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Diplocirrus nicolaji (Annelida: Flabelligeridae) from Japan, detailed morphological observation and DNA barcoding

Naoto Jimi[1,2*], Masaatsu Tanaka[3] and Yoshihiro Fujiwara[1,2]

Abstract

Diplocirrus nicolaji is a flabelligerid worm originally described from shallow waters of Peter the Great Bay, Sea of Japan. Later, this species was also recorded from Japan, but detailed data were not provided; thus the occurrence of *D. nicolaji* in Japan needs to be confirmed based upon additional material. The collection of 20 *D. nicolaji* individuals from four localities along Japanese coasts (the Sea of Japan and western Pacific Ocean), allowed us to provide detailed morphological observations using stereoscopic and scanning electron microscopy. Partial cytochrome c oxidase subunit I sequences were obtained for phylogenetic analysis. Although the morphological analysis detected a few variations in palp length and body colour in ethanol among the local populations, the phylogenetic analysis confirmed their conspecificity with little genetic divergence. This is the first report of *D. nicolaji* from the western Pacific Ocean and extends its distribution southward.

Keywords: Polychaete, *Diplocirrus nicolaji*, DNA barcoding, Japanese waters

Background

The annelid family Flabelligeridae is generally recognized as a sedentary group living within soft sediments or hard substrates (Rouse and Pleijel 2001); however, recent morphological and molecular phylogenetic studies show that the family also contains some holopelagic species (Burnette et al. 2005; Osborn and Rouse 2008; Salazar-Vallejo et al. 2008). In Japan, the records of flabelligerids are relatively few and scattered. To our knowledge, the following 13 species of Flabelligeridae have so far been recorded from Japanese waters: *Brada inhabilis*, *Br. mammillata*, *Br. ochotensis*, *Br. villosa*, *Buskiella vitjasi*, *Daylithos parmatus*, *Flabelligera affinis*, *Pherusa moorei*, *Ph. nipponica*, *Ph. papillata*, *Piromis suni*, *Semiodera nishii*, and *Stylarioides longisetosa* (Imajima 1964, 2006, 2009 and Hartman 1964; Uchida 1992; Jirkov 2001; Salazar-Vallejo 2011a, b, 2012, 2014; Miura 2014).

The flabelligerid genus *Diplocirrus* Haase, 1915 is characterized by having a club-shaped body, two types of retractable branchiae, and multiarticulate chaetae in both parapodia rami. After the comprehensive taxonomic revision by Salazar-Vallejo and Buzhinskaja (2011), the two monotypic genera *Bradiella* Rullier, 1965 and *Diversibranchius* Buzhinskaja, 1994 were synonymized with *Diplocirrus*; consequently, the genus currently consists of 13 species, of which three are still undescribed (Salazar-Vallejo and Buzhinskaja 2011), that live on soft bottoms in sublittoral depths worldwide.

Diplocirrus nicolaji (originally combined with the newly established genus *Diversibranchius*) was described based on specimens collected from shallow waters of Peter the Great Bay, the Sea of Japan, Russia (Buzhinskaja 1994). Thereafter, an unidentified "Flabelligeridae from Japan" was illustrated in Rouse and Pleijel (2001), which was later identified as *D. nicolaji* by Salazar-Vallejo and Buzhinskaja

* Correspondence: beniimo7010@gmail.com
[1]Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima 739-8528, Japan
[2]Department of Marine Biodiversity Research, Japan Agency for Marine-Earth Science and Technology, Yokosuka 237-0061, Japan
Full list of author information is available at the end of the article

(2011) on the basis of their reexamination of the photographed material of Rouse and Pleijel (2001) (Salazar-Vallejo, personal communication). However, neither Rouse and Pleijel (2001) nor Salazar-Vallejo and Buzhinskaja (2011) provided the precise collection sites of their material; no other record of this species has been reported from Japanese waters. Thus, the distribution of *D. nicolaji* in Japan needs to be clarified.

