**Trilobodrilus itoi** sp. nov., with a Re-Description of *T. nipponicus* (Annelida: Dinophilidae) and a Molecular Phylogeny of the Genus

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The marine interstitial annelid *Trilobodrilus itoi* sp. nov., the sixth member of the genus, is described on the basis of specimens collected intertidally at Ishikari Beach, Hokkaido, Japan; this is the second species in the genus described from the Pacific Rim. In addition, *T. nipponicus* Uchida and Okuda, 1943 is re-described based on fresh topotypic material from Akkeshi, Hokkaido, Japan. From both species, we determined sequences of the nuclear 18S and 28S rRNA genes, and the mitochondrial cytochrome c oxidase subunit I (COI) gene. Molecular phylogenetic trees based on concatenated sequences of the three genes showed that *T. itoi* and *T. nipponicus* form a clade, which was the sister group to a clade containing the two European congeners *T. axi* Westheide, 1967 and *T. heideri* Remane, 1925. The Kimura two-parameter distance for COI was 22.5–22.7% between *T. itoi* and *T. nipponicus*, comparable with interspecific values in other polychaete genera. We assessed the taxonomic utility of epidermal inclusions and found that the known six species can be classified into three groups.

**Key words:** North Pacific, marine invertebrates, archiannelid, mesopsammon, meiofauna, interstitial

**INTRODUCTION**

The dinophilid annelid genus *Trilobodrilus* Remane, 1925 comprises marine interstitial ‘archiannelids’ that reach approximately 2 mm in body length and lack typical polychaete features such as distinct body segmentation, parapodia, and chitinous chaetae. It currently contains five named species: *T. heideri* Remane, 1925 and *T. axi* Westheide, 1967 in European waters (e.g., Remane, 1925; Boaden, 1963; Westheide, 1967; Ax, 1968; Scharnofske, 1986); *T. indicus* Chandrasekhara Rao, 1973 from India (Chandrasekhara Rao, 1973); *T. nipponicus* Uchida and Okuda, 1943 from Japan (Uchida and Okuda, 1943), India (Chandrasekhara Rao and Ganapati, 1968), and the Pacific coast of the USA (Wieser, 1957); and *T. hermaphroditus* Riser, 1999 from the Atlantic coast of the USA (Riser, 1999). The hypothesis that these and other dinophilids have evolved from an ancestor most closely related to dorvilleid polychaetes in Eunicida (e.g., Westheide, 1987; Eibye-Jacobsen and Kristensen, 1994) was not supported by molecular phylogenetic analyses (Struck et al., 2002, 2005), and the sister taxon to Dinophilidae has not clearly been established (e.g., Rousset et al., 2007; Struck et al., 2008; Zrzavý et al., 2009). The family as currently diagnosed (Westheide, 1984) contains 16 species in three genera (Read, 2014). Dinophilids and other interstitial annelid families, such as Diurodrilidae (Worsaae and Rouse, 2008; Golombek et al., 2013), Nerillidae (Worsaae et al., 2005), Protodrilidae (Bailey-Brock et al., 2010; Di Domenico et al., 2013; Martínez et al., 2013, 2014), and Saccocirridae (Di Domenico et al., 2014a, b, c) are important for understanding annelid evolution overall (Worsaae and Kristensen, 2005; Struck, 2006; Westheide, 2008) and the biogeography (Curnin-Galletti et al., 2012) and macroevolutionary patterns (Rundell and Leander, 2010) of microscopic animals.

The Pacific species *Trilobodrilus nipponicus* needs re-description, as some of the morphological characters considered to be useful in distinguishing among congeners, e.g., the shape of ‘epidermal inclusions’ (see below), were not known for this species, nor were DNA sequence data available. Westheide (1967) pointed out that *T. nipponicus* is morphologically superficially similar to the European species *T. heideri*, and that a more detailed taxonomic description of the former is necessary to differentiate between them. While *T. nipponicus* has been reported from several localities distant from the type locality subsequent to the original description, insufficient original descriptions and lack of DNA data call into question previous species identifications of *Trilobodrilus* specimens worldwide (e.g., Rieger and Rieger, 1975).

Under light microscopy, specimens of *Trilobodrilus* show within the epidermis two types of small bodies up to 15 μm long: (1) a mosaic or aggregation of droplets, and (2) an elongated, darker body with granular contents. The terminology for these small bodies has been inconsistent in the literature. Westheide (1967) originally used the term “Epidermiseinschlüsse” [epidermis inclusions] collectively to...
refer to both types, but called the aggregations of droplets "polygonale Felder" [polygonal fields] and the other, granular type "spindelförmige Drüsen" [spindle-shaped glands]. Chandrasekhara Rao (1973) did not use a collective term to refer to the two types, but called the former type "epidermal glands" and the latter "bacillary glands". Riser (1999) likewise did not use a collective term, but called the former type "epidermal inclusions" and the latter "spindle glands". Westheide (1967), who initially discovered epidermal inclusions in Trilobodrilus, noted differences in the inclusions between T. axi and T. heideri, which suggested the small bodies might provide useful taxonomic characters. Although the "epidermis inclusions" sensu Westheide (1967) have been succinctly described in T. indicus (Chandrasekhara Rao, 1973) and T. hermaphroditus (Riser, 1999), their structure has not been explored in detail, nor has their taxonomic significance been rigorously assessed with statistical tests.

