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Newly detected lineage of *Branchiomma* and the first record of a fan worm *Branchiomma* sp. B sensu Capa et al. (2013) (Annelida: Sabellidae) from the Japanese Archipelago

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Abstract The fan worm genus *Branchiomma* (Annelida: Sabellidae) includes 30 valid species. A previous study of the molecular phylogenetic assessment of *Branchiomma* indicates problems in using traditional morphological diagnostic characters for identification of *Branchiomma* species. We performed phylogenetic analysis based on the cytb gene sequences of recently collected specimens of *Branchiomma* from an artificial seawater pond in Iwaki, Fukushima, and from a boat mooring rope at Tanabe Bay, Shirahama, Wakayama, Japan. The Fukushima specimen was identified as a distinct lineage, which was genetically different from the other species of *Branchiomma* recognized by the previous study. The Shirahama specimens were included in the clade of *Branchiomma* sp. B *sensu* Capa et al. (2013) and were identified as this species. The haplotype network illustrated low genetic differences among *Branchiomma* sp. B specimens collected from Japan, Australia, Hawaii, Saipan, and Florida. More specimens and loci with higher substitution rates would be needed to discuss whether they are spread by unintentional or natural dispersal. The Shirahama specimens of *Branchiomma* sp. B morphologically differ from *Branchiomma cingulatum*, the only congener recorded from Japan, in having macrostylodes. However, a substantial revision of the genus and a detailed morphological description of the two species would be necessary to determine whether these two species are indeed distinct. The results of the present study represent the first record of *Branchiomma* sp. B sensu Capa et al. (2013) from Asia and further extend the known geographic distribution of this wide-ranging species. The 16S rRNA gene sequences were also obtained as reference sequences of DNA barcoding for future studies.

Keywords: 16S rRNA, cryptic species, cytb, polychaetes, Sabellida, sessile organism

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Introduction

The genus *Branchiomma* (Annelida: Sabellidae) is characterized by paired compound radiolar eyes and stylodes on radioles (Tovar-Hernández, 2010). Currently, 30 valid species are recognized for the genus (Capa et al., 2020). Imajima and Hartman (1964) reported three species of *Branchiomma*, namely, *B. cingulatum*, *B. serratibranchis*, and *B. picta*, from Japan. However, the latter two were later moved to *Pseudobranchiomma* (Knight-Jones, 1994; Knight-Jones and Giangrande, 2003; see also Ishaq and Mustaquim, 1996; Tovar-Hernández and Fitzhugh, 2021). Consequently, *Branchiomma cingulatum* remains the only recognized species of the genus in Japan. A previous taxonomic study including molecular phylogenetic analysis revealed cryptic diversity in the genus and the limitation of the usefulness of traditional

diagnostic characters for distinguishing the species of *Branchiomma*, e.g., presence/absence of macrostylodes, due to homoplasy (Capa et al., 2013). Although genetic information, alongside morphological data, would be indispensable to understanding the true diversity of *Branchiomma*, the genetic data pertaining to the species of *Branchiomma* in Japan is not yet available.

We found a solitary tube made by an unidentified individual of *Branchiomma* at Iwaki, Fukushima, Japan, and aggregations of tubes on a boat mooring rope made by another *Branchiomma* sp. at Shirahama, Wakayama, Japan. For identification of specimens in those tubes, we sequenced the mitochondrial cytb, commonly used for DNA barcoding of *Branchiomma* (Capa et al., 2013), and 16S rRNA gene sequences of the specimens. Additionally, we conducted a brief morphological observation to compare them with *B. cingulatum*, a congeneric species reported from Japan.

Materials and Methods

Sampling and the preparation of DNA template

More than 30 specimens of *Branchiomma* were collected from tubes aggregated on a boat mooring rope at Tanabe Bay, Shirahama, Wakayama, Japan on 29 July 2021 (Figs. 1, 2). A solitary specimen of *Branchiomma* was collected from artificial open-air saltwater ponds, known as "Janome Beach," created in an aquarium, Aquamarine Fukushima in Fukushima, Japan, on October 29, 2018 (Fig. 1). This specimen had been previously reported as *Branchiomma* cf. *cingulatum* (Kanaya et al., in press) and was used in the present study. The Fukushima and Shirahama specimens were fixed with 99% and 80% ethanol, respectively, and preserved. Morphological examination of the Fukushima specimen and five Shirahama specimens was conducted using a stereomicroscope.