During the course of a survey of Japanese polychaete fauna by the first and second authors, some flabelligerid worms were collected from four localities of Japan (Oshoro, Tateyama, Misaki, and Komatsubara). After morphological observation, we identified these flabelligerids as *D. nicolaji*. In this paper, we report the morphology of these Japanese *D. nicolaji* specimens including observations of the detailed structure of the taxonomic characters using a scanning electron microscope (SEM). In addition, we also obtained partial mitochondrial cytochrome *c* oxidase subunit I (*COXI*) sequences for conducting phylogenetic analysis and for contributing to DNA barcoding of Flabelligeridae.

Methods

Materials

Worms were collected in Oshoro, Hokkaido (43°12'33"N, 140°51'30"E) (Fig. 1a) and Komatsubara beach, Hiroshima prefecture, Seto Inland Sea (34°16'42"N, 132°47'16"E) (Fig. 1b) by hand from shallow waters (2-m depth), and in Tateyama Bay, Chiba prefecture (34°59'04.7"N, 139° 48'57.4"E) (Fig. 1c) at a depth of 4–10 m and Misaki, Kanagawa prefecture, Sagami Bay (35°09'30"N, 139° 36'46"E) (Fig. 1d) at a depth of 4 m by a dredge. All the specimens were first anesthetized with menthol and then fixed and preserved in 70 % ethanol. The anesthesia durations were different among each specimen.

Morphological observations

Preserved specimens were observed with an MZ 16 F stereoscopic microscope (Leica, Germany), two of which were treated for SEM. The specimens for SEM observations were washed in filtered artificial seawater and post-fixed with 2 % OsO_4/artifical seawater for 2 h. After six washes with deionized water (DW), the specimens were incubated with 0.2 % aqueous tannic acid (pH 6.8) for 30 min for conductive staining. The specimens were washed again

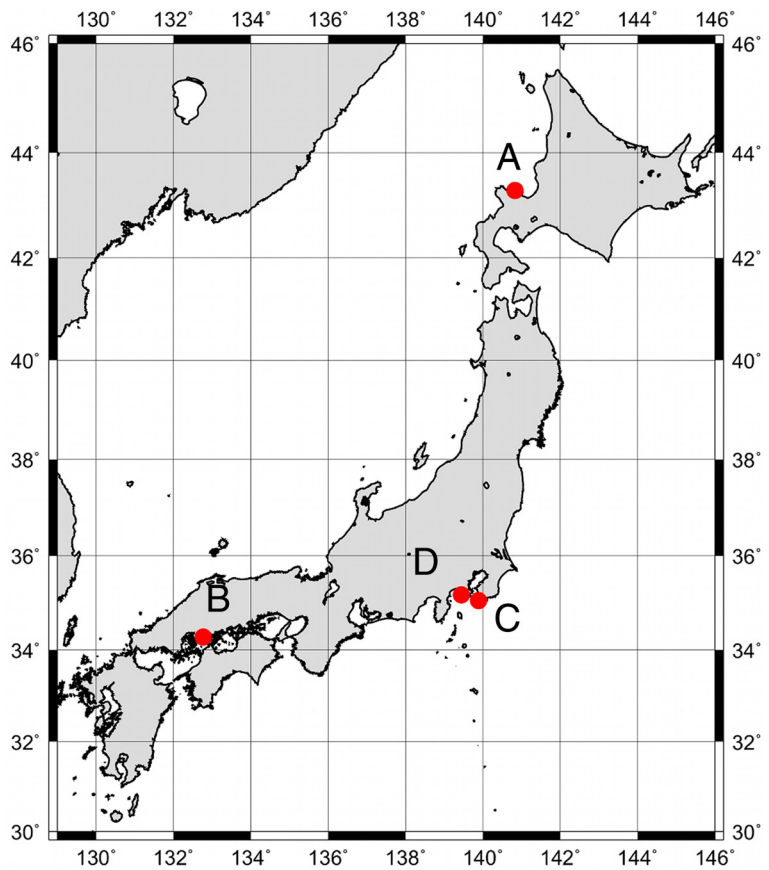

Fig. 1 Sampling locations of *Diplocirrus nicolaji* in the present study. **a**: Oshoro; **b**: Komatsubara; **c**: Tateyama; **d**: Misaki

with DW, treated with 1 % OsO_4/DW for 30 min, and then washed with DW. The specimens were dehydrated in a graded ethanol series, dried in a JCPD-5 (JEOL, Japan) critical point dryer using liquid CO_2, and coated with osmium in a POC-03 (Meiwafosis, Japan) for 5 s. SEM observations were conducted using a JSM-6700 F instrument (JEOL, Japan).

