian tree, Group I is the sister group of a portion of Group II that was collected from eastern Shikoku, and the basal position of the Bayesian tree consists of a paraphyletic assemblage composed of Group II, Group I, and all of the other groups (Figs. 1-3). Groups III and IV are composed of individuals from southeastern and northeastern Shikoku, respectively. Moreover, Groups V and VI consist of individuals from mainland Shikoku, collected from Chugoku and Kyushu; and southern Kyushu and small islands on

the Bungo Channel (Uguru and Okinoshima Islands), respectively. Group VII is composed of individuals from southeastern Kyushu and western Shikoku (Figs. 1-3).

Both trees indicated that individuals of *M. sieboldi* collected from Kinki and western Chubu make up a monophyletic group. Nevertheless, individuals from the three distinct sampling areas of Shikoku, Kyushu, and Chugoku did not form monophyletic groups. No individuals from Shikoku were included in Groups I, V, or VI. These groups were mostly allopatric, except for Group II. In southern Shikoku, there were two distinct lineages inhabiting the same areas (Group II and Group III in eastern Shikoku, and Group II and Group VII in southwestern and southern Shikoku).

4. **Discussion**

4.1. Distribution of *M. sieboldi*

The present sampling records indicate that the distribution area of *M. sieboldi* is broader than previously reported by Kobayashi [21] and Kita and Kawaguchi [19]. The following new distribution areas were found: coastal western Kyushu (sample nos. 17, 18), northern Chugoku (sample no. 12), northern Kinki (sample no. 08), Awaji island (sample no. 09), and western Chubu (sample no. 01) (Fig. 3, Table 1).

4.2. Population phylogeology in *M. sieboldi*

By recognizing historical patterns resulting from major geological events such as mountain uplifts, changes in sea level, and climatic shifts, it is possible to hypothesize how Japanese earthworms responded to such events in the past. The present-day form of Japan is the result of major geologic and climatic change since the Miocene (approx. 5.3-23 mya), and these events are thought to have had a significant influence on the composition of Japan's modern fauna and flora [22]. It is likely that such events also influenced the distribution of Japanese earthworms. Because *M. sieboldi* inhabits a shallow position in the A_0 layer and in the soil, it is

restricted from colonizing from Chugoku or Kyushu into Shikoku and vice versa because Shikoku is surrounded by the sea – to the north by the Inland Sea, to the east by the Kii Channel, to the west by the Bungo Channel, and to the south by the Pacific Ocean (Fig. 3). Such topographical barriers around Shikoku would have blocked colonization, with considerable effect on the genetic structure of earthworm populations. However, the phylogenetic trees indicated that individuals belonging to the same lineages are distributed among various islands (e.g., Chugoku and Kyushu in Group V, Shikoku and Kyushu in Group VII), suggesting that this species has expanded its distribution across the sea. Artificial distribution should be taken into consideration, and it is also one of the species incidentally or deliberately used by fisherman as bait, at least on southern coastal Shikoku (R. J. Blakemore, pers. obs.). However, it is reported that *M. sieboldi* inhabits only montane forests, not artificially disturbed areas [17]. This suggests that the present results are the result of natural expansion of *M. sieboldi*.

How did *M. sieboldi* colonize across the sea? To address this issue, it is necessary to have some information on various geographical aspects of these areas. As a result of the expansion of the Sea of Japan, the ancient Japanese archipelago was separated from the Asian continent approx. 15-20 mya [20]. Subsequently, the Japanese Islands formed a large island; Chugoku, Shikoku, and Kyushu had not yet separated [20].