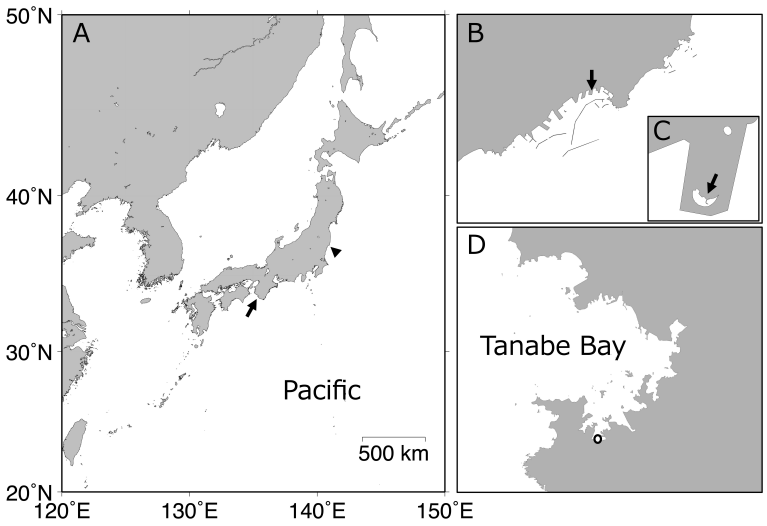

Figure 1. Locality of the sampling sites. **A**, map of Japan. An arrowhead indicates the locality of an aquarium, Aquamarine Fukushima, Iwaki, Fukushima, Japan (*Branchiomma* sp. I). An arrow indicates the locality of Tanabe Bay, Shirahama, Wakayama, Japan (*Branchiomma* sp. B). **B**, close-up view of Iwaki. An arrow indicates the place of Aquamarine Fukushima. **C**, The place of the Janome Beach at Aquamarine Fukushima (an arrow). **D**, close-up view of Tanabe Bay. A circle indicates the sampling site. The maps were produced using Generic Mapping Tools (GMT) v5.1.1. (Wessel et al., 2013).

Portions of the body wall of the five Shirahama and one Fukushima specimens were cut out and treated with the mixture of 9 μL of proteinase K solution (Nacalai Tesque, Kyoto, Japan) and 100 μL of 10% solution of Chelex 100 Resin (Bio-Rad, Hercules, CA) incubated at 56 °C for 30 min and then at 100 °C for 20 min, and the supernatant fluid was used as template DNA. The specimens were deposited at the National Museum of Nature and Science, Tokyo (NSMT, NSMT-Pol113490–113494 for the Shirahama specimens and NSMT-Pol113495 for the Fukushima specimen). The DNA template was deposited at the Center for Molecular Biodiversity Research, NSMT (NSMT-DNA56569–56573 for the Shirahama specimens and NSMT-DNA56574 for the Fukushima specimen).