DNA extraction, amplification, sequencing, and phylogenetic analysis

The total genomic DNA was extracted from eight specimens using a DNeasy Blood & Tissue Kit (Qiagen, USA) following the manufacturer's protocol. Partial *COXI* (ca. 650-bp) sequences were amplified by the polymerase chain reaction (PCR) with the primers polyLCO (5'-GAYTATWTTCAACAAATCATAAAGATATTGG-3') and polyHCO (5'-TAMACTTCWGGGTGACCAAAR AATCA-3') (Carr et al. 2011). The reaction mixture consisting of 0.25 μl TaKaRa Ex Taq (Takara, Japan), 5 μl of 10 × Ex Taq Buffer (Takara), 4.0 μl dNTP mixture (Takara), 5 μl of each primer pair (10 μM), 0.75 μl of extracted DNA, and 35 μl of distilled water was prepared. The PCR protocol consisted of an initial denaturation step at 94 °C for 1 min, followed by 35 cycles of 30-s denaturation at 94 °C, 60-s annealing at 55 °C, and 1-min extension at 72 °C, with a final extension at 72 °C for 10 min. To confirm successful amplifications, PCR products were visualized using 1.2 % Agarose S (Nippon Gene, Japan) gel electrophoresis. Successful PCR products were purified by Wizard SV Gel and PCR Clean-Up System (Promega, USA) following the manufacturer's protocol. The DNA sequencing reaction of the PCR products was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Direct sequencing was performed using a 3130xl Genetic Analyzer (Applied Biosystems). Sequencing reactions were conducted using the 1-μM primers applied for the PCR amplifications. The newly obtained sequences were deposited in DDBJ/EMBL/Gen Bank (accession nos. LC056917–LC056924).

Additional Flabelligeridae and Acrocirridae *COXI* sequences were also obtained from DDBJ/EMBL/Gen Bank (Table 1). All sequences were aligned using Mesquite version 2.75 (Maddison and Maddison 2011), taking each codon position into account. A phylogenetic tree was constructed using the maximum likelihood (ML) method. The ML tree was obtained using the partitioned method (partitions per each codon position) as implemented in RAxML-VI-HPC (Stamatakis 2006) and its graphical interface raxmlGUI version 1.3 (Silvestro and Michalak 2012). As recommended in the RAxML-VI-HPC manual (Stamatakis 2006), the general time-reversible model with sites following a discrete gamma distribution (GTR + Γ) (Tavaré 1986; Yang 1993) for each partition was used without incorporating a proportion of invariant sites. Rapid bootstrap analysis was conducted with 1,000 replicates (–f a option). Pairwise genetic distances [± standard deviation (SD)] were calculated on the basis of the *COXI* sequence using the uncorrected *p*-distance and Kimura's two-parameter (K2P) model (Kimura, 1980), as implemented in MEGA6.06 (Tamura et al. 2013).

Results

SYSTEMATICS

Family Flabelligeridae de Saint-Joseph, 1894

Genus *Diplocirrus* Haase, 1915

(New Japanese name: Konbouhabouki-zoku)

Diplocirrus nicolaji (Buzhinskaja, 1994)

(New Japanese name: Bouzu-habouki)

(Figs. 2, 3)

Diversibranchius nicolaji Buzhinskaya, 1994: 231, figures 2–7; Darbyshire and Mackie 2009: 97, Table 1.

Flabelligeridae from Japan: Rouse and Pleijel 2001, plate 11, figure f.

Diplocirrus nicolaji: Salazar-Vallejo and Buzhinskaja 2011, 31–33 figure 9.

