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# Morphological and Genetic Characterization of the First Species of *Thalassodrilides* (Annelida: Clitellata: Naididae: Limnodriloidinae) from Japan

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A species of marine limnodriloidine oligochaete, *Thalassodrilides* cf. *briani* Erséus, 1992, is recorded from gravelly sand sediments of the subtidal zone in Ehime Prefecture, Japan. The present material agrees with the original description of *T. briani*, which was first found at Hong Kong, with the exception that the copulatory sacs are oval; not slender. Despite the lack of genetic data for the Hong Kong population, we conclude that the Japanese specimens are conspecific with it, or at least very closely related, based on morphological considerations. This is the first record of the genus *Thalassodrilides* Brinkhurst and Baker, 1979 in Japan. The phylogenetic relationships between *T.* cf. *briani* and three other species of *Thalassodrilides* are estimated, based on partial DNA sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and the complete nuclear ribosomal Internal Transcribed Spacer (ITS) region, using two members of the closely related genus *Doliodrilus* Erséus, 1984 as outgroups. The genetic analysis shows that *T.* cf. *briani* is a species delimited by both mitochondrial and nuclear data, and clearly separated from at least its closely related congeners in the Northwest Atlantic (Caribbean and adjacent areas).

**Key Words:** Oligochaeta, marine subtidal sediments, new record, taxonomy, molecular systematics, COI, ITS.

## Introduction

*Thalassodrilides* Brinkhurst and Baker, 1979 is a relatively small genus of marine or brackish-water Naididae in the subfamily Limnodriloidinae. It was established by Brinkhurst and Baker (1979), and later Erséus (1990) revised it to include species of Limnodriloidinae with a reticulate blood plexus surrounding a widened and thick-walled part of the oesophagus in segment IX and a pair of complex, muscular, eversible pseudopenes. Erséus (1981, 1990) transferred *Curacaodrilus* Righi and Kanner, 1979 and *Kaketio* Righi and Kanner, 1979, to *Thalassodrilides* on the basis of this emended definition (see Erséus 1990). Six species of *Thalassodrilides* are recognized today as follows; *T. belli* (Cook, 1974), *T. briani* Erséus, 1992, *T. bruneti* Erséus, 1990, *T. gurwitschi* (Hrabě, 1971) (the type species, originally described as *Limnodriloides gurwitschi* Hrabě, 1971), *T. ineri* (Righi and Kanner, 1979), and *T. milleri* Brinkhurst and Baker, 1979. Among these, Erséus (1981, 1990) regarded *T. milleri* as *incertae sedis* or a *nomen dubium*.

Fifteen species of brackish-water or marine Naididae have been reported from Japan up to the present (Ohtaka 1987; Takashima and Mawatari 1996, 1998; Takashima 2000, 2001), but none of them belongs to this genus. This study documents the first Japanese record of *Thalassodrilides*, based on morphological and genetic information.

## Materials and Methods

**Specimen collecting.** Worms were collected from bottom sediment next to a fish farm in an embayment on the Pacific coast of southwestern Shikoku, Japan. Bottom samples (depth 36.6 m) were taken with an Ekman-Birge grab, and worms suspended in water in a tray were sucked up directly with pipettes. Sampling was qualitative. Live and fixed specimens were examined using a compound microscope. Specimens for morphological observation were fixed with either 10% formalin or 70% ethanol solutions, after being anesthetized in low concentrations of ethanol, and dehydrated in a graded ethanol series, cleared in methyl salicylate, and mounted whole onto slides in Canada balsam. Unless otherwise specified in the description, measurements refer to whole-mounted specimens. The drawings were made freehand with the aid of traced from a photo using a light box. Specimens for molecular phylogenetic study were fixed in 99% ethanol.

**DNA sequencing and assembly.** For the genetic analyses ten Japanese specimens of the species here described as *Thalassodrilides* cf. *briani* were selected together with four specimens of *T. bruneti*, two of *T. belli*, and two of an unidentified *T.* sp. For rooting the trees one specimen each of *Doliodrilus fibrisaccus* Wang and Erséus, 2004 and *D. tener* Erséus, 1984 were included. See Table 1 for details of all

Table 1. List of material included in the molecular genetic study of *Thalassodrilides* spp., with specimen identification numbers, collection data, GPS coordinates, GenBank accession numbers, and voucher numbers. Accession numbers in bold are newly generated sequences.