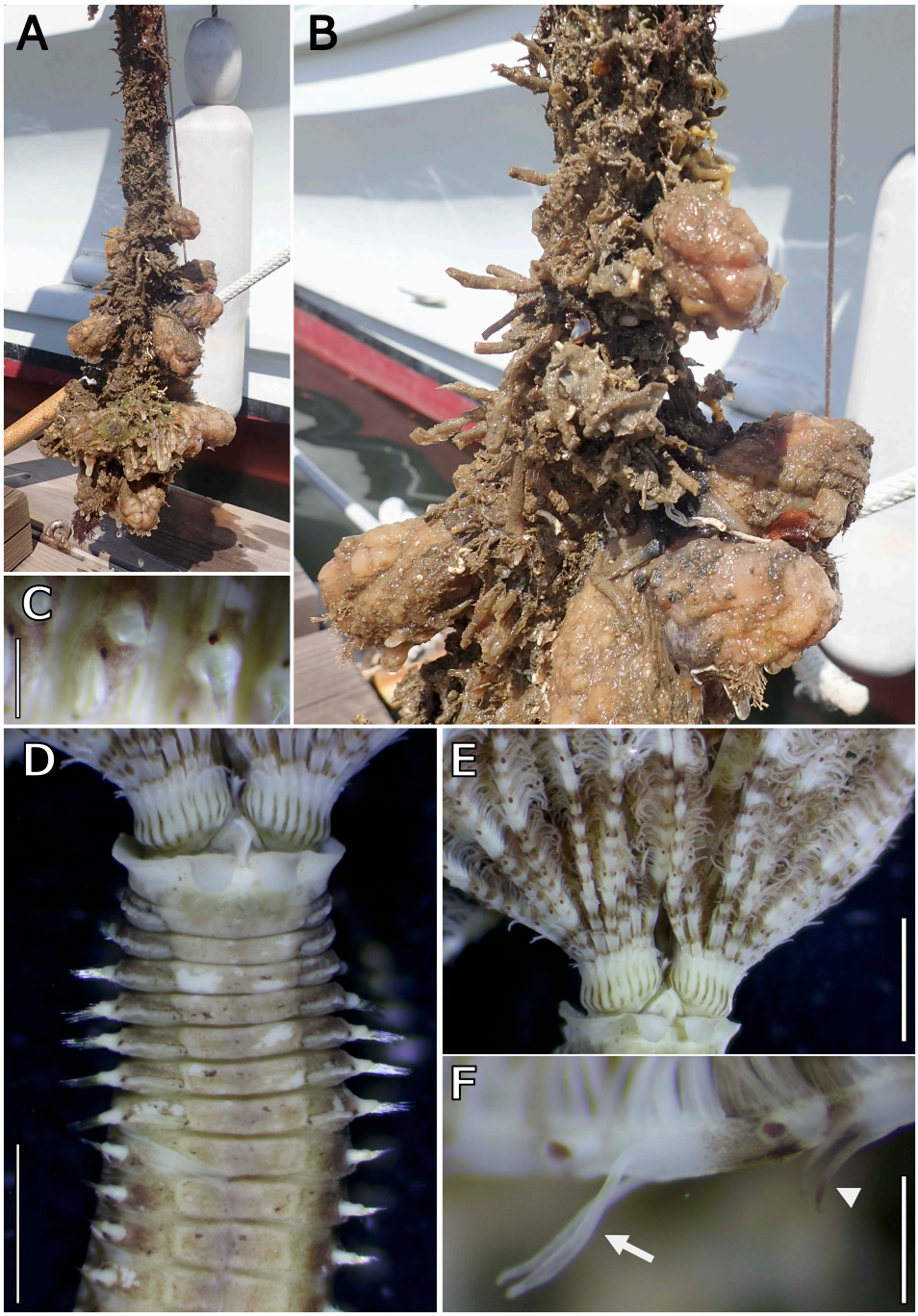

Figure 2. Photographs of *Branchiomma* sp. B sensu Capa et al. (2013). **A** & **B**, aggregation of the tubes on a boat mooring rope (photos courtesy of Luna Yamamori and Ryutaro Goto). **C**–**F**, ethanol fixed specimen (identifier GK2276). **C**, interramal eyespots, lateral view. **D**, trunk and the base of a branchial crown, ventral view. **E**, branchial crown, ventral view. **F**, close-up view of macrostylodes (an arrow) and microstylodes (an arrowhead) on a radiole. Scale bars = 2 mm (D and E) and 500 µm (C and F).

Polymerase chain reactions and sequencing

The partial sequences of the mitochondrial cytb gene of six specimens (346–355 bp) were determined. Additionally, the partial sequences of 16S rRNA gene (16S) were obtained from the Fukushima specimen (402 bp) and two Shirahama specimens (399 bp) as reference sequences for DNA barcoding for future studies. The primer sets CYTBfm/CYTBrm or cytb424F/cobr825 for cytb and 16Sann-f2/16Sb-ann for 16S were used for PCR (Table 1). The newly designed CYTBfm/CYTBrm primers include 5' tails for 2nd PCR, which adds indices and binding sites for sequencing with a next generation sequencer (Kobayashi, unpublished). The PCR mixtures were as follows: (1) 4.81 µL of sterilized water, 0.25 µL of MightyAmp DNA Polymerase Ver. 3 (TaKaRa Bio, Kusatsu, Japan), 6.25 µL of MightyAmp Buffer, 0.19 µL of 20

μM forward and reverse primers (the cytbF/cobr825 and 16Sann-f2/16Sb-ann primer sets), and 1.0 μL of template DNA; or (2) 2.25 μL of sterilized water, 3.13 μL of KOD One PCR Master Mix (TOYOBO, Osaka, Japan), 0.19 μL of 10 μM forward and reverse primers (CYTBfm/CYTBrm), and 1.0 μL of template DNA. PCR amplifications were performed as follows: (1) initial denaturation at 94 °C for 120 s; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 40 s, and extension at 72 °C for 20 s; and then a final extension at 72 °C for 300 s (MightyAmp) or (2) 35 or 40 cycles comprising denaturation at 98 °C for 10 s, annealing at 42 °C for 5 s, and extension at 68 °C for 1 s (KOD One PCR Master Mix). The PCR results were checked by electrophoresis on a 2% agarose gel. PCR products were purified using ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA) or FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). Sequencing was outsourced to Eurofins Genomics (Tokyo, Japan). The obtained nucleotide sequences were deposited through the DNA Data Bank of Japan (DDBJ) with DDBJ/EMBL/GenBank accession numbers LC801520–LC801525 (cytb) and LC801517–LC801519 (16S).