Material examined

Oshoro: NSMT-Pol-113032, One of unknown sex, Body length 36.2 mm, Body width 1.9 mm, 49 chaetigers, 20 October 2014, coll. N. Jimi, 2 m depth, gravelly sand.

Tateyama: NSMT-Pol-113033, One of unknown sex (anterior fragment), Body length 16.0 mm, Body width 3.0 mm, 19 chaetigers, 6 March 2015, coll. M. Tanaka, 4–10 m depth, sand.

Misaki: NSMT-Pol-113034, 113036, 12 of unknown sex (one complete specimen and 11 anterior fragments), Body length 8.0–18.6 mm, Body width 1.8 mm, 29 chaetigers, 16 October 2014, coll. N. Jimi, 4 m depth, sand.

Komatsubara: NSMT-Pol-113035, Six of unknown sex (two complete specimens and four anterior fragments), Body length 17.0–26.2 mm, Body width 1.2–1.7 mm, 31–51 chaetigers, 2 September 2014, coll. N. Jimi, 2 m depth, sandy mud.

Description

Body length 8.0–36.2 mm and width 1.2–1.9 mm, chaetigers 29–51, and orange yellow in colour. Anterior and last few segments slightly swollen. Cephalic cage absent (1st chaetiger notochaeta 0.2–0.5 mm). Tunic densely covered by digitate or bowling-pin shaped papillae which arranging as 10–17 rows per segment (Fig. 2a). Cephalic hood white colour and thinly covered by papillae. Eyes absent (Fig. 3a). One pair of palps, which 0.8–8.3 mm long, grooved. Lobes reduced and rounded. Two types of

Table 1 List of flabelligerid and acrocirrid species included in the phylogenetic analysis, together with accession numbers in DDBJ/EMBL/GenBank

Taxon_#	Accession number	Collection site	Reference
Flabelligeridae			
*Brada villosa*_1	HM473328	British Columbia, Sechelt, Possie Island, Canada	Carr et al. (2011)
*Brada villosa*_2	HQ024268	Nunavut, Resolute, Resolute Bay, Canada	do.
*Brada villosa*_3	HQ024269	do.	do.
*Brada villosa*_4	HQ024270	do.	do.
*Brada villosa*_5	HQ024271	do.	do.
Brada sp._1	HQ023866	Newfoundland and Labrador, Saglek Fjord, Canada	do.
Brada sp._2	HQ024267	Nunavut, Resolute, Resolute Bay, Canada	do.
Brada sp._3	HQ326970	Central California, USA	Osborn and Rouse (2011)
Coppingeria cf. *longisetosus*[1]	HQ326971	Spencer Gulf, South Australia	do.
*Diplocirrus longisetosus*_1	HQ024289	Nunavut, Resolute, Resolute Bay, Canada	Carr et al. (2011)
*Diplocirrus longisetosus*_2	HQ024290	do.	do.
*Diplocirrus longisetosus*_3	HQ024291	do.	do.
*Diplocirrus longisetosus*_4	HQ024292	do.	do.
*Diplocirrus longisetosus*_5	HQ024293	do.	do.
*Diplocirrus longisetosus*_6	HQ024294	do.	do.
*Diplocirrus longisetosus*_7	HQ024295	do.	do.
*Diplocirrus longisetosus*_8	HQ024296	do.	do.
*Diplocirrus longisetosus*_9	GU672433	Kandalaksha Bay, Velikaya Salma Strait, Russia, 10 m depth	Hardy et al. (2011)
*Diplocirrus longisetosus*_10	GU672586	Kandalaksha Bay, Velikaya Salma Strait, Russia, 35 m depth	do.
*Diplocirrus nicolaji*_1	LC056917	Oshoro, Hokkaido, Japan	This study
*Diplocirrus nicolaji*_2	LC056924	Tateyama Bay, Chiba Pref., Japan	do.
*Diplocirrus nicolaji*_3	LC056918	Misaki, Kanagawa Pref, Japan	do.
*Diplocirrus nicolaji*_4	LC056919	do.	do.
*Diplocirrus nicolaji*_5	LC056920	do.	do.
*Diplocirrus nicolaji*_6	LC056921	Komatsubara Beach, Hiroshima Pref, Japan	do.
*Diplocirrus nicolaji*_7	LC056922	do.	do.
*Diplocirrus nicolaji*_8	LC056923	do.	do.
Flabelliderma ockeri	EU694127	La Jolla, California, USA	Osborn and Rouse (2008)
*Flabelligera affinis*_1	HQ024304	Nunavut, Resolute, Resolute Bay, Canada	Carr et al. (2011)
*Flabelligera affinis*_2	HQ024305	Nunavut, Igloolik, Canada	do.
*Flabelligera affinis*_3	HQ024306	do.	do.
*Flabelligera affinis*_4	GU672447	Kandalaksha Bay, Velikaya Salma Strait, Russia, 10 m depth	Hardy et al. (2011)
Flabelligera infundibularis	EU694131	Astoria, Oregon, USA	Osborn and Rouse (2008)
Flabelligera mundata	HQ326969	South Orkney Islands, Antarctica	Osborn and Rouse (2011)
Flabelligera sp._1	HQ024307	Nunavut, Resolute, Canada	Carr et al. (2011)
Flabelligera sp._2	HQ024308	Nunavut, Resolute, Resolute Bay, Canada	do.
Flabelligera sp._3	HQ024309	do.	do.
*Pherusa affinis*_1	HQ024180	New Brunswick, St. Andrews, Indian Point, rocky intertidal, Canada	do.
*Pherusa affinis*_2	HQ024181	do.	do.
*Pherusa affinis*_3	HQ024182	New Brunswick, St. Andrews, Ministers Island, intertidal, Canada	do.
*Pherusa affinis*_4	HQ024183	New Brunswick, St. Andrews, between harbor/DFO, subtidal, Canada	do.
*Pherusa affinis*_5	HQ024184	do.	do.