In the late Pliocene to early Pleistocene, transgression had occurred in the present-day inland sea region, but eastern Shikoku and southern Kinki were still connected, as the Kii Channel had not yet formed [24]. The Kii Channel formed approx. 1.0-1.2 mya [24], dividing eastern Shikoku and southern Kinki. The estimated divergence time between Groups I and II in the present study is roughly estimated to be 1.3 mya, using the reported COI gene divergence rate of 4.8% per million years for the congeneric Taiwanese earthworm *M. yuhsii* (Tsai, 1964) [9]. This result is consistent with the geological data, suggesting that Groups I and II were divided by the formation of the Kii Channel. After the separation of Groups I and II, the lineage of Group II diversified in eastern and southern Shikoku (about 1.0 mya). Moreover, the phylogenetic tree indicated that *M. sieboldi* expanded its distribution north and westward, from Shikoku to Chugoku and Kyushu (about 0.8-1.0 mya), as individuals from Chugoku and Kyushu have a common ancestor from Shikoku. Subsequently, individuals of Group VII accidentally colonized western Shikoku from Kyushu (about 0.4-0.5 mya). Studies have reported that there have been repeated glacial/interglacial cycles after 0.8 mya, and that Shikoku and Kyushu were connected at least in the most recent glacial period, which ended about 10,000 years ago [20, 22]. Therefore, the present results suggest that *M. sieboldi* may have expanded its distribution through land bridges between Kyushu and Shikoku; moreover, there are two different

lineages of *M. sieboldi* in Shikoku. Such historical differentiation among geographically disparate populations suggests that Shikoku is a secondary contact zone; contact zones between populations of varying degrees of differentiation provide a potentially important source of information on how populations interact. In the future, *M. sieboldi* collected from such zones could provide valuable opportunities for exploring the nature and extent of contact zones and the processes that may have formed them.

In summary, the mtDNA analyzed in the present study provides a phylogeographic hypothesis for the evolution of *M. sieboldi* in western Japan. This hypothesis, based on molecular information, allows unbiased interpretation of the evolutionary history of this species. Additional molecular markers may lend support to the scenario inferred here, and multiple markers, in particular nuclear DNA, should be surveyed to identify hybridizations between various groups within this species. Such research will assist in reappraising existing mtDNA data sets, and will facilitate new interpretations.

Acknowledgements

The authors wish to thank Drs. Tsukamoto J., Akita Y., Ichizawa K., Imayasu T., Nagai H., Ichie T., Ito K., Kawase J., Kawano K., Nakamura M., Hirata A., and Muramatsu Y. for providing much assistance, and Dr. S. Gale for reviewing this English manuscript. This study was partly supported by the Sasakawa Scientific Research Grant from The Japan Science Society (to Y.M.) and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (to T.F.).

References

[1] J.C. Avise, Phylogeography: the History and Formation of Species, Harvard University Press, London, 2000.

[2] J.C. Avise, J. Arnold, R.M. Ball, E. Bermingham, T. Lamb, J.E. Neigel, C.A. Reeb, N.S. Saunders, Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics, Ann. Rev. Ecol. Syst. 18 (1987) 489–522.

[3] R.J. Blakemore, Japanese earthworms (Annelida: Oligochaeta): a review and checklist of species, Org. Divers. Evol. 3, (Electr. Suppl.) 11 (2003) 1-43.

[4] R.J. Blakemore, A Series of Searchable Texts on Earthworm Biodiversity, Ecology and Systematics from Various Regions of the World, YNU CD-ROM Publication and Online Supplement, 2007: http://bio-eco.eis.ynu.ac.jp/eng/database/earthworm/.

[5] R.J. Blakemore, Cosmopolitan Earthworms CD. (3rd Edition). VermEcology, Yokohama, Japan. Pp.757+~243 Figs. & Internet links. [Published Dec., 2008]

[6] J.L. Boore, W.M. Brown, Complete sequence of the mitochondrial DNA of the annelid worm *Lumbricus terrestris*, Genetics 141 (1995) 305-319.

[7] C.H. Chang, J.H. Chen, Taxonomic status and intraspecific phylogeography of two sibling species of *Metaphire* (Oligochaeta: Megascolecidae) in Taiwan, Pedobiologia 49 (2005) 591-600.

[8] C.H. Chang, Y.H. Lin, I.H. Chen, S.C. Chuang, J.H. Chen, Taxonomic re-evaluation of the Taiwanese montane earthworm *Amynthas wulinensis* Tsai, Shen & Tsai, 2001 (Oligochaeta: Megascolecidae): Polytypic species or species complex? Org. Divers. Evol. 7 (2007) 231-240.

[9] C.H. Chang, S.M. Lin, J.H. Chen, Molecular systematics and phylogeography of the gigantic earthworms of the *Metaphire formosae* species group (Clitellata, Megascolecidae), Mol. Phylogenet. Evol. 49 (2008) 958-968.

