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Meiobenthic Polychaete *Dinophilus* sp. cf. *gyrociliatus* (Annelida: Dinophilidae) from Japan with SEM Observation and DNA Barcodes

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Tiny annelids identified as the marine meiobenthic polychaete *Dinophilus gyrociliatus* (Schmidt, 1857) have been reported not only from shallow water sediments but also artificial environments such as experimental aquaria almost all around the world; the species has thus been regarded to show a cosmopolitan distribution. However, various types of ciliary-band arrangements and genetic sequences have been reported from different populations, leading to a doubt on the species' taxonomic identity. In this study, we present results of our SEM observation of *Dinophilus* sp. cf. *gyrociliatus* from Japan and provide mitochondrial 16S ribosomal RNA and cytochrome *c* oxidase subunit I gene sequences of Japanese populations as DNA barcodes for contributing to resolving the taxonomic uncertainty of "*D. gyrociliatus*".

Key Words: Asamushi, DNA barcoding, meiobenthos, Misaki, Polychaeta, taxonomy.

Introduction

Dinophilus gyrociliatus (Schmidt, 1857) is an interstitial annelid species of extremely tiny worms. While the adult females look similar to polychaete juveniles (Worsaae and Kristensen 2005; Struck *et al.* 2015), the dwarf males show a resemblance to trochophore larvae (Nelson 1907). The genus *Dimorphilus* Worsaae, Kerbl, Vang, and Gonzalez, 2019 was erected by Worsaae *et al.* (2019) for dinophilid species that bear strongly dimorphic dwarf males. However, the online publication (Worsaae *et al.* 2019) does not contain any evidence in the work itself that its ZooBank registration has occurred, and thus violates amended Article 8.5 of the International Code of Zoological Nomenclature (International Commission on Zoological Nomenclature 2012). Therefore, the genus-group name *Dimorphilus* is deemed to be unavailable; "*Dimorphilus*" species *sensu* Worsaae *et al.* (2019) should be placed under *Dinophilus* until when the genus-group name "*Dimorphilus*" is validated under the provisions of the Code.

Dinophilus gyrociliatus has been used in morphological and developmental studies as a model organism owing to their rapid life cycle and easiness in breeding (Kerbl *et al.* 2016). This species was originally described from Tórshavn (Thorshaven), the Faroe Islands (Schmidt 1857), and has subsequently been reported from all over the world (*e.g.*, Wu *et al.* 1980; Lisitskaya and Boltachova 2016), including Japan (Sudzuki and Sekiguchi 1972). Uchida (1972) reported dinophilids from Japan under the name *D. conklini* (Nelson, 1907), which was established based on material collected from sea-water aquaria of the University of Pennsylvania (Nelson 1907), but has been synonymized with *D. gyrociliatus* (Shearer 1912; Fauvel 1927). However, there are differences in the pattern of the ciliary-band arrangement among previously reported populations of "*D. gyrociliatus*" (*e.g.*, Jones and Ferguson 1957), indicating that it may contain several cryptic species. Comparative studies about the pattern of the ciliary bands and DNA sequences among "*D. gyrociliatus*" populations from all over the world are necessary to resolve this taxonomic uncertainty.

The second and third authors found tiny polychaete worms that resembled *Dinophilus gyrociliatus* from experimental aquaria. In this study, we report the worms as *Dinophilus* sp. cf. *gyrociliatus* along with photographs of our SEM observation. We provide DNA sequences of the mitochondrial 16S ribosomal RNA gene (*16S*) and cytochrome *c* oxidase subunit I (*COI*) regions as DNA barcodes for contributing to the future taxonomic revision of "*D. gyrociliatus*".