Table 1 List of flabelligerid and acrocirrid species included in the phylogenetic analysis, together with accession numbers in DDBJ/EMBL/GenBank *(Continued)*

*Pherusa affinis*_6	HQ024185	New Brunswick, St. Andrews, Bar Road, intertidal mudflat, Canada	do.
Flabelligeridae sp.	HM375490	Canada (58.798 N, 94.15 W)	do.
*Pherusa plumosa*_1	HQ023901	Newfoundland and Labrador, Nachvak Fjord, Canada	do.
*Pherusa plumosa*_2	HQ023902	do.	do.
Acrocirridae (outgroup)			
Acrocirrus validus	FJ944525	Hayama, Sagami Bay, Japan	Osborn et al. (2009)
Macrochaeta clavicornis	EU791463	Vattenholmen, Sweden	Osborn and Rouse (2008)

[1] Salazar-Vallejo (2011a) considered the genus *Coppingeria* Haswell, 1892 as a junior synonym of *Stylarioides* delle Chiaje, 1831

branchiae present, green colour when alive, but faded to white in ethanol preserved specimens (Fig. 3b, c). Four branchiae in posterior row; 0.5–1.4 mm long and lamellate. Lamella reaching tips, lamellate surface ciliated, and two sucker-like sockets on lateral side (Fig. 3d). Four branchiae in anterior row; 0.7–1.9 mm long and lamellate. Lamella reaching to one-third of its branchial length. Branchial surface ciliated (Figs. 2b, 3e). Chaetiger 1 notosetae emerging from dorsal side contrary to following setigers. Chaetigers 3 to 8 or 9 swollen, without clear segmentation of tunic between them. Gonopores orange red, present in chaetigers 2 to 23–48 (Fig. 3f). Parapodia poorly developed. Chaetae emerging directly from body wall. Notochaeta and neurochaeta multiarticulated, 5–7 per bundles (Fig. 2a); 23–26 articles on notochaeta and 9–11 articles on neurochaeta in median chaetigers.

Variation among four localities

The palp length of the specimen from Oshoro was much longer than that of specimens from the other localities (Fig. 3c). The preserved branchial colour of the specimens from Misaki was often green as in its live state, but the colour of the specimens from other localities are soon faded to white.