[10] C.H. Chang, R. Rougerie, J.H. Chen, Identifying earthworms though DNA barcodes: Pitfalls and promise, Pedobioloiga 52 (2009) 171-180.

[11] L. Dupont, Perspectives on the application of molecular genetics to earthworm ecology, Pedobiologia 52 (2009) 191-205.

[12] P.D.N. Hebert, A. Cywinska, S.L. Ball, J.R. deWaard, Biological identifications through DNA barcodes, Pro. R. Soc. Lond., Ser. B 270 (2003) 313–321.

[13] M. Heethoff, K. Etzold, S. Scheu, Mitochondrial COII sequences indicate that the parthenogenetic earthworm *Octolasion tyrtaeum* (Savigny 1826) constitutes of two lineages differing in body size and genotype, Pedobiologia 48 (2004) 9-13.

[14] J. Huang, X. Qin, J.S. Zhen, L.T. Gui, Y.S. Zi, Identifying earthworms through DNA barcodes, Pedobiologia 51 (2007) 301-309.

[15] J.P. Huelsenbeck, F. Ronquist, MRBAYES. Bayesian inference of phylogeny. Bioinformatics 17 (2001) 754–755.

[16] B.G.M. Jamieson, S. Tillier, A. Tillier, J.L. Justine, E. Ling, S. James, K. McDonald, A.F. Hugall, Phylogeny of the Megascolecidae (Oligochaeta, Annelida): combined versus partitioned analysis using nuclear (28s) and mitochondrial (12s, 16s) rDNA, Zoosystema 24 (2002) 707-734.

[17] Y. Kamihira, Earthworms from Miyazaki prefecture in Kyushu district, Japan, together with the consideration of their geographical distribution, The Review of Hakodate Univ. 35 (2004) 91-102 (in Japanese).

[18] R.A. King, A.L. Tibble, W.O.C. Symondson, Opening a can of worms: unprecedented sympatric cryptic diversity within British lumbricid earthworms. Mol. Ecol. 17 (2008) 4684-4698.

[19] E. Kita, S. Kawaguchi, Note on distribution of *Metaphire sieboldi*, Kagawa Seibutsu 25 (1998) 21-24 (in Japanese).

[20] T. Koaze, Archipelago surrounding sea, in: H. Ono, H. Hagiwara, K. Uemura, H. Baba (Eds.), Natural History of the Japanese islands, Tohkai University Press, Kanagawa, 2006, pp. 11-22 (in Japanese).

[21] S. Kobayashi, The distribution of terrestrial earthworms of western Japan, Zool. Mag. 53 (1941) 371-384 (in Japanese).

[22] K. Nakamura, Z. Uchijima, Climate changes, in: K. Nakamura, R. Kimura, Z. Uchijima (Eds.), Nature in Japan V, Iwanami-shoten, Tokyo, 1985, pp. 180-193 (in Japanese).

[23] K. Nishikawa, M. Matsui, S. Tanabe, S. Sato, Geographic enzyme variation in a Japanese salamander, *Hynobius boulengeri* Thompson (Amphibia: Caudata), Herpetologica 57 (2001) 281-294.

[24] Y. Ohta, T. Naruse, S. Tanaka, A. Okada, Geomorphology of Kinki, Chugoku and Shikoku, Tokyo University Press, Tokyo, 2004 (in Japanese).

[25] M. Pérez-Losada, J. Eiroa, S. Mato, J. Domínguez, Phylogenetic species delimitation of the earthworms *Eisenia fetida* (Savigny, 1826) and *Eisenia andrei* Bouché, 1972 (Oligochaeta, Lumbricidae) based on mitochondrial and nuclear DNA sequence, Pedobiologia 49 (2005) 317-324.

[26] A.A. Pop, M. Wink, V.V. Pop, Use of 18S, 16S rDNA and cytochrome c oxidase sequences in earthworm taxonomy (Oligochaeta, Lumbricidae), Pedobiologia 47 (2003) 428-433.

[27] D. Posada, K.A. Crandall, MODELTEST: testing the model of DNA substitution, Bioinformatics 14 (1998) 817–818.

[28] F. Ronquist, J.P. Huelsenbeck, MrBayes 3: Bayesian phylogenetic inference under mixed models, Bioinformatics 19 (2003) 1572–1574.