| Species | Spm. no. | Locality | Leg. and coll. date | GPS coordinates |  | GenBank Accession no. |  | Museum voucher no. |
|---|---|---|---|---|---|---|---|---|
|  |  |  |  | N | E/W | COI | ITS |  |
| *Thalassodrilides* cf. *briani* (Japan) | CE11667 | Japan, Ehime-Pref., Minami-Uwa county, Ainan town, Fukuura Bay | K. Ito, 30 May 2011 | 32°55.142′N | 132°31.804′E | KX235884 | — | SMNH 153627 |
|  | CE11668 | Japan, Ehime-Pref., Minami-Uwa county, Ainan town, Fukuura Bay | K. Ito, 30 May 2011 | 32°55.142′N | 132°31.804′E | KX235886 | KX235901 | SMNH 153628 |
|  | CE11669 | Japan, Ehime-Pref., Minami-Uwa county, Ainan town, Fukuura Bay | K. Ito, 30 May 2011 | 32°55.142′N | 132°31.804′E | KX235887 | KX235902 | SMNH 153629 |
|  | CE11690 | Japan, Ehime-Pref., Minami-Uwa county, Ainan town, Fukuura Bay | K. Ito, 30 May 2011 | 32°55.142′N | 132°31.804′E | KX235888 | KX235903 | NSMT-An 501 |
|  | CE11691 | Japan, Ehime-Pref., Minami-Uwa county, Ainan town, Fukuura Bay | K. Ito, 30 May 2011 | 32°55.142′N | 132°31.804′E | KX235885 | KX235904 | NSMT-An 502 |
|  | CE26405 | Japan, Ehime-Pref., Minami-Uwa county, Ainan town, Fukuura Bay | K. Ito, 8 Aug 2015 | 32°55.142′N | 132°31.804′E | KX235879 | KX235897 | SMNH 153204 |
|  | CE26406 | Japan, Ehime-Pref., Minami-Uwa county, Ainan town, Fukuura Bay | K. Ito, 8 Aug 2015 | 32°55.142′N | 132°31.804′E | KX235880 | KX235900 | SMNH 153205 |
|  | CE26407 | Japan, Ehime-Pref., Minami-Uwa county, Ainan town, Fukuura Bay | K. Ito, 8 Aug 2015 | 32°55.142′N | 132°31.804′E | KX235881 | KX235898 | NSMT-An 506 |
|  | CE26408 | Japan, Ehime-Pref., Minami-Uwa county, Ainan town, Fukuura Bay | K. Ito, 8 Aug 2015 | 32°55.142′N | 132°31.804′E | KX235882 | KX235905 | NSMT-An 507 |
|  | CE26409 | Japan, Ehime-Pref., Minami-Uwa county, Ainan town, Fukuura Bay | K. Ito, 8 Aug 2015 | 32°55.142′N | 132°31.804′E | KX235883 | KX235899 | NSMT-An 508 |
| *Thalassodrilides belli* | CE951 | USA, Florida, St. Lucie Co., Indian River, South Hutchinson Island, N of St. Lucie Nuclear Power Plant, W side of road A1A, 2 km N of Little Mud Creek bridge, high intertidal, poorly oxygenated sand | C. Erséus, 8 Apr 2005 | 27°23.71′N | 80°15.82′W | KX235891 | KX235908 | SMNH 153609 |
|  | CE2494 | USA, Virginia, Middlesex Co., Urbanna, Chesapeake Bay, Rappahannock River at Lagrange Creek, 1–2 m, 8 ppt salinity | S. Kvist, May 2007 | 37°40′N | 75°35′W | KX235892 | KX235909 | SMNH 153610 |
| *Thalassodrilides bruneti* | CE79 | Bahamas, Exuma, Lee Stocking Island, subtidal muddy sand | C. Erséus, 20 Apr 1999 | 23°46′N | 76°06′W | GU902113 | KX235894 | No voucher |
|  | CE17585 | Bahamas, Exuma, between Darby Island and Little Darby Island sand bar in middle of cut, coarse sand, 5–6 m | C. Erséus, 4 Apr 2013 | 23°51.354′N | 76°13.488′W | KX235877 | KX235895 | SMNH 153611 |
|  | CE17586 | Bahamas, Exuma, between Darby Island and Little Darby Island sand bar in middle of cut, coarse sand, 5–6 m | C. Erséus, 4 Apr 2013 | 23°51.354′N | 76°13.488′W | KX235878 | KX235896 | SMNH 153612 |
|  | CE18140 | Belize, off Dangriga, Carrie Bow Cay, W side of island, 0.7 m, sand | C. Erséus, 12 Apr 2013 | 16°48.126′N | 88°04.926′W | KX235876 | — | SMNH 153613 |
| *Thalassodrilides* sp. | CE888 | USA, Florida, St. Lucie Co., Indian River, edge of Harbor Branch Channel, lower intertidal, poorly oxygenated sand | C. Erséus, 1 Apr 2005 | 27°32.1′N | 80°20.9′W | KX235889 | KX235906 | SMNH 153614 |
|  | CE952 | USA, Florida, St. Lucie Co., Indian River, South Hutchinson Island, N of St. Lucie Nuclear Power Plant, W side of road A1A, 2 km N of Little Mud Creek bridge, high intertidal, poorly oxygenated sand | C. Erséus, 8 Apr 2005 | 27°23.71′N | 80°15.82′W | KX235890 | KX235907 | SMNH 153615 |
| *Doliodrilus fibrisaccus* | CE146 | China, S coast of Hainan, lower end of estuary SE of Teng Qiao town | H. Z. Wang, C. Erséus, 18 Mar 2000 | 18°23.18′N | 109°45.71′E | KX235875 | KX235893 | No voucher |
| *Doliodrilus tener* | CE1385 | Australia, Queensland, Great Barrier Reef, Lizard Island, One Tree Coconut Beach, inside mangroves, upper to middle intertidal, coarse heterogeneous sand | C. Erséus, 14 Feb 2006 | 14°40.8′S | 145°27.4′E | KX235874 | KX235910 | SMNH 153616 |

Table 2. Uncorrected pairwise genetic distances (in %) for COI (below diagonal) and ITS (above diagonal), mean distances in parentheses.