Materials and Methods

Worms were collected from experimental aquaria at the Research Center for Marine Biology (RCMB), Tohoku University, Japan, and the Misaki Marine Biological Station (MMBS), The University of Tokyo, Japan. The aquarium at the RCMB contained sediment from a tidal flat in

Asadokoro, Aomori, Japan, and was filled with sea water drawn from Asamushi, Aomori, Japan. The aquarium at the MMBS contained egg masses of a cladobranchian sea slug *Pteraeolidia semperi* (Bergh, 1870) (Mollusca: Gastropoda: Nudibranchia) collected from Misaki, and was filled with sea water drawn from Misaki, Kanagawa, Japan. The live specimens were anesthetized and fixed in 10% formalin-seawater. Live or preserved specimens were observed with a stereomicroscope (Nikon SMZ1500) and compound microscopes (OLYMPUS BX51, Nikon Optiphot-2, and Leica DM IL), 12 (RCMB) and six (MMBS) of which were processed for SEM. Specimens for SEM observations were washed in deionized water and dehydrated in a graded ethanol series, dried in a critical-point dryer (HITACHI HCP-2) using liquid CO_2, and coated with gold in an ion sputter (HITACHI E-1045). Observations were conducted using a SEM instrument (HITACHI S-3000N).

Some of the worms were cultured from one parent for molecular work. DNA extraction and sequencing were carried out following the method of Jimi and Fujiwara (2016). Genetic distance calculation followed Jimi *et al.* (2016). Newly obtained sequences have been deposited in the DNA Data Bank of Japan (DDBJ).

Results and Discussion

Dinophilus* sp. cf. *gyrociliatus Schmidt, 1857
(Figs 1–4)

?*Dinophilus conklini*: Uchida 1972: 29–34, fig. 1 [from an aquarium in Hokkaido University, which contained bryozoans collected from Akkeshi, Hokkaido, Japan, 2 m in depth].

Material examined. ICHUM 6114, 12 female specimens, mounted on a stub for SEM observation, 25 May 2017, collected by the second author, from an aquarium of the Research Center for Marine Biology, Tohoku University. ICHUM 6115, 6 female specimens, mounted on a stub for SEM observation, 2 March 2019, collected by third author, from an aquarium of the Misaki Marine Biological Station, The University of Tokyo. Non-deposited specimens, 20 female specimens, whole specimens were used for molecular work, cultured from one parent specimen that was collected from the aquarium by the second author.

Brief description. *Female.* Body about 500–900 µm long, 100–250 µm wide in the cephalic part, cylindrical, segmentation obscure (Figs 1A, 2A, 3A), whitish or transparent in life (Fig. 1A, B), whitish in ethanol, no pigmentation. Prostomium rounded, palps and tentacles absent. Eyes present, one pair, reddish in life, oval (Fig. 1A). One pair of prosto-