[29] M.E. Siddall, K. Apakupakul, E.M. Burreson, K.A. Coates, C. Erséus, S.R. Gelder, M. Källersjö, H. Trapido-Rosenthal, Validating Livanow: molecular data agree that leeches, Branchiobdellidans and *Acanthobdella peledina* form a monophyletic group of Oligochaetes. Mol. Biol. Evol. 21 (2001) 346-351.

[30] D.L. Swofford, Paup*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4, Sinauer Associates, Sunderland, MA., 2002.

[31] K. Tamura, M. Nei, Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees, Mol. Biol. Evol. 10 (1993) 512-526.

[32] K. Tamura, M. Nei, S. Kumar, Prospects for inferring very large phylogenies by using the neighbor-joining method, PNAS 101 (2004) 11030-11035.

[33] S. Tavaré, Some probabilistic and statistical problems on the analysis

of DNA sequences. In: Miura R.M. (Ed.) Some Mathematical Questions in Biology—DNA sequence Analysis, Lec. Math. Life Sci. Vol. 17, Providence, R.I., 1986, pp. 57–86.

[34] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice, Nucleic Acids Research 22 (1994) 4673-4680.

[35] M.J. Wishart, J.M. Hughes, Genetic population structure of the net-winged midge, *Elporia barnardi* (Diptera: Blephariceridae) in streams of the south-western Cape, South Africa. Implications for dispersal, Freshwater Biology 48 (2003) 28–38.

[36] Y. Yasuda, N. Miyoshi, Illustration for Japanese Historical Botany, Asakura Shoten, Tokyo, 1998.

[37] M. Yamada, E. Hosoi, H.B. Tamate, J. Nagata, S. Tatsuzawa, H. Tado, S. Ozawa, Distribution of two distinct lineages of sika deer (Cervus nippon) on Shikoku Island revealed by mitochondrial DNA analysis, Mammal Study 31 (2006) 23-28.

[38] Y. Yasukochi, S. Nishida, S.H. Han, T. Kurosaki, M. Yoneda, H. Koike, Genetic structure of the Asiatic black bear in Japan using mitochondrial DNA analysis. J. Heredity 100 (2008) 297-308.

Figure Legends

Fig. 1. Phylogenetic tree of *Metaphire sieboldi* (Horst, 1883) and outgroups using the neighbour-joining (NJ) method. The numbers below the branches indicate the bootstrap value. For abbreviations, see Table 1.

Fig. 2. Phylogenetic tree of *Metaphire sieboldi* (Horst, 1883) and outgroups using the Bayesian analyses. The numbers below the branches indicate the bootstrap value. For abbreviations, see Table 1.

Fig. 3. Geographical distribution of *Metaphire sieboldi* (Horst, 1883) used in this study. Shading indicates the previously reported distribution range of *M. sieboldi* [19, 21].