Table 1. List of primers used in the present study.

Locus	Primer	Sequence (5'–3')	Reference
cytb	CYTBfm[1, 2]	GTATAAGAGACAGGACAGTGRGGAGCIACIGTWATYACHAA	This study
	CYTBrm[1, 2]	GTATAAGAGACAGTTCTCAARTAYCAYTCIGGYWKRATRTG	This study; modified from cobr825
	cytb424F	GGWTAYGTWYTWCCWTGRGGWCARAT	Boore and Brown (2000)
	cobr825	AARTAYCAYTCYGGYTTRATRTG	Burnette et al. (2005)
16S	16Sann-f2	CCTGACYGTGCWAAGGTAGC	Kobayashi and Kojima (2021)
	16Sb-ann	CGGTCTRAACTCARCTCAYG	Kobayashi et al. (2023)

[1] I (inosine) can be replaced with N.

[2] Underlines indicate binding sites for sequencing analysis using next generation sequencer (see details for Materials and Methods).

Phylogenetic analysis and haplotype network analysis

To reveal the phylogenetic position of the specimens collected in the present study, a molecular phylogenetic analysis of *Branchiomma* spp. based on the cytb gene sequences was conducted, using *Pseudobranchiomma* sp. and *Sabellastarte australiensis* as outgroup (Table 2). Tentative names of unidentified species (*Branchiomma* sp. A–D and F–H) are after Capa et al. (2013). All sequences, except for those from our specimens, were obtained from GenBank (Table 2). The sequences were aligned using MAFFT v7.294b (Katoh and Standley, 2013), and primer regions in the alignment were manually removed. Maximum likelihood (ML) analysis was conducted with 1000 ultrafast bootstrap replicates with IQ-TREE v1.6.12 (Nguyen et al., 2014). The best-fit substitution model (TN+F+I+G4) was selected using ModelFinder (Kalyaanamoorthy et al., 2017) implemented in IQ-TREE. The resultant tree was edited using FigTree v1.4.3 (http://tree.bio. ed.ac.uk/software/figtree/).

To visualize the relationships among the cytb haplotypes, a haplotype network of a lineage of *Branchiomma* (i.e., *Branchiomma* sp. B) including the sequences obtained from the Shirahama specimens and GenBank was constructed with the TCS algorithm (Clement et al., 2002) using PopART (Leigh and Bryant, 2015). The positions that include ambiguous bases or gaps at 5' and 3' ends were manually removed (the resultant dataset with 344 characters). Nucleotide sequences were translated into amino acid sequences using the invertebrate mitochondrial code (-T 5) with SeqKit v0.12.0 (Shen et al., 2016) to examine amino acid substitutions among haplotypes.

Table 2. List of species used for phylogenetic analysis based on cytb gene sequences in the present study. Bold indicates specimens whose sequences were newly obtained in this study.

Species	Locality	Accession number
Branchiomma bairdi	Pacific: Australia: Queensland	KF429117
	Atlantic: USA: Florida	KF429103
	Atlantic: USA: Florida	KF429105
Branchiomma bombyx	Mediterranean Sea: Spain: Catalonia	KF429101
Branchiomma luctuosum	Indian Ocean: Pakistan: Karachi	KF429113
Branchiomma lucullanum	Mediterranean Sea: Spain: Catalonia	KF429098–KF429100
Branchiomma sp. A	Pacific: Australia: Western Australia	KF429119, KF429120
Branchiomma sp. B	Pacific: Northern Mariana Islands: Saipan	KF429141
	Atlantic: USA: Florida	KF429102, KF429104
	Pacific: USA: Hawaii	KF429107
	Pacific: Australia: Northern Territory	KF429148
	Pacific: Japan: Shirahama, Wakayama	**LC801520–LC801524**
Branchiomma sp. C	Pacific: Australia: Western Australia	KF429124, KF429137, KF429138, KF429140
Branchiomma sp. D	Pacific: Australia: Queensland	KF429093, KF429144–KF429147
	Pacific: Australia: Western Australia	KF429139
	Pacific: USA: Hawaii	KF429106, KF429111, KF429112
Branchiomma sp. F	Pacific: Australia: Western Australia	KF429118, KF429121, KF429125
	Pacific: Australia: New South Wales	KF429132, KF429133
Branchiomma sp. G	Pacific: Australia: New South Wales	KF429094–KF429097, KF429114, KF429115, KF429122, KF429123, KF429126–KF429131, KF429135, KF429136, KF429142, KF429143
Branchiomma sp. H	Pacific: Australia: Northern Territory	KF429116
***Branchiomma* sp. I**	**Pacific: Japan: Iwaki, Fukushima**	**LC801525**
Pseudobranchiomma sp.	Pacific: USA: Hawaii	KF429108
Pseudobranchiomma sp.	Pacific: USA: Hawaii	KF429109
Pseudobranchiomma sp.	Pacific: USA: Hawaii	KF429110
Sabellastarte australiensis	Pacific: Australia: New South Wales	KF429134