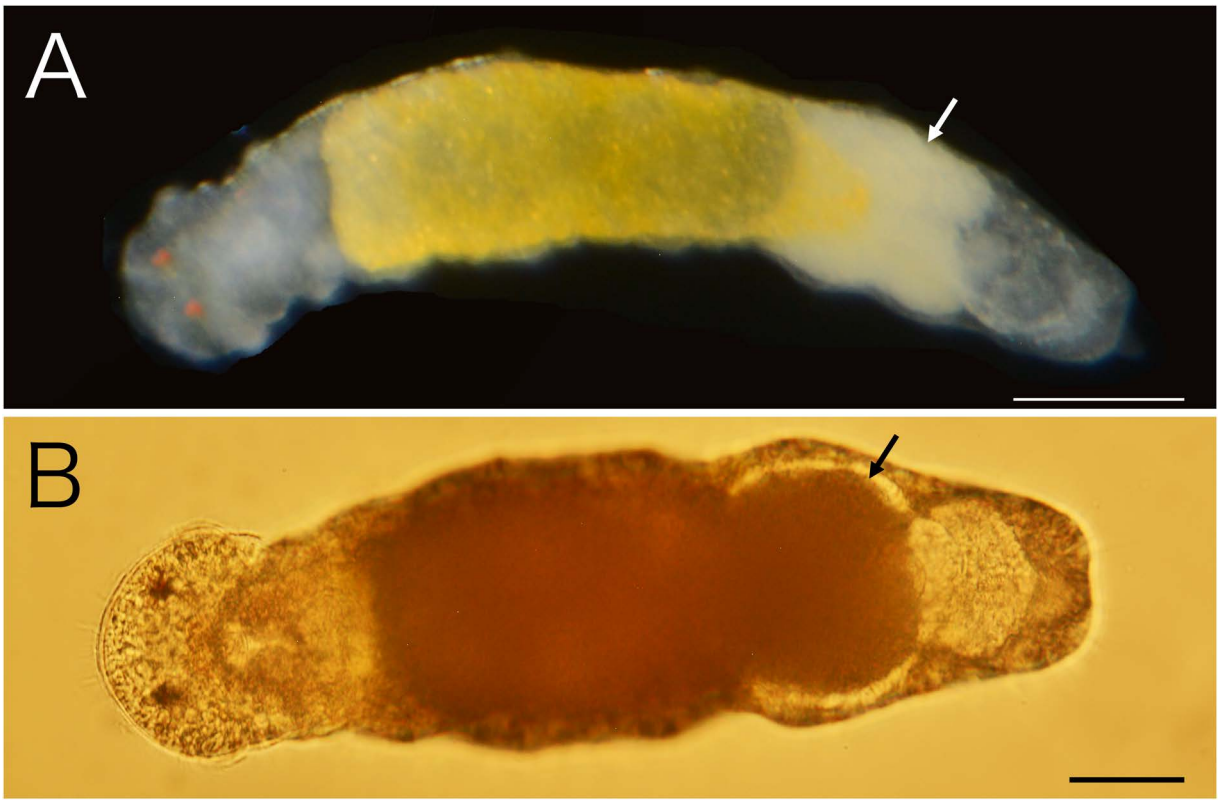

Fig. 1. *Dinophilus* sp. cf. *gyrociliatus*, live female specimen (RCMB, non-deposited specimen, cultured from the same parent of the specimens that was used for the molecular work). A, Stereoscopic microscope image, dorsal view; B, light microscopic image, dorsal view. Arrows indicate eggs. Scale bars: A, B, 100 µm.