Fig. 1

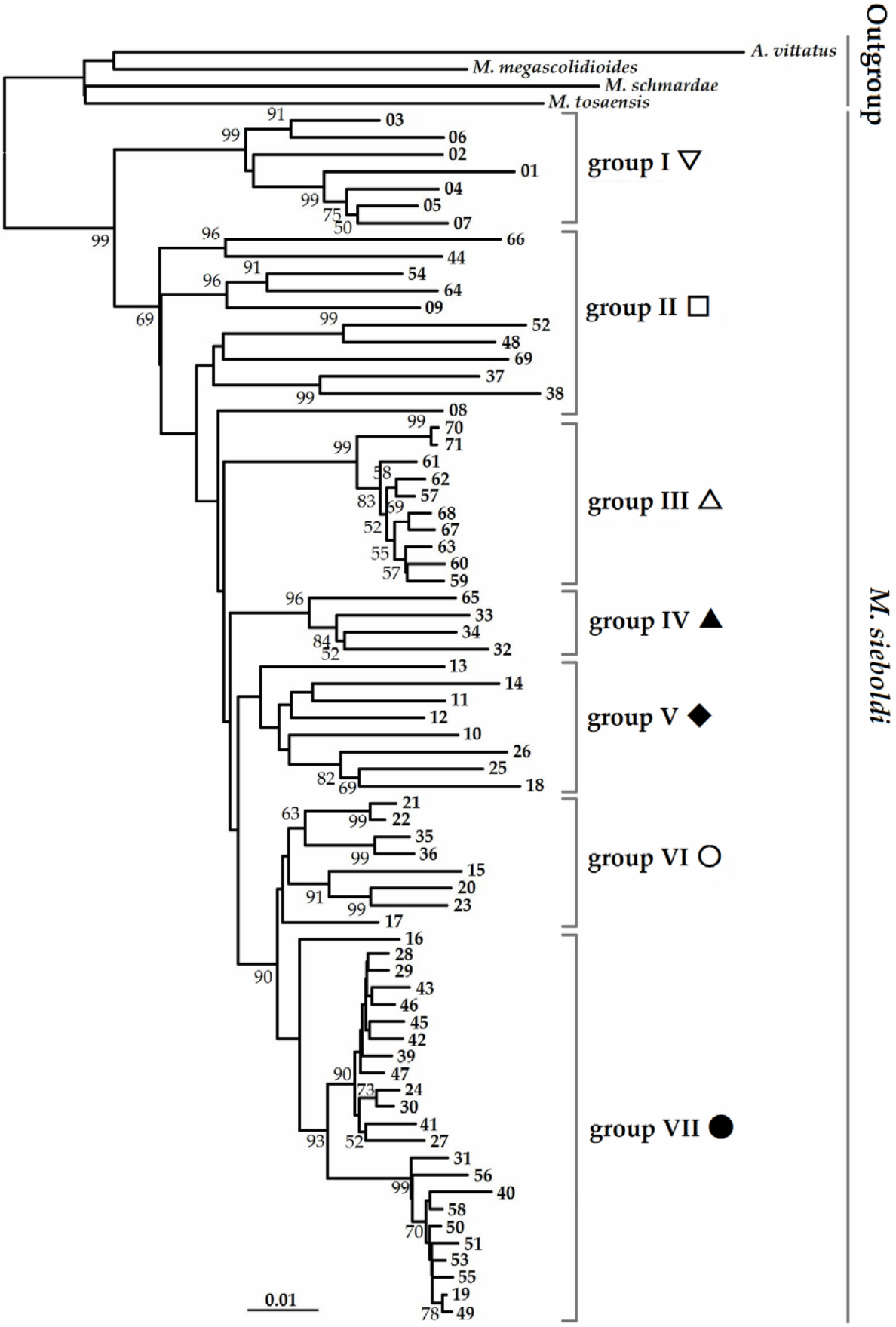
Outgroup
A. vittatus
M. megascolidioides
M. schmardae
M. tosaensis
M. sieboldi
group I ▽
group II □
group III △
group IV ▲
group V ◆
group VI ○
group VII ●
0.01