Results

The Shirahama specimens consisted of seven or eight thoracic and 69–77 abdominal chaetigers in complete specimens. Whole bodies (including branchial crowns) and branchial crowns were 39.8–51.6 mm and 8.9–11.1 mm in length, respectively. Each half of the branchial crown included 17–22 radioles. Approximately 16–20 paired and basal unpaired filiform stylodes were present per radiole. The stylodes include short and thin microstylodes, and long and thick macrostylodes (Fig. 2F). Color of the fixed specimens was brownish yellow. Dark reddish brown spots were present on the whole body surface except for the branchial crown (Fig. 2D). Interramal eyespots (Fig. 2C), which were quite small compared to body width, were present from chaetiger 2 (except for identifier GK2276) or 3 (GK2276) to subsequent chaetigers.

The whole body of the Fukushima specimen was 13.0 mm in length with eight thoracic and 41 abdominal chaetigers (Fig. 3). Branchial crowns were 3.0 mm in length. Each half of the branchial crown included 16 radioles. Approximately ten paired stylodes and basal unpaired stylodes were present on each radiole. Stylodes were short, broad basally, and not as slender as the Shirahama specimens (Fig. 3D). The tip of stylodes was rounded. The size differs slightly among stylodes (Fig. 3D). The body color changed by fixation; the body of the living specimen was orangish yellow while rather whitish after fixation by 99% ethanol (Fig. 3A, 3F). Purplish spots, which were rather evident in the fixed specimen, were sporadically present on the whole body surface except for the branchial crown (Fig. 3E, F). Interramal eyespots were present from chaetiger two and subsequent chaetigers; small compared to body width before fixation (Fig. 3B) whereas larger after fixation (Fig. 3C).