| | Species | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| 1 | *D. tener* (*n*=1) | — | 15.0 (15.0) | 12.9–15.3 (13.7) | 16.0–16.1(16.0) | 13.1 (13.1) | 12.9 (12.9) |
| 2 | *D. fibrisaccus* (*n*=1) | 19.0 (19.0) | — | 13.3–14.3 (13.7) | 15.1–15.2 (15.1) | 14.5 (14.5) | 14.4–14.5 (14.4) |
| 3 | *T. bruneti* (*n*=4/3) | 15.7 (15.7) | 18.2–18.5 (18.3) | — | 4.4–5.8 (5.0) | 0.5–0.8 (0.7) | 1.6–2.1 (1.8) |
| 4 | *T. belli* (*n*=2) | 16.2–18.2 (17.2) | 18.3–19.0 (18.7) | 10.4–12.1 (10.9) | — | 4.8–4.9 (4.8) | 4.8–5.0 (4.8) |
| 5 | *T.* sp. (*n*=2) | 16.7 (16.7) | 18.5–18.7 (18.6) | 8.8–9.3 (9.0) | 9.8–11.6 (10.7) | — | 1.5–1.6 (1.6) |
| 6 | *T.* cf. *briani* (Japan) (*n*=10/9) | 14.7–15.4 (15.3) | 19.9–20.2 (20.1) | 5.6–7.3 (6.3) | 10.9–12.4 (11.6) | 8.3–8.6 (8.5) | — |

specimens. DNA was extracted from the posterior ends of ethanol-preserved worms. DNA extraction, PCR amplification, and primers follow Martinsson *et al.* (2013). Two markers, the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and the complete nuclear ribosomal Internal Transcribed Spacer (ITS) region, were amplified. One of the COI sequences of *T. bruneti* was from Erséus *et al.* (2010) and downloaded from GenBank. Sequencing was carried out by Macrogen Inc. (Seoul, Korea) and Eurofins MWG Operon (Ebersberg, Germany). Sequences were assembled and aligned in Geneious Pro v. 7.1. (Biomatters Ltd.; http://www.geneious.com). All sequences produced in this study are deposited in GenBank (Accession numbers in Table 1). Vouchers of sequenced specimens (*i.e.*, anterior body parts mounted on microscope slides) are deposited in the National Museum of Nature and Science (NSMT), Tsukuba, Japan, and in the Swedish Museum of Natural History (SMNH), Stockholm, Sweden (see Table 1).

**Distance analysis.** Uncorrected pairwise genetic distances, including within-species and between-species means, were calculated for both the COI and ITS datasets in MEGA 6 (Tamura *et al.* 2013), using pairwise deletion for missing data (Table 2).

**Gene tree estimation.** Gene trees were estimated using Maximum Likelihood; the analyses were performed with PhyML 3.0 (Guindon and Gascuel 2003; Guindon *et al.* 2010) as implemented at the South of France Bioinformatics platform (http://www.atgc-montpellier.fr/). The automatic model selection using SMS (Smart Model Selection) with BIC (Bayesian Information Criterion) as the selection criterion was used; SPR+NNI was used for tree improvement. Branch support was calculated with the Chi-squared-based approximate Likelihood Ratio Test (aLRT) (Anisimova and Gascuel 2006) in PhyML. The same settings were used for both the COI and the ITS analyses. The trees were drawn in FigTree 1.4.2 (Rambaut 2014) and further edited in Adobe Illustrator.

**Abbreviations in figures.** aa: atrial ampulla; ad: atrial duct; cs: copulatory sac; eg: egg; ov: ovary; pr: prostate gland; sa: spermathecal ampulla; sb: sperm bundles; sd: spermathecal duct; sf: sperm funnel (with mass of spermatozoa); sp: spermathecal pore; vd: vas deferens.

### Genus ***Thalassodrilides*** Brinkhurst and Baker, 1979
[New Japanese name: Naiwan–ito–mimizu-zoku]

### ***Thalassodrilides* cf. *briani*** Erséus, 1992
[New Japanese name: Hime–naiwan–ito–mimizu]
(Figs 1–2)

*Thalassodrilides* sp.: Ito *et al.* 2016: 340, fig. 1.
*Thalassodrilides briani* Erséus, 1992: 172–173, fig. 10 (morphologically similar).

**Material examined.** 4 mature specimens from the Seto Inland Sea, Fukuura Bay, Ainan town, Minami-Uwa county, Ehime Prefecture, Japan, 32°55′14.2″N, 132°31′8.0″E, 36.6 m depth, 17 November 2010 (NSMT-An 497–500); 5 mature specimens, 30 May 2011, other data as for NSMT-An 497–500 (NSMT-An 501–505); 4 mature specimens, 8 August 2015, other data as for NSMT-An 497–500 (NSMT-An 506–509, SMNH 153204–153205).

**Description of new material.** Body color reddish, mostly reflecting blood color. Fixed and mounted specimens 6.8–9.1 mm long (5 specimens), 200–260 µm wide at clitellum. Segments 48–58. Prostomium round or somewhat triangular. Body wall naked, *i.e.*, devoid of cuticular papillation. Clitellum extending over XI to mid XII. Chaetae sigmoid and bifid with upper tooth shorter and narrower than lower, with nodulus about 1/3 of way from distal tip (Figs 1A, 2A); 3–5 chaetae per bundle anteriorly, 2–3 per bundle in postclitellar segments, but chaetae absent ventrally from XI in mature specimens. Ventral and dorsal chaetae shortest in II, longest in VI, gradually shortening from VIII on. Hair chaetae and modified genital chaetae absent. Male pores paired in line with ventral chaetae, posteriorly in XI. Spermathecal pores in X.