Phylogenetic analysis

The final length of the aligned *COXI* sequence was 531 bp; this contained 230 variable sites, of which 213 sites were parsimony informative. The mean base composition was 28.3 % (A), 20.2 % (C), 16.4 % (G), and 35 % (T).

The ML tree obtained (Fig. 4) showed that the monophyly of Flabelligeridae was strongly supported (bootstrap [BS] value = 95 %); however, the internal relationships within the family were totally unresolved. All the sequences of *Diplocirrus nicolaji* formed a monophyletic clade with high support value (BS value = 94 %) and were well discriminated from the remains (Fig. 4). The average genetic divergence of the sequences among *D. nicolaji* was 2.1 ± 1.6 % in *p*-distance (2.2 ± 2 % in K2P). Monophyly of the two *Diplocirrus* species included, *D. longisetosus* and *D. nicolaji*, was not supported.

Discussion

The present specimens agreed well with the original and subsequent descriptions of *Diplocirrus nicolaji* in following diagnostic features: 1) notochaetae in anterior

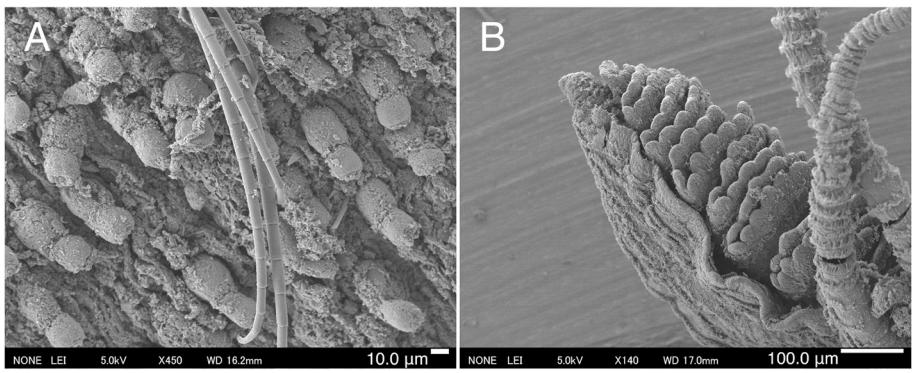

Fig. 2 Scanning electron micrographs of *Diplocirrus nicolaji* from Misaki (NSMT-Pol-113036): (**a**) Lateral view of neurochaetae and papillae on the tunic surface in a median chaetiger; (**b**) frontal view of anterior and posterior branchiae