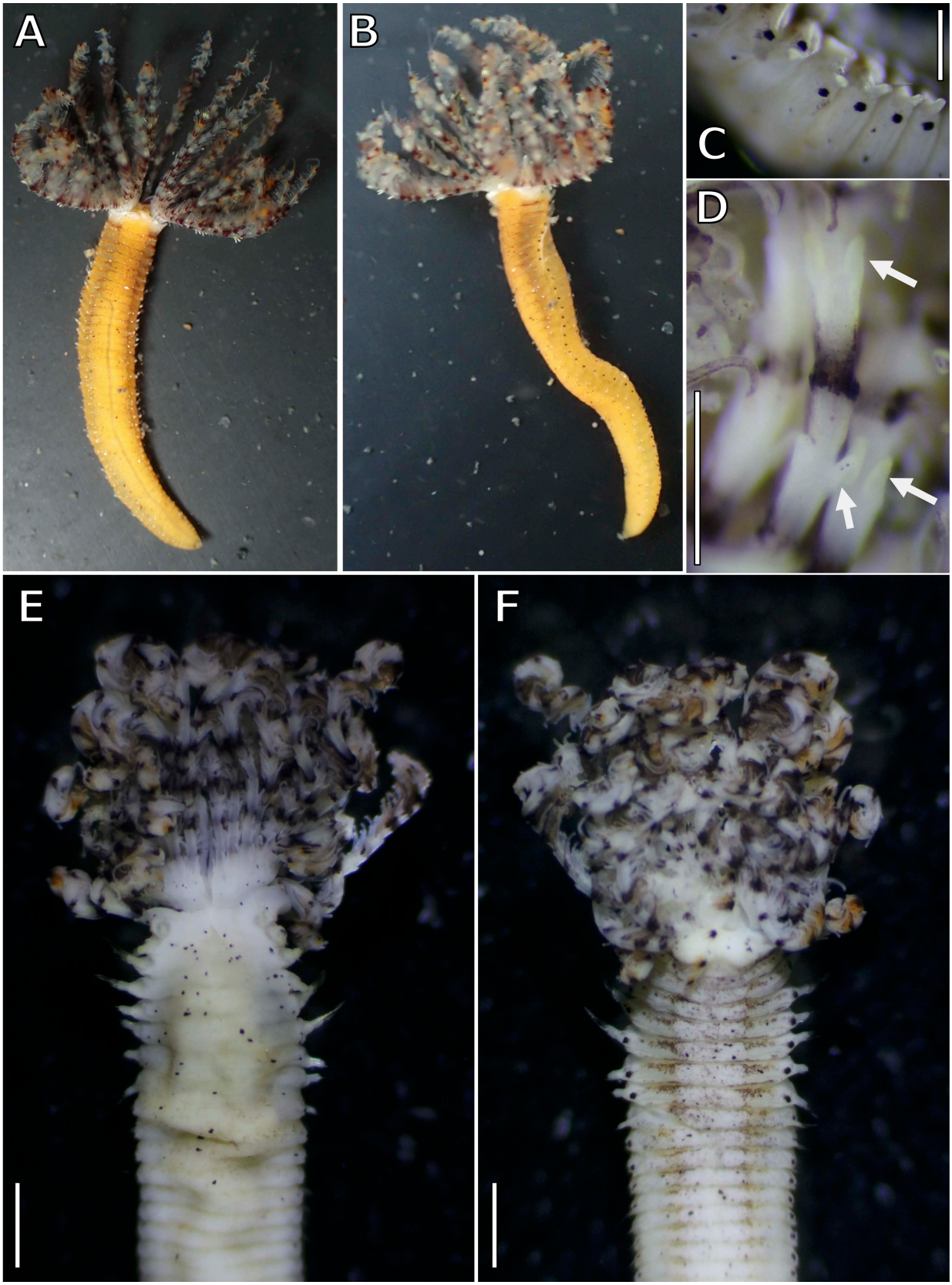

Figure 3. Photographs of *Branchiomma* sp. I. **A**, **B**, living specimen; **C**–**F**, fixed specimen. **A**, whole specimen, ventral view. **B**, whole specimen, lateral view. **C**, close-up view of the lateral body, showing interramal eyespots. **D**, close-up view of stylodes (arrows) on radioles. **E**, anterior part of the body, dorsal view. **F**, anterior part of the body, ventral view. Scale bars = 500 µm (C, D) and 1 mm (E, F) (no scales for A and B).

The monophyly of each ingroup species (eight clades) was highly supported by the phylogenetic analysis based on the cytb gene sequences (ultrafast bootstrap value, ufBS = 93–100) except for four singleton taxa, *Branchiomma bombyx*, *Branchiomma luctuosum*, *Branchiomma* sp. H, and the Fukushima specimen (Fig. 4). The specimens collected from Shirahama were included in the clade of *Branchiomma* sp. B (ufBS = 99) (Fig. 4). *Branchiomma* sp. B was sister to *B. luctuosum* (ufBS = 96), and this clade was sister to *Branchiomma* sp. A (ufBS = 81). The Fukushima specimen was sister to *B. lucullanum* in the phylogenetic tree (p-distance between the two species was 34%), but its phylogenetic position remained uncertain due to the low support value (ufBS = 75).