Brain in I–II, concave posteriorly, approximately 1.5 times as long as wide, length 165–180 µm, posterior width 88–94 µm. Pharyngeal glands in IV–V. Oesophagus enlarged in IX, and in this segment thick-walled with conspicuous blood plexus.

Sperm funnel (Figs 1B, C, 2B) 49–60 µm long and 36–45 µm wide at ental margin, funnel wall becoming thinner posteriorly. Vas deferens thick-walled, rather short, entering apical end of atrium in XI. Atrial ampulla somewhat spindle-shaped, 48–67 µm long, 20–32 µm wide, with thin outer muscular layer and rather wide lumen, latter partly divided by thin horizontal septum into dorsal and ventral compartments (Figs 1B, C, 2B). In some pre-copulatory specimens, atrial ampulla somewhat lobed. Prostatic pad

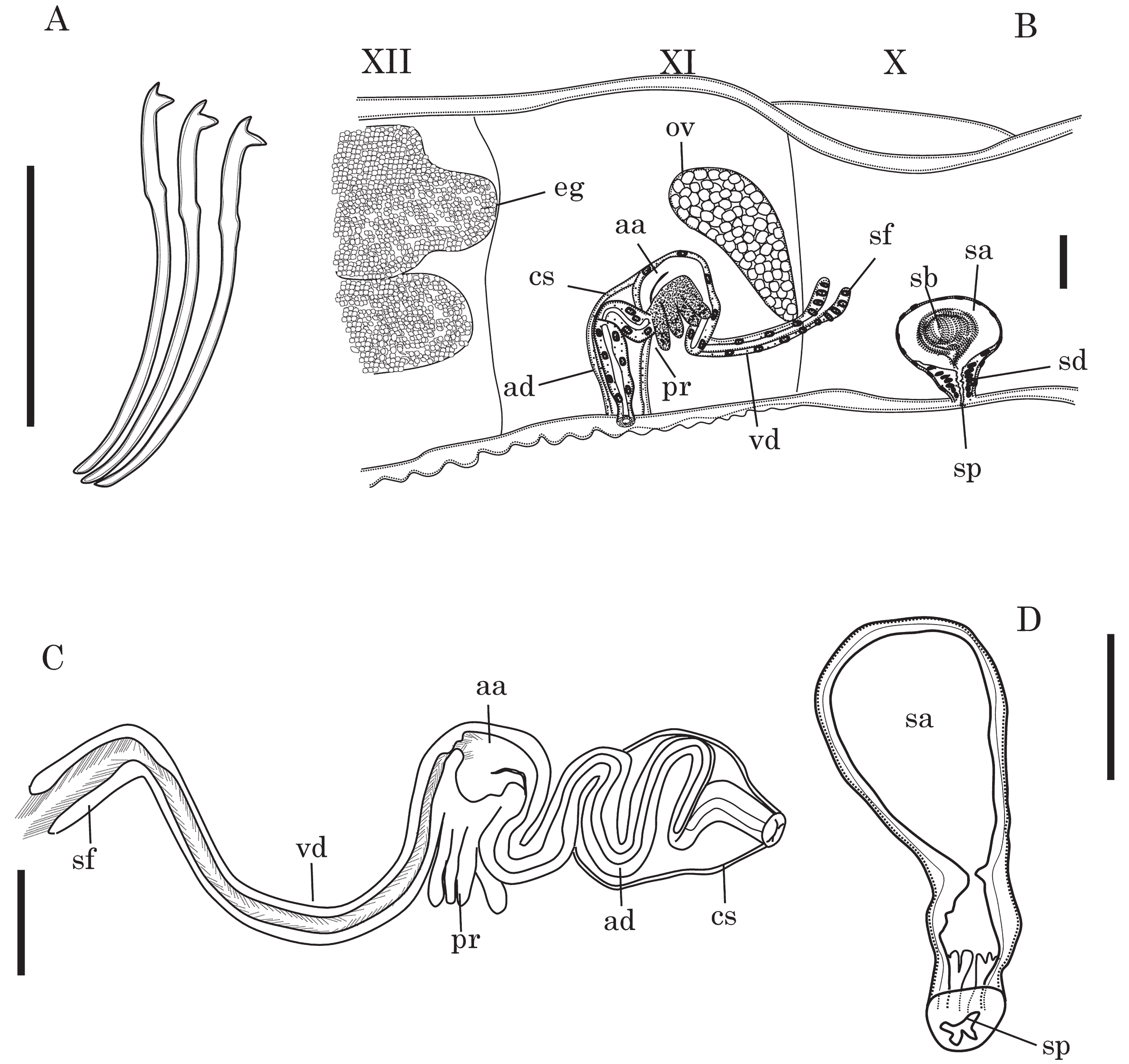

Fig. 1. *Thalassodrilides* cf. *briani* from Ehime Prefecture, Japan (NSMT-An 507), drawings from whole mounts. A, ventral chaetae from anterior segments; B, reproductive system in segments X–XII; C, detail of male reproductive structures from a live specimen; D, detail of spermatheca from a live pre-copulatory specimen. Scale bars: 50 μm.