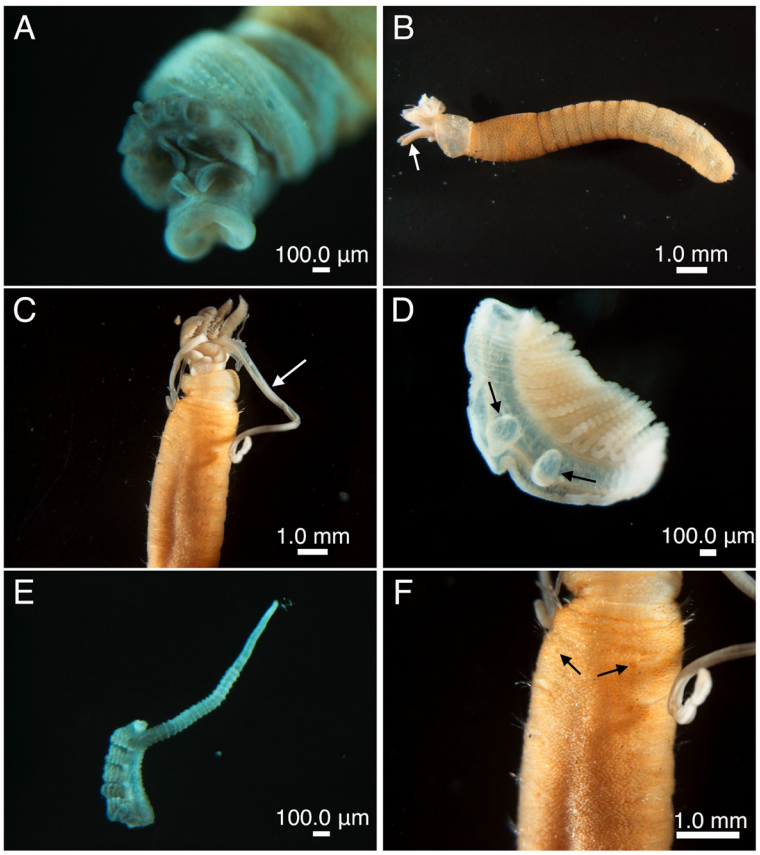

Fig. 3 Stereoscopic micrographs of *Diplocirrus nicolaji*: (**a**) Frontal view of a preserved specimen from Misaki (NSMT-Pol-113034) without head appendages for clarity; (**b**) dorsolateral view of a preserved specimen from Komatsubara (NSMT-Pol-113035) with palps (1.2 mm in length) (*arrow*); (**c**) ventral view of a preserved specimen from Oshoro (NSMT-Pol-113032) with palps (8.3 mm in length) (*arrow*); (**d**) lateral view of a posterior branchia taken from a specimen from Komatsubara (NSMT-Pol-113035) showing the two sucker-like sockets (*arrow*); (**e**) lateral view of an anterior branchia taken from a specimen from Misaki (NSMT-Pol-113034); (**f**) anteroventral view of a preserved specimen from Oshoro (NSMT-Pol-113032) showing the gonopores in each segment (*arrow*)

setigers are very short and do not form a cephalic cage; 2) papillae on the tunic are relatively short, digitate or bowling-pin shaped, and arranged in 10–17 rows per segment; 3) ventrolateral gonopores are present from chaetiger 2; 4) neurochaetae in median chaetigers are barely tapered with markedly falcate tips; and 5) the lamellate region of the anterior cirriform branchiae extends to one-third of the branchial length (Buzhinskaja 1994; Salazar-Vallejo and Buzhinskaja 2011). They were thus identified as *D. nicolaji* with little hesitation. Although a few morphological variations were found among the local populations (see above), our phylogenetic analysis confirmed that the present specimens are conspecific with little genetic divergence (Fig. 4). The differences of palp length among each population might be attributable to the various anesthesia durations. Meanwhile, our analysis based only on *COXI* sequences did not resolve the inter-family relationships of Flabelligeridae as shown in Osborn and Rouse (2011), which might reflect the fact that the *COXI* is relatively unconserved. More gene sequences from both the nuclear and mitochondrial genomes, as well as increased taxonomic samplings, will be needed for the better resolution.

Imajima (1992) and Uchida (1992) also reported the genus *Diplocirrus* from Japanese waters; the former recorded *Diplocirrus* sp. from shallow waters off Sarufutsu, northern Hokkaido, and the latter merely included this genus in his key of shallow-water flabelligerids in Japan. Unfortunately, we could not ascertain their identification nor compare these records with *D. nicolaji* since they gave no specific accounts of their species. Consequently, this is the first report of the detailed description of the morphology and distribution of the genus *Diplocirrus*, as well as *D. nicolaji*, from Japan.

In this study, *D. nicolaji* was collected from shallow waters (up to a depth of 10 m) along Japanese coasts (Fig. 1). This is the first report of *D. nicolaji* from the Pacific Ocean and extends its distribution southward (Komatsubara, Seto Inland Sea). Therefore the distribution