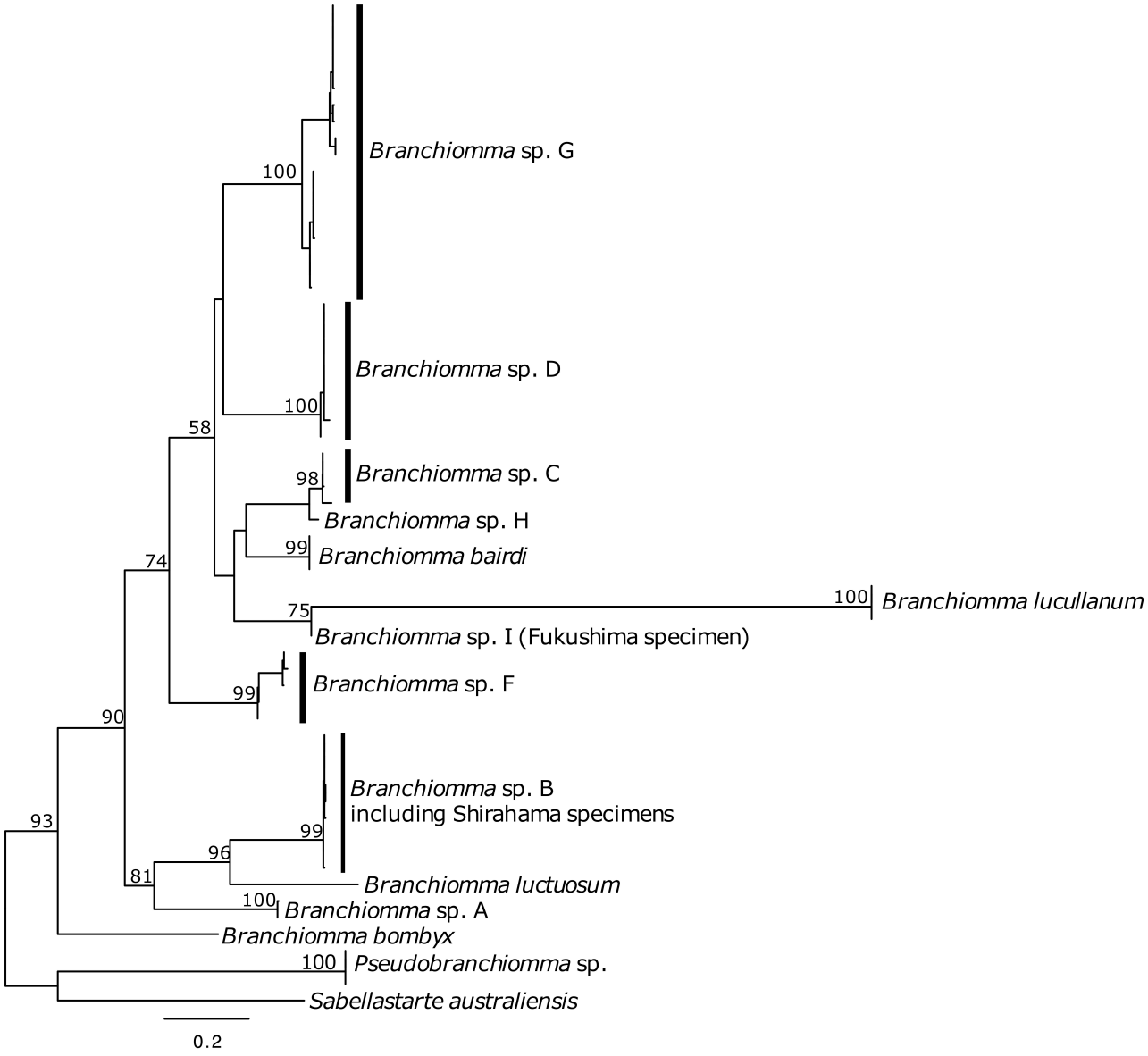

Figure 4. ML phylogenetic tree of *Branchiomma* spp. based on the partial nucleotide sequences of the mitochondrial cytb gene (387 characters). Numbers above the branches indicate ultrafast bootstrap values (≥ 50%; values for intra-species abbreviated).

Four haplotypes were obtained from five cytb sequences *Branchiomma* sp. B collected from Shirahama. The haplotype network revealed low genetic variation among *Branchiomma* sp. B specimens collected from Florida, Hawaii, Saipan, Australia, and Japan (Fig. 5). One of the variable sites, at position 254 of the alignment (C in identifier GK1876 or T in the other specimens), was the 1st codon and resulted in an amino acid substitution (lysine and phenylalanine, respectively).

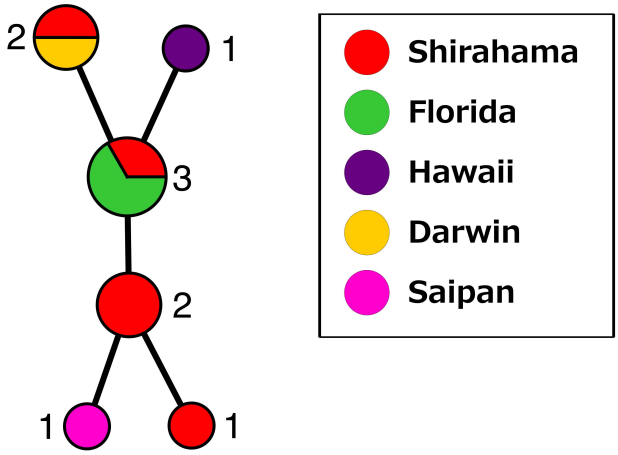

Figure 5. Haplotype network of *Branchiomma* sp. B sensu Capa et al. (2013) based on the mitochondrial cytb gene partial nucleotide sequences with the degenerate bases in the GenBank sequences were trimmed (344 characters). A line indicates a single nucleotide substitution. Numbers indicate sample size.

Discussion

In the present study, the gene sequences of *Branchiomma* spp. were obtained for the first time from Japan. The single specimen collected from Fukushima was genetically distinct from all the *Branchiomma* sequences available in GenBank (Fig. 4). The lineage is here tentatively designated as "*Branchiomma* sp. I," which is an addition to the tentative names for the unidentified species of *Branchiomma* given by Capa et al. (2013). Molecular phylogenetic analysis based on the mitochondrial cytb gene confirmed the specimens of *Branchiomma* collected at Shirahama, Japan as an unidentified species "*Branchiomma* sp. B" sensu Capa et al. (2013). This species has been widely recorded from the Pacific (Australia, Hawaii, and Saipan) and the Atlantic (Florida) (Capa et al. 2013). The result of the present study represents the first record of *Branchiomma* sp. B from Asia and further expands its geographic range to north.