Fig. 2

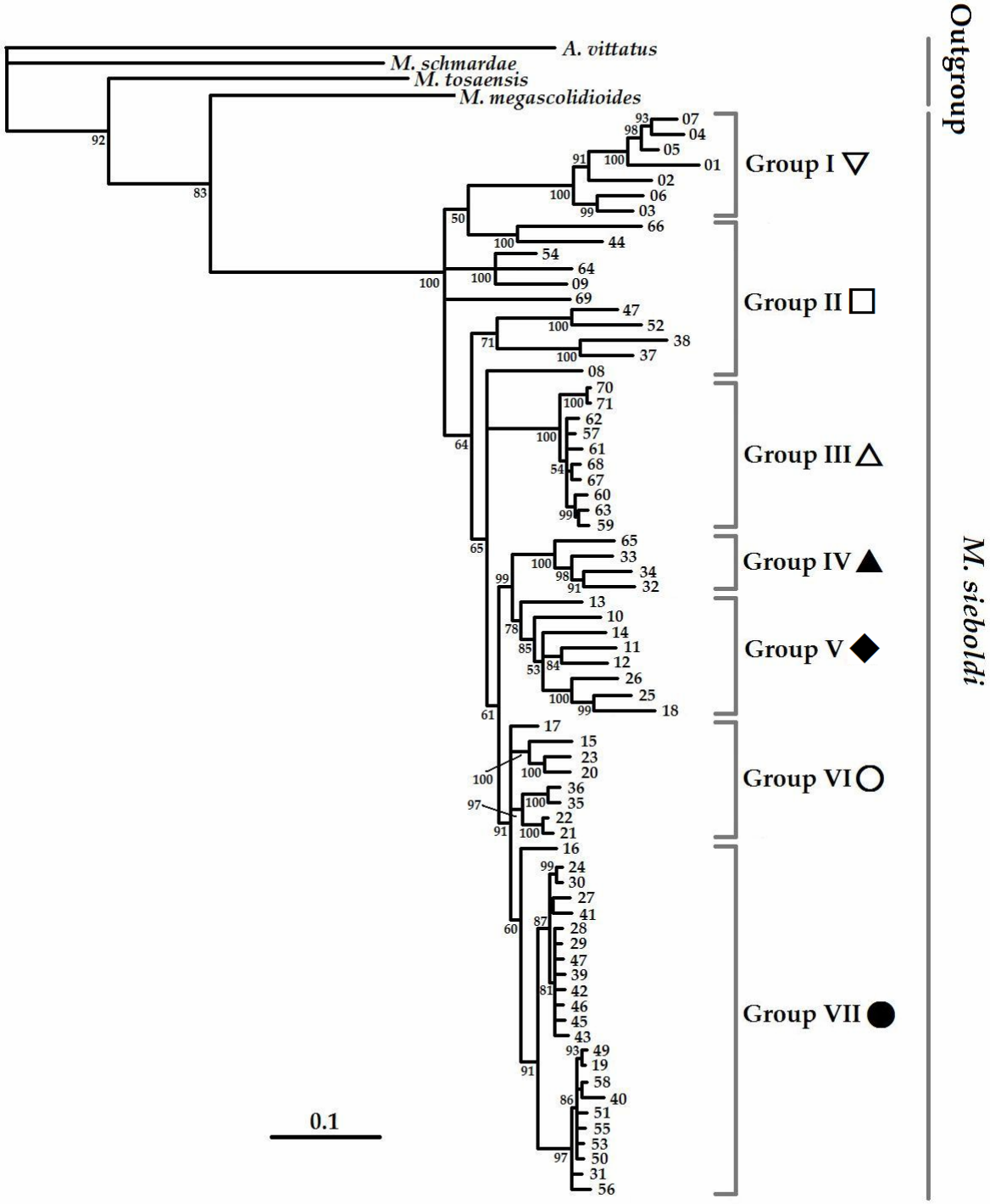
A. vittatus
M. schmardae
M. tosaensis
M. megascolidioides
Outgroup
M. sieboldi
Group I
Group II
Group III
Group IV
Group V
Group VI
Group VII
0.1