inconspicuous, but extending along most of length of atrial ampulla. Prostate gland small, lobed. Atrial duct slender, slightly longer than ampulla, running inside somewhat pear-shaped, moderately muscular copulatory sac. Ental part of duct granulated, ectal part forming pseudopenis. Male pores not on protuberances when pseudopenes retracted. Egg sac unpaired in XIII–XV, well–developed, sometimes extending back to XVIII–XXI. One pair of testes in spermathecal segment X and one pair of ovaries in atrial segment XI. Spermatheca composed of thick-walled, conical duct and thin-walled, oval ampulla. Duct usually shorter than wide (34–60 μm long, 48–62 μm maximum diameter), with no ectal glands. Ampulla 1/3 as wide as segment (120–282 μm maximum diameter in post-copulatory worms) (Figs 1D, 2C, D), ectal duct sometimes with vestibule (Figs 1D, 2C). Ball-shaped mass (or bundles) of sperm in ampullar lumen (Figs 1D, 2D).

**Habitat.** The Japanese specimens were collected in the Pacific coast, next to a fish farm, in gravelly sand (5.4% gravel, 92.5% sand, and 2.1% silt and clay). Acid volatile sulfide and loss on ignition (600°C for 2 h) of the surface layer sediment were 0.2 mg S/g and 8.6%, respectively. It is very probable that fish farm waste had settled into the bottom.

**Remarks.** The new material agrees well with the original description of *Thalassodrilides briani*, a species so far only known from Hong Kong (southern China), with the exception that the copulatory sacs of our worms are not as slender as those described by the author (Erséus 1992). The horizontal atrial septum present in *T. briani* and *T.* cf. *briani* appears to be an unusual character, but it may have been overlooked in *T. bruneti* and *T. gurwitschi*. On morphological grounds only, we find it difficult to decide whether our *T.* cf. *briani* is the same as the proper *T. briani*; this can only be finally resolved by genetic information. Our Japanese