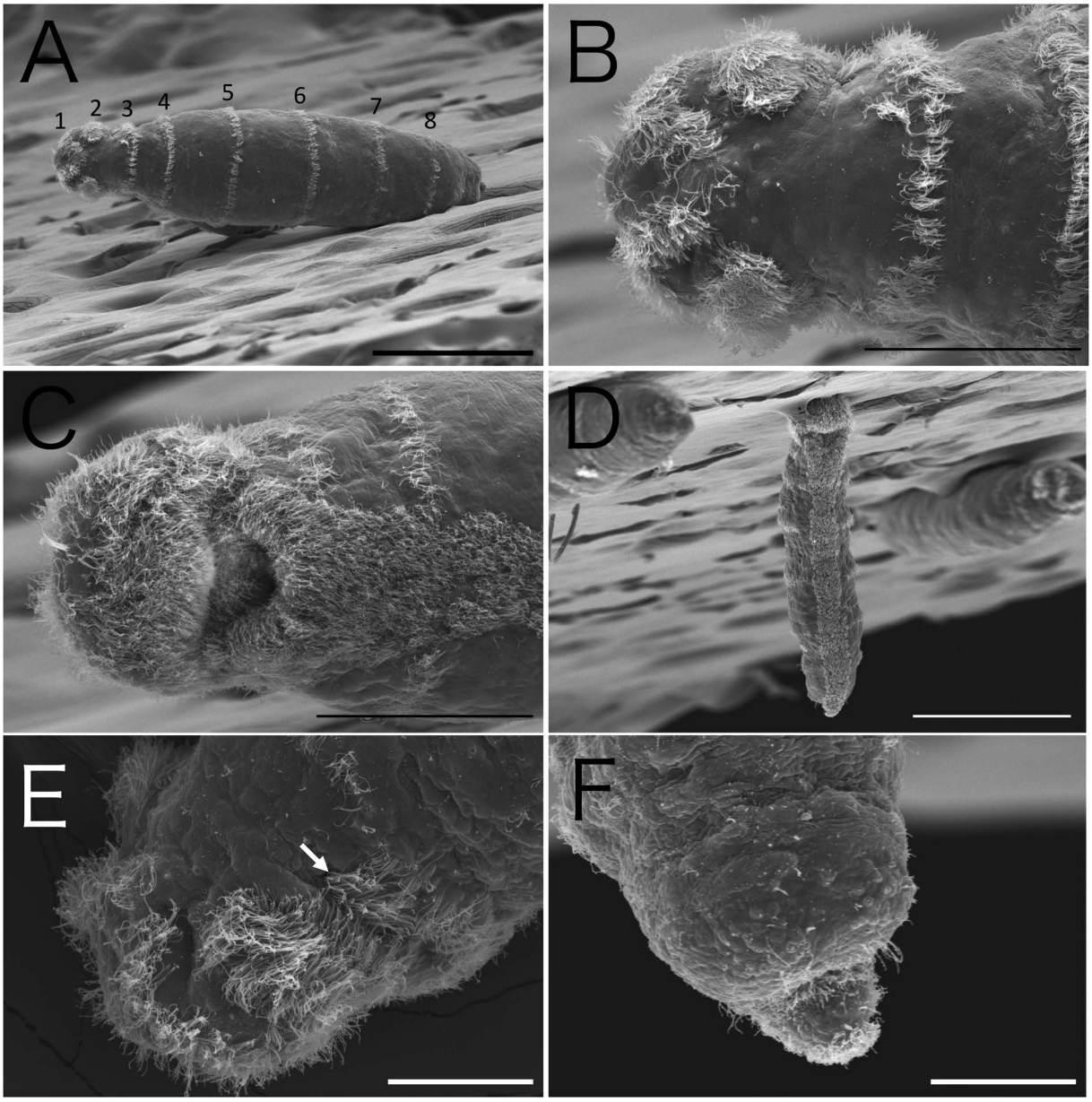

Fig. 2. SEM images of *Dinophilus* sp. cf. *gyrociliatus* (RCMB, ICHUM-6114). A, Whole specimen, dorsal view, numbers indicate ciliary bands; B, anterior end, dorsal view; C, anterior end, ventral view; D, whole specimen, ventral view; E, anterior end, dorsolateral view, showing nuchal organ (arrow); F, posterior end, dorsal view. Scale bars: A, D, 300 µm; B, C, 100 µm; E, F, 50 µm.

mial compound cilia present on apical head (Figs 2B, 3B). Eight transverse ciliary bands on dorsal side: first band V-shaped from dorsal view, with its backward-directing corner having only a few cilia on median line; second band having wide gap on its mid-dorsal portion, band present only outside of two eyes (Figs 1A, 2B, 3B); six posterior bands, with one band in each segment, having no mid-dorsal gap (Figs 2A, 3A). Ventral side of cephalic region ciliated (Figs 2C, 3C). Single, continuous, mid-ventral, longitudinal ciliary band running throughout body length (Figs 2D, 3D). A pair of nuchal organs present between second and third ciliary bands (Fig. 2E). Pygidium conical; anus situated dorsally; several cilia present around anus (Fig. 2F). Stomach yellowish. Eggs in posterior part of body (Fig. 1A).

Male. Body about 30–50 µm long, 20–40 µm wide, transparent in life, oval, like a trochophore larva of annelids (Fig. 4A). Anterior end and all ventral side ciliated (Fig. 4B). Male inhabits in an egg capsule together with a female larva (Fig. 4C) and remains after the female gets out of the capsule (Fig. 4D).

Genetic sequence. Sequences of *16S* (RCMB, DDBJ LC545953, 455 bp; MMBS, DDBJ LC545954, 455 bp) and *COI* (RCMB, DDBJ LC545951, 658 bp; MMBS, DDBJ LC545952, 658 bp) were determined from a female in the present material of *Dinophilus* sp. cf. *gyrociliatus*. Sequences of RMCB specimens differ by 0.009 (*16S*) and 0.118 (*COI*) in terms of K2P distance from MMBS specimens. Our sequences differ by 0.019 (*16S*) and 0.148–0.174 (*COI*) in terms of K2P distance from China and USA specimens (Table 1) (Dahlgren *et al.* 2001; David and Halanych 2017).