Fig. 3

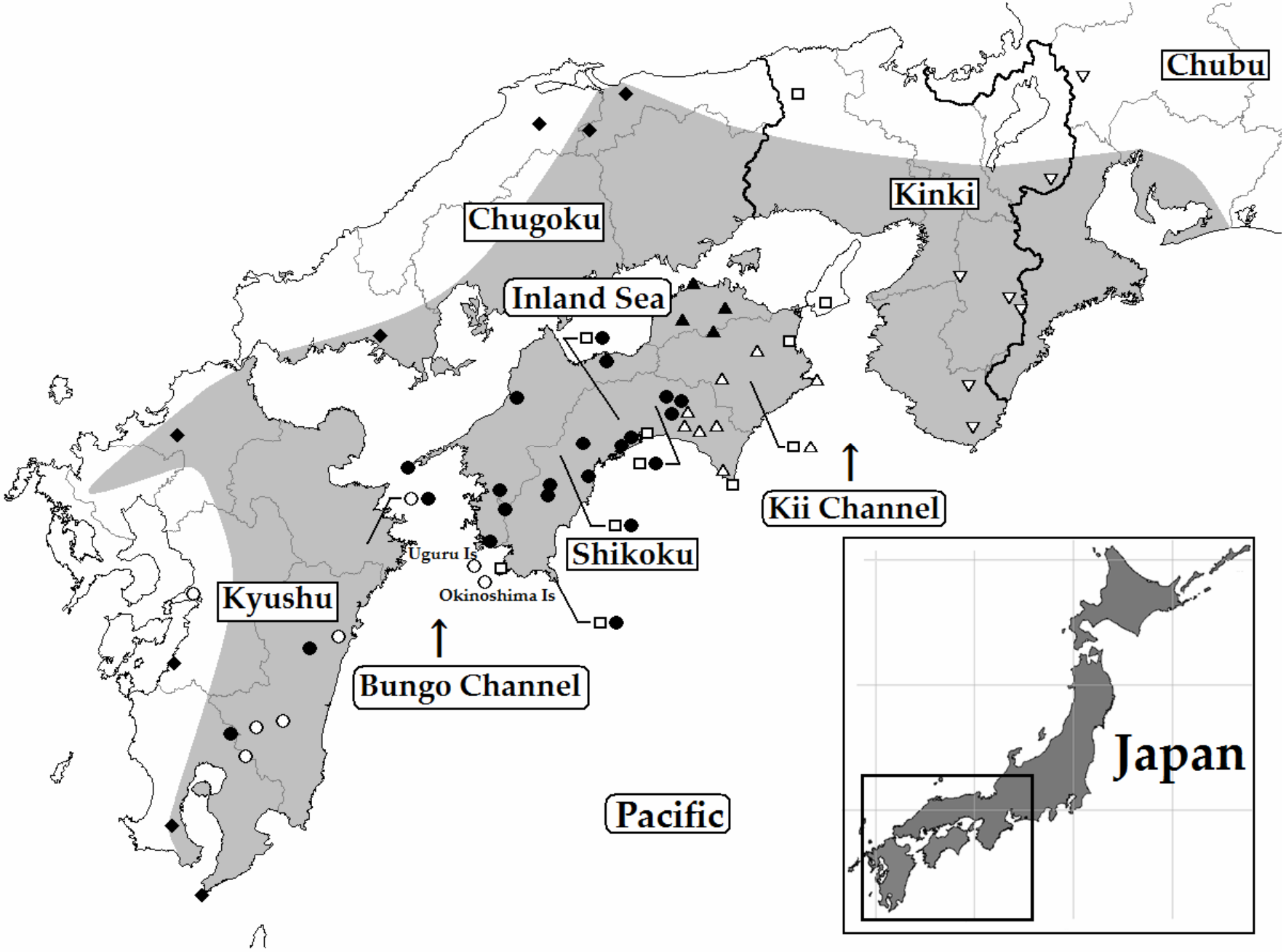
Chubu
Kinki
Chugoku
Inland Sea
Kii Channel
Shikoku
Uguru Is
Okinoshima Is
Kyushu
Bungo Channel
Pacific
Japan

Table 1 Samples used in the phylogenetic study and corresponding GenBank accession numbers.