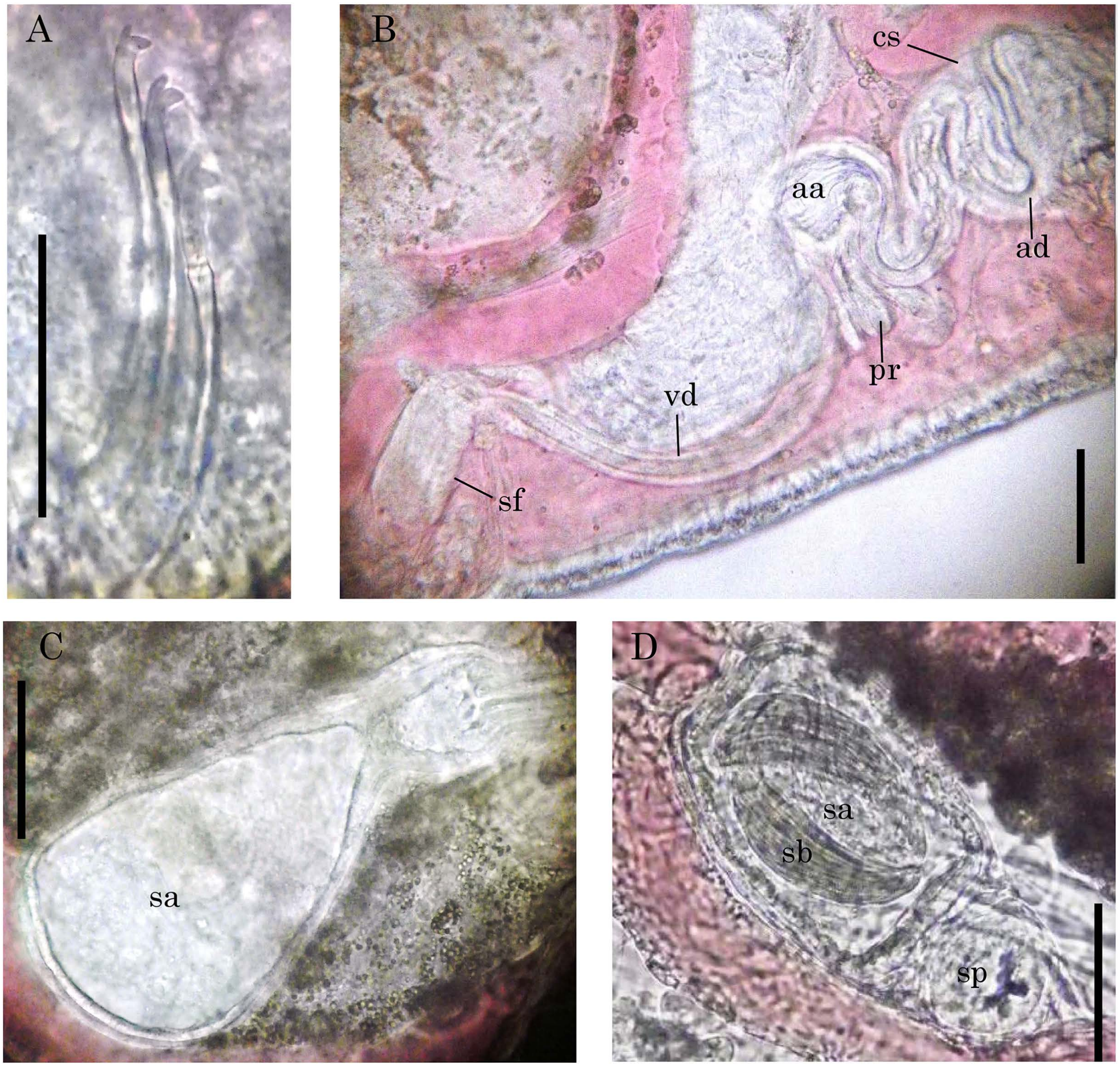

Fig. 2. *Thalassodrilides* cf. *briani* from Ehime Prefecture, Japan, microscopic photographs from live specimens. A, ventral chaetae from anterior segments; B, male reproductive structures; C, pre-copulatory spermatheca; D, mature (post-copulatory) spermatheca. Scale bars: 50 µm.

species also resembles the Belizean *T. bruneti*, with which it shares similar male ducts (including the simple, weakly muscular copulatory sacs). However, the absence of spermathecae is a useful character to identify *T. bruneti* as a different morphospecies. A morphological comparison between all the members of *Thalassodrilides* is shown in Table 3. Except for *T. ineri*, a large species with exceptionally elaborate atrial, penial, and spermathecal structures, and the fact that two nominal taxa (*T. bruneti* and *T. gurwitschi*) and one unidentified species (*T.* sp.) lack spermathecae completely, the various species described to date are extremely difficult to discriminate morphologically from each other.

**Distribution.** Our *T.* cf. *briani* has so far only been found in Ehime Prefecture in southern Japan. *Thalassodrilides briani per se* is only known from a single locality in Hong Kong (Erséus 1992).

## Genetic Analysis

**DNA sequencing and assembly.** COI was successfully sequenced from all 20 specimens, and ITS from 18 specimens. After trimming, the COI alignment was 605 bp long, and the ITS alignment 1386 bp long.

**Distance analysis.** The results are summarized in Table 2. In COI, no variation was observed among the Japanese specimens, which were separated from the specimens of the other included species of *Thalassodrilides* by 5.6–12.4% pairwise distances. The closest species to *T.* cf. *briani* was *T. bruneti*; these two species had a mean pairwise distance of 6.3%. Mean pairwise distances among *Thalassodrilides* species varied from 6.3% to 11.6%. Deep splits were observed within two of the included species, *viz.*, *T. bruneti* with a maximum intraspecific pairwise distance of 5.0%, and *T.*

Table 3. Morphological comparison of different species of *Thalassodrilides*. Shaded areas indicate differentiating characteristics.

| Species | Chaetae | Copulatory sacs | Male protuberances | Spermathecae | Sperm in spermathecae | Geographic distribution | References |
|---|---|---|---|---|---|---|---|
| *Thalassodrilides belli* (Cook, 1974) | upper tooth as long as but thinner than lower tooth | oval, not heavily muscular | lacking | 70–110 µm diameter, small, globular to pear-shaped,with short and relatively wide ducts, ducts indistinctly separated | bundled sperm random | Virginia, Louisiana, Texas, Puerto Rico, Trinidad, Bermuda, Pacific coast of Mexico | Cook (1974)<br>Erséus (1981) |
| *Thalassodrilides briani* Erséus, 1992 | upper tooth shorter and narrower than lower tooth | oval or slender, with the ducts less coiled inside than in *T. belli*, not heavily muscular | lacking | 120–150 µm long, 110–130 µm wide, with characteristically funnel-shaped duct, and globular, thin-walled ampullae, spermathecal ducts distinctly separated | bundled sperm random | Southern China, Hong Kong | Erséus (1992) |
| *Thalassodrilides* cf. *briani* Erséus, 1992 | upper tooth shorter and narrower than lower tooth | more oval than those first described for the specimens from Hong Kong | lacking | 120–282 µm diameter, with conical duct and globular, thin-walled ampullae, spermathecal ducts distinctly separated | ball-shaped mass (or bundles) of sperm random | Japan | Present study |
| *Thalassodrilides bruneti* Erséus, 1990 | upper tooth shorter and narrower than lower tooth | slender, not heavily muscular | lacking | absent | — | Belize, Curaçao | Erséus (1990) |
| *Thalassodrilides gurwitschi* (Hrabě, 1971) | upper tooth shorter and narrower than lower tooth | oval, heavily muscular | prominent | absent | — | widespread in the Caribbean (Aruba, Curaçao, Bonaire, Panama, Puerto Rico, Florida, Belize), Black Sea, Mediterranean Sea, Persian Gulf, southern China, Hawaii, Western Australia | Righi and Kanner (1979)<br>Erséus (1981)<br>Erséus (1990) |
| *Thalassodrilides ineri* (Righi and Kanner, 1979) | upper tooth thinner and somewhat shorter than lower tooth | oval, not heavily muscular | prominent | *ca.* 500 µm long, 250 µm wide, voluminous and egg-shaped, with short ducts | one large spermatozeugma in each spermatheca | Belize, Curaçao, Bonaire, Florida, Bermuda | Righi and Kanner (1979)<br>Erséus (1981)<br>Erséus (1990) |
| *Thalassodrilides milleri* Brinkhurst and Baker, 1979 | upper tooth shorter and narrower than lower tooth | ? | ? | small, bilobed, 173 µm long. 67 µm wide sperm in oriented bundles | bundled sperm random | Delaware | Brinkhurst and Baker (1979) |
| *Thalassodrilides* sp. unidentified* | upper tooth shorter and narrower than lower tooth | oval, heavily muscular, with short ducts inside | lacking? | absent | — | Florida | — |

* Specimens CE888 and CE952 in current study.