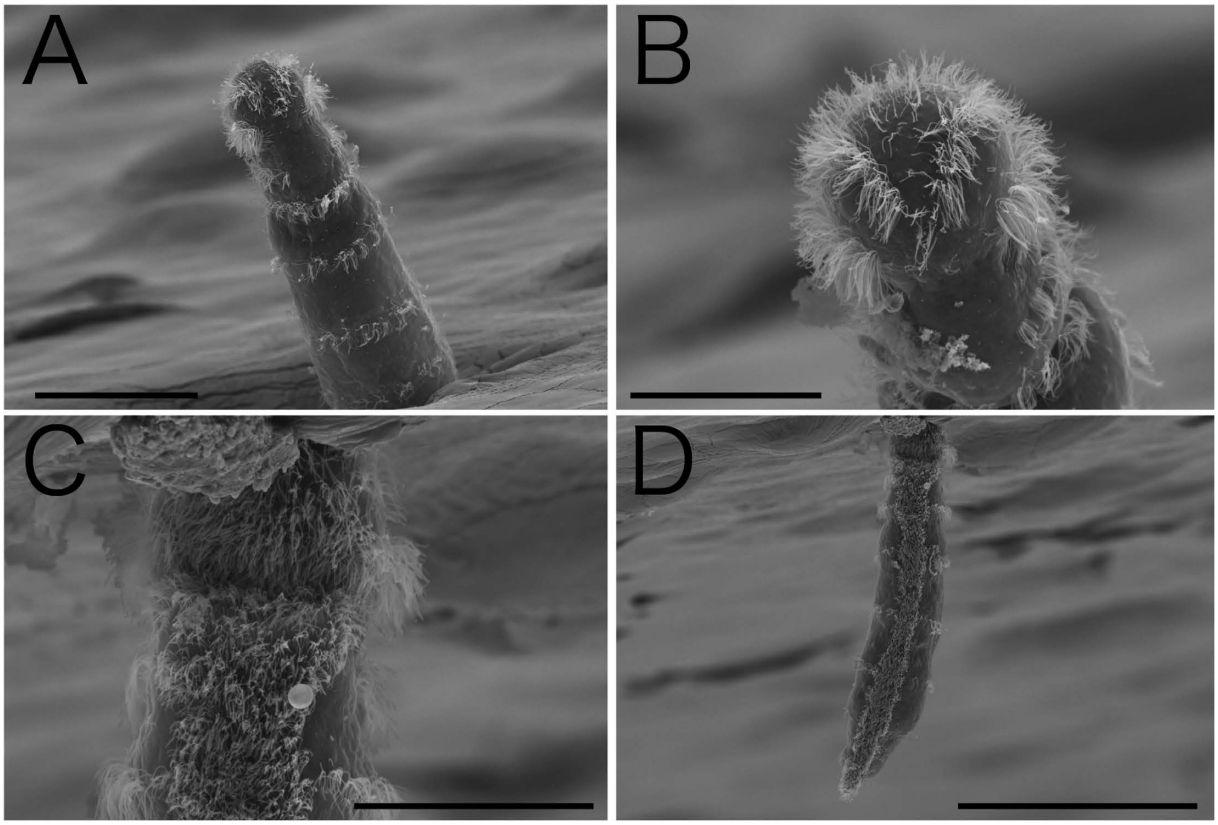

Fig. 3. SEM images of *Dinophilus* sp. cf. *gyrociliatus* (MMBS, ICHUM-6115). A, Whole specimen, dorsal view; B, anterior end, dorsal view; C, anterior end, ventral view; D, whole specimen, ventral view. Scale bars: A, 100 μm; B, C, 50 μm; D, 200 μm.