Species name	Source locality			OUT name	Accession No.	
	District	Prefecture	City/ Town		COI	16S
Metaphire sieboldi	Chubu	Gifu	Ibigawa town, Sototsuoi	01	AB482074	AB482109
	Kinki	Wakayama	Kozagawa town, Hirai	02	AB482075	AB482110
			Tanabe city, Heijigawa fall	03	AB482076	AB474353
		Nara	Kamikitayama village, Ohdaigahara	04	AB482077	AB482111
			Kawakami village, Shionoha	05	AB482078	AB482112
			Gose city, Sekiya	06	AB482079	AB482113
		Shiga	Koga city, Ohkawara	07	AB482080	AB482114
		Hyogo	Toyooka city, Ase	08	AB482081	AB482115
			Minamiawaji city, Mihara	09	AB482082	AB482116
	Chugoku	Tottori	Daisen town, Joujuku	10	AB425719	AB482117
			Nichinan town, Funadori	11	AB425720	AB482118
		Shimane	Unnan city, Kakeai, Yae fall	12	AB482083	AB482119
		Yamaguchi	Shunan city, Hachikubo	13	AB425722	AB482120
	Kyushu	Fukuoka	Umi town, Shiouji	14	AB482084	AB482121
		Oita	Saiki city, Yagata	15	AB425726	AB482122
				16	AB425724	AB482123
		Kumamoto	Uto city, Mt. Ootake	17	AB482085	AB482124
			Minamata city, Hama	18	AB482086	AB482125
		Miyazaki	Misato town, Tatsume	19	AB425727	AB482126
			Hyuga city, Yamanota	20	AB425729	AB482127
			Aya town, Hirase	21	AB425733	AB474355
			Miyakonojo city, Miike	22	AB425734	AB482128
		Kagoshima	Soo city, Ohkawarakyo	23	AB425737	AB482129
			Kirishima city, Mt. Kirishima	24	AB425736	AB482130
			Minamiosumi town, Cape Sata	25	AB482087	AB482131
			Minamikyushu city, Kidoko	26	AB482088	AB482132
	Shikoku	Ehime	Ikata town, Cape Sada	27	AB425738	AB482133
			Uwajima city, Yakushidani	28	AB425739	AB482134
			Ainan town, Johen Park	29	AB482089	AB482135
			Iyo city, Otaniike	30	AB425743	AB482136
			Niihama city, Ashima	31	AB425744	AB482137
		Kagawa	Sakaide city, Goshikidai	32	AB482090	AB482138
			Takamatsu city, Sato	33	AB482091	AB482139
			Manno town, Kamino	34	AB482092	AB482140
		Kochi	Sukumo city, Okinoshima Island	35	AB482093	AB482141
			Ugurushima Island	36	AB425747	AB482142
			Otsuki town, Odo	37	AB482094	AB482143
			Tosashimizu city, Cape Ashizuri	38	AB482095	AB482144
				39	AB425765	AB482145
				40	AB425764	AB482146
			Shimanto city, Kuroson	41	AB425748	AB482147
			Shimanto town, Ichinomata	42	AB425763	AB482148
			Shimodo	43	AB425754	AB482149
			Tsuno town, Funato	44	AB482096	AB482150
				45	AB425750	AB482151
			Susaki city, Koyaiga	46	AB425770	AB482152
			Ochi town, Mt. Yokogura	47	AB425771	AB482153
			Kochi city, Anagawa	48	AB482097	AB482154
				49	AB425775	AB482155
			Kochi city, Nishibun	50	AB425787	AB482156
			Hitsuzan	51	AB425778	AB482157
			Mt. Godaisan	52	AB482098	AB482158
			Kami city, Ananai	53	AB425796	AB482159
				54	AB482099	AB482160

Table 1 (continued)

Species name	Source locality			OUT name	Accession No.	
	District	Prefecture	City/ Town		COI	16S
			Ohira	55	AB425801	AB482161
			Shigetou	56	AB425803	AB482162
			Meikai	57	AB482100	AB482163
			Taniai	58	AB425804	AB482164
			Konan city, Kitatani	59	AB482101	AB482165
			Kunimitsu	60	AB425806	AB482166
			Aki city, Harikawa	61	AB425807	AB482167
			Umaji village, Yanase	62	AB425812	AB482168
			Muroto city, Haji	63	AB425813	AB482169
			Cape Muroto	64	AB482102	AB482170
		Tokushima	Mima city, Mt. Ohtaki	65	AB482103	AB482171
			Tokushima city, Mt. Bizan	66	AB482104	AB482172
			Kamiyama city, Anoochi	67	AB425815	AB482173
			Naka town, Takanose	68	AB425814	AB482174
			Yuridani	69	AB482105	AB482175
				70	AB425816	AB482176
			Anan city, Cape Kamoda	71	AB482106	AB482177
Outgroup						
Amynthas vittatus		Miyazaki	Miyakonojo city, Miike		AB425818	AB474324
M. megascolidioides		Kochi	Nankoku city, Monobe, Kochi University		AB482107	AB474345
M. schmardae		Kochi			AB425820	AB474347
M. tosaensis		Kochi	Konan city, Kunimitsu		AB482108	AB474359

Table 2 Mean infraspecific sequence divergences between groups within *Metaphire sieboldi* calculated using the most-appropriate model of DNA substitution based on the COI sequences (lower left) and the combined sequences of COI and 16S rDNA (upper right).

	Group I	II	III	IV	V	VI	VII
Group I		0.105	0.102	0.100	0.102	0.096	0.099
II	0.159		0.083	0.087	0.087	0.080	0.080
III	0.156	0.123		0.066	0.067	0.062	0.060
IV	0.147	0.127	0.097		0.069	0.064	0.064
V	0.156	0.129	0.099	0.100		0.061	0.063
VI	0.145	0.114	0.088	0.086	0.086		0.041
VII	0.157	0.120	0.090	0.093	0.095	0.057	