*belli* with an intraspecific distance of 11.4%, *i.e.*, a distance of the same magnitude as seen between species in this genus. The out-groups *Doliodrilus fibrisaccus* and *D. tener* were well separated from the *Thalassodrilides* species, with average mean distances between 15.3 and 20.2%, and the out-groups were also well separated from each other (19%).

In ITS, there were no differences among the specimens from Japan, and in general the distances between *Thalassodrilides* species were smaller than for COI, varying between 0.7 and 4.8%. The Japanese specimens (*T.* cf. *briani*) were closest to *T.* sp., with a mean difference of 1.6%, and also close to *T. bruneti*, with a mean difference of 1.8%. *T. bruneti* and *T.* sp. were only 0.7% different from each other, and the distances between *T. belli* and the other *Thalassodrilides* species were greater (mean differences of 4.8–5%). The variation within species was small, with a maximum of 0.2% in *T. belli*. The out-groups were well separated from the in-group, with mean differences of 12.9–16%, and also well separated from each other (15%).

**Gene tree estimation.** For the COI analysis the TN93 substitution model with 6 gamma shape parameters was selected, whereas for the ITS analysis the HKY85 model with 6 gamma shape parameters was selected. Both analyses (Fig. 3) found *Thalassodrilides* to be monophyletic with maximum aLRT support, but because we include only a limited sample of out-groups, this was not a proper test of the monophyly of the genus. In the ITS tree (Fig. 3B), all species were recovered as monophyletic with maximum aLRT support, and in the COI tree (Fig. 3A), all species except *T. belli* were also recovered as monophyletic with maximum support. In the COI tree (Fig. 3A), (1) all relationships between species were unsupported, (2) a well-supported split was observed within *T. bruneti*, and (3) *T. belli* was not recovered as monophyletic and its terminal branches were (unexpectedly) long. In the ITS tree, *T. belli* was found as the sister to the remaining *Thalassodrilides* species, and *T.* cf. *briani* was found as the sister to a clade consisting of *T. bruneti* and *T.* sp. The splits observed within *T. bruneti* and *T. belli* in the COI analysis were not found in the ITS tree, but this may be due to our failure to obtain an ITS sequence for *T. bruneti* specimen CE18140, which was found as sister to the remaining *T. bruneti* in the COI tree.

## Discussion

Based on morphological data, a species of *Thalassodrilides* that resembles the taxon *T. briani*, which was previously known from only a single site near Hong Kong (southern China), is here reported and described from Japan for the first time. We have shown that this Japanese species is distinctly delimited from many congeners by both mitochondrial and nuclear molecular markers. For the time being, however, we have no access to genetic information about specimens from the vicinity of *T. briani*'s type locality, and we prefer to regard our new material as *T.* cf. *briani* rather than conclude it is identical to *T. briani sensu* Erséus (1992), especially as we noted some morphological differences between the copulatory sacs of the two populations. Nevertheless, our results will facilitate further studies to ascertain the taxonomic status of *T.* cf. *briani*, once someone obtains the missing genetic information.

Vivien *et al.* (2015) recently showed that morphological studies may underestimate the diversity of aquatic oligochaetes inferred from genetic data. Within the limited samples of *Thalassodrilides* included in this study, we noted two cases of mitochondrial (COI) divergence within a morphospecies. In *T. bruneti* there was an approximately 5% split between a specimen from Belize (CE18140) and the remaining three specimens from the Bahamas. In *T. belli* there was a 11.4% split between specimens from Florida and Virginia (US east coast), and *T. belli* was not recovered as monophyletic in the COI tree. This may serve as a warning that *T. briani* and *T.* cf. *briani* are part of a complex of very similar species. On the other hand, the two *T. belli* specimens only differ by 0.2% in ITS, and we lack ITS data (see above) for the CE18140 individual of *T. bruneti*. It is possible that these two cases involve cryptic species, but without more data it is impossible to rule out deep intraspecific mitochondrial divergence, a phenomenon not uncommon for clitellates (*e.g.*, Achurra and Erséus 2013; Martinsson *et al.* 2013).

