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	
<i>Thalassodrilides</i> <i>cf. briani</i> (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
<i>Thalassodrilides</i> <i>belli</i>	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
	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
<i>Thalassodrilides</i> <i>bruneti</i>	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
<i>Thalassodrilides</i> 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
	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
<i>Doliodrilus fibrissatus</i>	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 <i>D. tener</i> (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 <i>D. fibrissacus</i> (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 <i>T. bruneti</i> (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 <i>T. belli</i> (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 <i>T. sp.</i> (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 <i>T. cf. briani</i> (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 spermatzoa); 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. *brianii* 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 μ m.

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 μ m long, 48–62 μ m maximum diameter), with no ectal glands. Ampulla 1/3 as wide as segment (120–282 μ m 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 brianii*, 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. brianii* and *T. cf. brianii* 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. brianii* is the same as the proper *T. brianii*; 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
<i>Thalassodrilides belli</i> (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) Erséus (1981)
<i>Thalassodrilides briani</i> Erséus, 1992	upper tooth shorter and narrower than lower tooth	oval or slender, with the ducts less coiled inside than in <i>T. belli</i> , 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)
<i>Thalassodrilides</i> cf. <i>briani</i> 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
<i>Thalassodrilides bruneti</i> Erséus, 1990	upper tooth shorter and narrower than lower tooth	slender, not heavily muscular	lacking	absent	—	Belize, Curaçao	Erséus (1990)
<i>Thalassodrilides gurwitschi</i> (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) Erséus (1981) Erséus (1990)
<i>Thalassodrilides ineri</i> (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) Erséus (1981) Erséus (1990)
<i>Thalassodrilides milleri</i> 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)
<i>Thalassodrilides</i> 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 fibrissatus* 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. brianii*) 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. brianii* 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. brianii*, 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. brianii*'s type locality, and we prefer to regard our new material as *T. cf. brianii* rather than conclude it is identical to *T. brianii sensu* Erséus (1992), especially as we noted some morphological differences be-

tween the copulatory sacs of the two populations. Nevertheless, our results will facilitate further studies to ascertain the taxonomic status of *T. cf. brianii*, 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 morpho-species. 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. brianii* and *T. cf. brianii* 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. brianii* (=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. brianii*. 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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