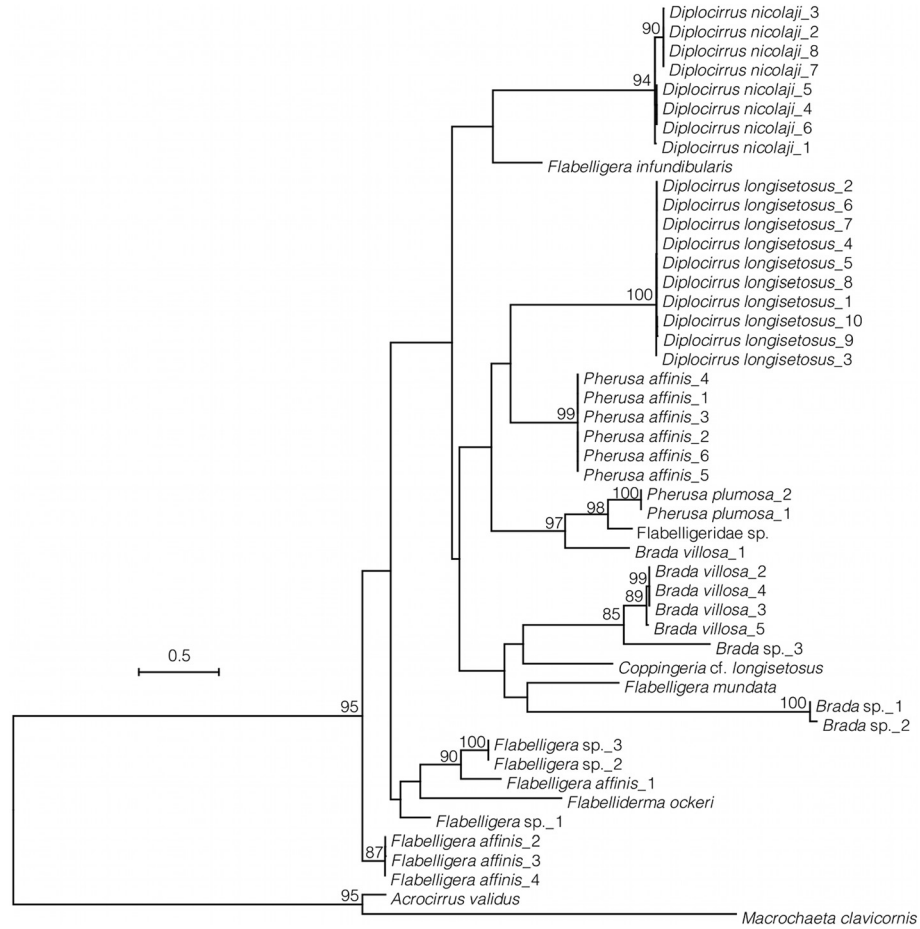

Fig. 4 Maximum-likelihood (ML) tree based on mitochondrial *COXI* sequence. *Acrocirrus validus* and *Macrochaeta clavicornis* (Acrocirridae) were used as outgroups. Nodal support values (bootstrap support) higher than 70 % are indicated above the branches

of *D. nicolaji* is not restricted to northern cold waters in the Sea of Japan. Although intertidal to shallow-water polychaete faunae in central to southern Japan have been relatively well surveyed (e.g., Inaba 1988; Nishi et al. 2001; Nishi and Kudo 2003, 2005; Sato 2000; Yamanishi and Sato 2007; Nishi and Kupriyanova; 2011), *D. nicolaji* was not recorded in those studies and may thus have been overlooked. Future surveys will add its new distributional records in Japanese waters and the vicinity.

This study contributes to the pool of knowledge on Japanese coastal biodiversity and will help generate more accurate environmental assessments. Furthermore, the given *COXI* sequences will be helpful for the future accurate identification of this species, that is, DNA barcoding. Recently, a number of cryptic species have been discovered through DNA barcoding analyses, even among polychaetes (see Nygren 2014). We have taken a first step toward unraveling the cryptic diversity of Flabelligeridae occurring in Japanese waters.

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Authors' contributions
NJ and MT designed the study, collected the specimens, and drafted the manuscript. NJ conducted the morphological analysis and performed the molecular experiments. MT conducted the phylogenetic analysis. YF helped to improve the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima 739-8528, Japan. [2]Department of Marine Biodiversity Research, Japan Agency for Marine-Earth Science and Technology, Yokosuka 237-0061, Japan. [3]Department of Biology, Faculty of Science, Toho University, Funabashi, Chiba 274-8510, Japan.

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