With respect to their previously uninvestigated phylogenetic relationships, our analysis of four *Thalassodrilides* species based on the mitochondrial cytochrome *c* oxidase subunit I and nuclear ribosomal internal transcribed spacer (ITS) region has shown that they form a genetically coherent group, which supports inferences based on morphological observations and taxonomic conclusions in the revision by Erséus (1990). In terms of ecological function, it is worth noting that Ito *et al.* (2016) recently revealed the ability of Japanese *T.* cf. *briani* (=their "*Thalassodrilides* sp.") to biotransform 1-nitronaphthalene, a nitrated polycyclic aromatic hydrocarbon. The worms, which were collected from the same site that was sampled in the present study, proved to have a superior ability to convert 1-nitronaphthalene into substances that were nontoxic to sensitive larval fish [*Fundulus heteroclitus* (Linnaeus, 1766), the mummichog], and furthermore, estimates of the worm population density at their sampling site were over 100,000 individuals per square meter. It is very probable that the very high densities of this species are related to effluent from the fish farm. In general, several Naididae are considered to have a high tolerance to organic toxins and may have a strong ability to degrade industrial pollutants. For example, Vorob'ev *et al.* (2010) reported that the aquatic naidid worm *Limnodrilus hoffmeisteri* Claparède, 1862 survived highly contaminated oil sediment and also had the ability to bioremediate oil from bottom sediment. In the case of microorganisms, efficient bioremediation is dependent on achieving adequate population density, metabolic capability, and physiological activity at the contaminated site (*e.g.*, Sayler *et al.* 1982; Dinesh *et al.* 2015). It is very likely that the same holds true for the Japanese *T.* cf. *briani*. Further taxonomic and physiological studies of this species may thus facilitate its usefulness as a bioindicator of water quality, and its involvement in the de-

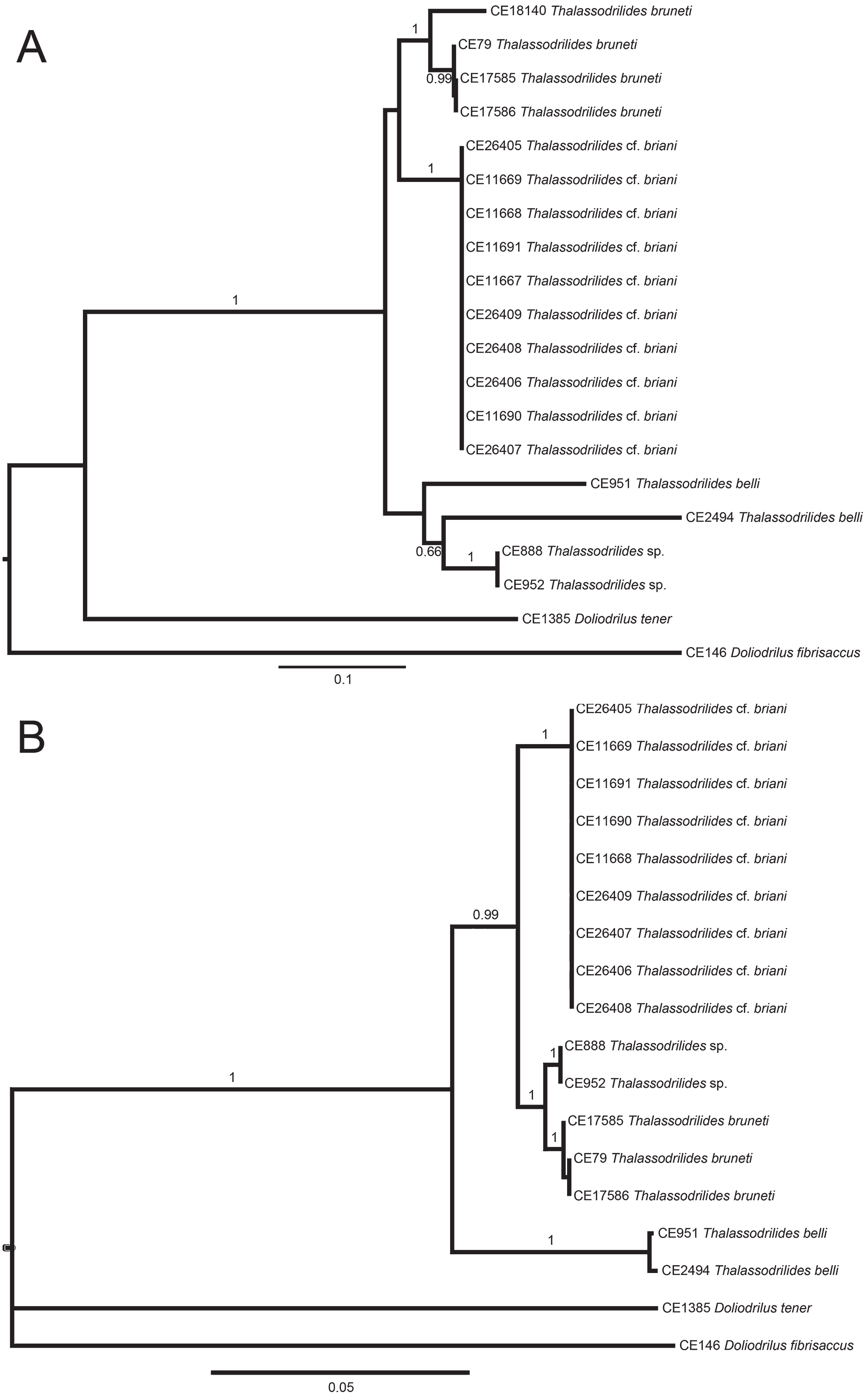

Fig. 3. Maximum likelihood gene trees estimated using PhyML. A, COI; B, ITS. Numbers at branches denote aLRT branch support. Scale shows estimated numbers of nucleotide substitutions per site.

velopment of practical applications for environmental measures such as bioremediation.

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