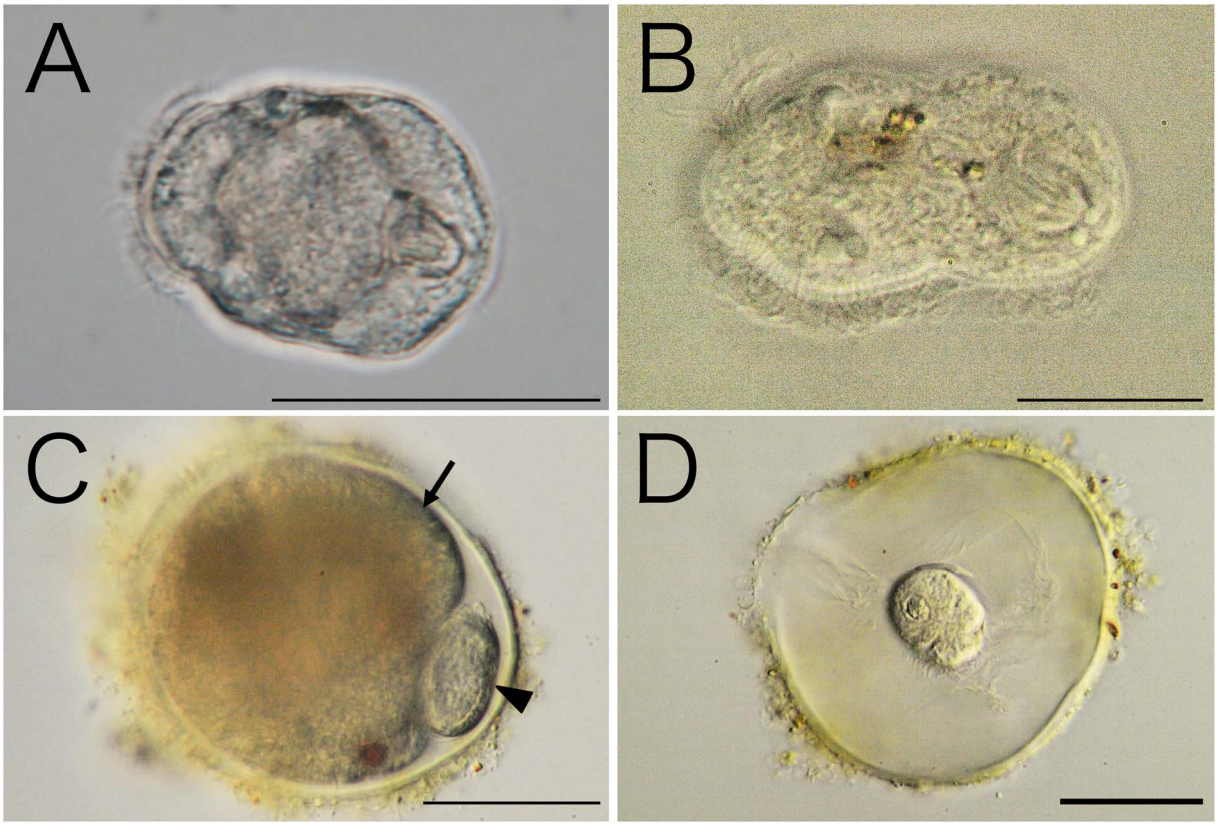

Fig. 4. *Dinophilus* sp. cf. *gyrociliatus*, live specimens (RCMB, no voucher remains) cultured from the same parent of the specimens that was used for the molecular work. A, Dwarf male, dorsal view; B, dwarf male, lateral view; C, dwarf male (arrowhead) in an egg capsule together with a female (indicated by an arrow); D, dwarf male in an egg capsule left by a female. Scale bars: A, C, D, 50 μm; B, 20 μm.

Table 1. Genetic distances of *Dinophilus* sp. cf. *gyrociliatus*.

Collection locality (COI/16S)	RCMB	MMBS	USA	China
RCMB	—	—	—	—
MMBS	0.118/0.009	—	—	—
USA	0.174/0.019	0.148/0.019	—	—
China	—/0.019	—/0.019	—/0	—

Remarks. We could not find any morphological difference between the RMCB and MMBS specimens. Our morphological data suggest that our material is likely to be the same as *Dinophilus conklini sensu* Uchida (1972), but may be different from *D. conklini s. str.* and *D. gyrociliatus s. str.* Assessing the precise taxonomic identity of our material requires detailed information about morphology and DNA barcode for *D. conklini s. str.* and *D. gyrociliatus s. str.* These '*s. str.*' data should preferably be based on topotype specimens for each nominal species; namely, from Northeastern US (*D. conklini*) and the Faroe Islands (*D. gyrociliatus*), respectively. Unfortunately, such data are currently unavailable, making our taxonomic decision inconclusive. Meanwhile, we follow Shearer (1912) and Fauvel (1927) to regard *D. conklini* as synonymous with *D. gyrociliatus*.