Since the traditional morphological diagnostic characters for the species of *Branchiomma*, including the presence/absence of macrostylodes (stylodes more than twice as long as contiguous ones; Capa et al., 2013), are considered unuseful due to the intraspecific variation and/or interspecific similarity (Capa et al., 2013), species identification based solely on morphology proved unfeasible in the present study. The morphology of both *Branchiomma* sp. B and *Branchiomma* sp. I differs from *B. cingulatum*, the only species previously recorded from Japan, particularly in the shape of stylodes; they are slender in both *Branchiomma* sp. B and *B. cingulatum* but stout in *Branchiomma* sp. I; macrostylodes are present in *Branchiomma* sp. B of the present study but they are lacking in *Branchiomma* sp. I and *B. cingulatum* (see Fitzhugh, 2002; Ishaq and Mustaquim, 1996; Knight-Jones et al., 1991). On the other hand, the size of the stylodes of the specimen of *B. cingulatum* illustrated by Okuda (1939; fig. 14 in p. 242), which possesses both macrostylodes and microstylodes, resembles those of *Branchiomma* sp. B observed in the present study. The relative size of interramal eyespots to body size differs between *Branchiomma* sp. B and *Branchiomma* sp. I, although the relative size is larger after fixation in *Branchiomma* sp. I (not examined in *Branchiomma* sp. B) and should be careful when considering it as a taxonomically informative character. The comprehensive taxonomic revision of the genus and subsequent detailed morphological examination of the Shirahama specimens would therefore be necessary to determine whether *B. cingulatum* previously recorded from Japan and *Branchiomma* sp. B are indeed conspecific.

Although *B. lucullanum*, which was sequenced by Capa et al. (2013), was sister to *Branchiomma* sp. I with low support value, they were regarded as a distinct species, since the genetic distance between these two lineages was large. *Branchioma lucullanum* sensu Capa et al. (2013) and *Branchiomma* sp. I were also distinct in morphology; the former possesses flattened, tongue-like, and/or dendritic stylodes and 4–5 thoracic chaetigers and these morphological characters differ from those of the latter. *Branchiomma lucullanum* reported in the previous studies, however, is noted to possess slender stylodes (both slender and flattened stylodes depicted in fig. 68 of Rioja, 1923) and eight thoracic chaetigers (Fauvel, 1927; Knight-

Jones et al., 1991; Licciano and Giangrande, 2008; Rioja, 1923). Such inconsistency in the morphological characters of *B. lucullanum* suggests the possibility that the species in Capa et al. (2013) and the other studies are different species or it includes intraspecific variations in these morphological characters.

Branchiomma sp. B of Japan and other oceans (Florida, Hawaii, Saipan, and Australia) exhibit no distinctive differences in population genetics using cytb gene sequences (Fig. 5). To answer whether the widely distributed *Branchiomma* sp. B is native to Japan or not, further population genetic analysis with highly informative genetic markers and encompassing more specimens would be necessary. The nucleotide sequences of individuals collected from Florida, Hawaii, Saipan, Australia, and Japan displayed minor variations, with 3 bp differences in 344 bp at most (0.87%). Although two different amino acid sequences with one substitution were found in *Branchiomma* sp. B, this variation may not be attributable to geographic differentiations, because both sequences were found at Shirahama.

Some species of *Branchiomma* are considered invasive, which can explain their wide geographic distributions despite the low dispersal potential associated with the known developmental modes of *Branchiomma* spp. [lecithotrophic development with short larval stage (Berrill, 1977; Del Pasqua et al., 2017; Licciano et al., 2002) or brooders (Dragesco-Kernéis 1980; Tovar-Hernández et al. 2011); see Capa et al., 2013]. Asexual reproduction by scissiparity as reported for *Branchiomma bairdi* (Tovar-Hernández et al., 2009) is suggested as possible mechanism leading to the formation of the large aggregations of the species on artificial surfaces, including buoys, hulls of ships, or docs in ports. This mode of reproduction may also facilitate the aggregation of other *Branchiomma* spp. (Capa et al., 2013), including *Branchiomma* sp. B in Tanabe Bay. This feature may contribute to the successful invasion of this species as an exotic species into other waters. If *Branchiomma* sp. B is indeed an invasive species in Japan, understanding and monitoring its distribution in Japan would be crucial to mitigate its spread and ecological impacts; that is, *Branchiomma* sp. B aggregates on hard substrates (a mooring rope in the case of the present study) and can trigger the environmental modification to create microhabitats for other species.

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