The morphological similarity between our material and *Dinophilus conklini sensu* Uchida (1972) pertains the arrangement of ciliary bands in that *i*) the first band is V-shaped from dorsal view; *ii*) mid-dorsally, there is only a few cilia at the corner of the V-shaped first band; *iii*) there is a distinct gap on the mid-dorsal portion of the second band; and *iv*) the eyes are situated within the range of this gap, although slightly anterior to the level of the second band. The last character state is different from that of *D. conklini s. str.*, in which the eyes may be situated outside the range of the gap (Nelson 1907). Our present specimens from Asamushi showed sexual dimorphism, and thus are referable to *D. gyrociliatus s. lat.* Morphologically, our material possessed *i*) one ciliary band per a segment and *ii*) six posterior bands, which are also characteristic of *D. gyrociliatus s. lat.* (Nelson 1907; Jones and Ferguson 1957). *Dinophilus conklini* was supposed to be different from *D. gyrociliatus* by having a mid-dorsal gap on the first and second ciliary bands, but some of the previous researchers (*e.g.*, Shearer 1912; Fauvel 1927) regarded these gaps as representing intraspecific variation and synonymized *D. conklini* with *D. gyrociliatus*.

As of writing, there are two *16S* entries (AF380116: Dahlgren *et al.* 2001; MG428625, partial mitochondrial genome: David and Halanych 2017) available in public databases that are referable to the genus *Dinophilus*. Their source materials, both identified as *D. gyrociliatus*, were collected in Xiamen, China (Dahlgren *et al.* 2001) and Beaufort, North Carolina, USA (David and Halanych 2017), instead of the Faroe Islands, the type locality of the species (Schmidt 1857); the sequences from China (AF380116) and the USA (MG428625) are exactly the same. The genetic distance between the *16S* sequences from Asamushi and China/USA was 0.019 (K2P), approaching those values for interspecific variations recorded for nine other annelid families (0.012–0.26) (Nygren 2014). These values imply that those dinophilids may represent biologically different 'species,' but more data should corroborate any conclusion. Measuring intra- and interspecific variations—in terms of both molecular sequence and morphology—is indispensable for future DNA taxonomy in *Dinophilus*. Besides, the species may be truly cosmopolitan, invasive species, or contamination (*cf.* Worsaae *et al.* 2019) occurring in China and USA. In this paper, we regarded the Japanese specimens as a single species. However, future studies are necessary for verifying the taxonomic identity of "*D. gyrociliatus*" worldwide.

Sudzuki and Sekiguchi (1972) reported dinophilids under the name of *D. gyrociliatus* from an aquarium of Tokyo University of Education (now University of Tsukuba), Tokyo, Japan. Sudzuki and Sekiguchi (1972) wrote that "All the ventral side of the body are densely ciliated", which is also illustrated with line drawings (Sudzuki and Sekiguchi 1972: text-fig. 10F, H). They are thus different from our specimens, in which the mid-ventral ciliary band is more or less restricted to the median line (Fig. 2C, D).

Uchida (1972) argued that the ciliary-band morphology would be consistent within a species, and that it could be a useful character to discriminate different species in *Dinophilus*. To test Uchida's (1972) hypothesis, DNA-taxonomic studies should be performed based on a number of specimens from as wide geographic area as possible, each of which must be examined in terms of the ciliary pattern with SEM. Such studies will confirm the taxonomic utility of the ciliary-band pattern, elucidating the extent of the intra- and interspecific variation. For proper application of the species names, topotype specimens for the existing nominal taxa should also be included in future studies.

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