While Yamanishi (1983) listed 16 species of marine interstitial annelids reported from Japanese waters, representing 11 genera, these tiny worms remain understudied in this region. During the course of a faunal survey of marine interstitial animals around Hokkaido, northern Japan, we recognized two species of Trilobodrilus, one of which was referable to T. nipponicus, whereas the other turned out to be new to science. In this paper, we describe and illustrate these two species, and report the results of molecular phylogenetic analyses to infer their systematic position, making use of dinophilid sequences available in public databases. We also report intra- and interspecific variation in epidermis inclusions of the mosaic type (the "polygonale Felder" of Westheide [1967]), which we henceforth refer to as 'epidermal inclusions' sensu Riser (1999), and discuss the taxonomic utility of variation in these structures.

**MATERIALS AND METHODS**

**Sampling and observation**

Sampling was conducted at two localities, Ishikari Beach and Akkeshi Bay, Hokkaido, northern Japan (Fig. 1). Specimens were extracted from intertidal sediment samples by fresh-water shock followed by stirring and decantation: sediments were brought back to the laboratory and agitated briefly but intensively in tap water in a bucket; the suspension was passed through a Gwen's mermaid bra (Nybakken and Higgins, 2007) with a 32-μm mesh net; and the residue was immediately transferred to seawater to avoid rupture of the soft-bodied animals. Living worms were collected with a Pasteur pipette under a dissecting microscope and photographed with a Nikon D5200 digital camera attached to a Nikon SMZ 1500 microscope by adapters (NYPIXS-3166, NY1S-FA, and NY1S-1501750, Micronet), with a pair of external strobe lights (Hikaru Komachi Di, Morris, Japan). For light microscopy, specimens were observed under an Olympus BX51 compound microscope after being anaesthetized in a MgCl2 solution isotonic to seawater and photographed with the same digital camera. For scanning electron microscopy (SEM), specimens were anaesthetized as just described and then fixed in formalin–seawater, dehydrated in an ethanol series, dried in a Hitachi HCP-2 CO2 critical-point drier, mounted on an aluminium stub, coated with gold in a JEOL JFC-1100 ion sputter coater, and observed with a Hitachi S-3000N scanning electron microscope at 15-kV accelerating voltage. Measurements were taken from scaled digital images using ImageJ ver. 1.48 (Rasband, 1997–2014). Type and voucher material has been deposited in the Hokkaido University Museum (ZIHU), Sapporo, Japan.

Fig. 1. Map of Hokkaido, northern Japan, showing the sampling sites and the surrounding ocean currents.

Fig. 2. Schematic drawing of an epidermal inclusion, defining length and width, and the terminology for the components.

**Analyses of epidermal inclusions**

Terminology and standard measurements pertaining to the mosaic-type epidermal inclusions treated here are given in Fig. 2. Each epidermal inclusion consists of an 'envelope' containing a number of 'spherules'. The envelope is embedded in the epidermis, but may connect with the exterior via a small opening. The spherule situated closest to the opening is usually (but not always) the largest; we call the largest spherule the 'major spherule', corresponding to the "cap-like unit" of Riser (1999). Within the envelope there may be a 'vacuolar region' that lacks spherules, usually at the end opposite the major spherule. Because the envelope is so thin and the spherules are packed so tightly within it, the envelope itself cannot be observed by light microscopy; its outline distinct from the spherules is evident only in the vacuolar region. In the drawings of Westheide (1967, fig. 5) and Chandrasekhara Rao (1973, figs. 5–6), the envelope is not illustrated at all; in the photographs of Westheide (1967, fig. 6) and Riser (1999, fig. 1), the outline of the envelope is unclear. In this paper, to compare the measurements from T. itoi and T. nipponicus with those from other species, the length of the epidermal inclusion is defined as the long axis of the aggregation of spherules, rather than of the envelope itself, and the
width of the epidermal inclusion as the short axis of the spherule aggregation (Fig. 2).

To assess intraspecific variation in epidermal inclusions, the length and width of 11–26 epidermal inclusions were measured for each of five specimens of *T. itoi* sp. nov. Measurements were made from digital images, and differences in the variance and mean of the length-to-width ratio among the five specimens were examined by Bartlett’s test and Tukey’s HSD test, respectively. Interspecific variation in the epidermal inclusions was assessed in the same manner. For previously described species, lengths and widths were measured from figures in the published literature: Westheide (1967, figs. 5, 6) for *T. axi* (*n* = 13) and *T. heideri* (*n* = 15), Chandrasekhar Rao (1973, fig. 4) for *T. indicus* (*n* = 2), and Risér (1999, fig. 1) for *T. hemaphroditus* (*n* = 15). For *T. nipponicus*, 21 epidermal inclusions were measured from one specimen. Statistical tests were performed with R ver. 3.1.0 (R Core Team, 2014).

**DNA extraction, PCR amplification, and sequencing**

Total DNA was extracted by using a DNeasy Tissue Kit (Qiagen, USA). Fragments of the nuclear 18S and 28S rRNA genes (18S and 28S, respectively), and the mitochondrial cytochrome c oxidase subunit I gene (*COI*), were amplified by polymerase chain reaction (PCR) with the primer pairs listed in Table 1. PCR conditions were 95°C for 1 min; 35 cycles of 95°C for 30 sec, 45°C for 90 sec, and 72°C for 3 min (90 sec for *COI*); and 72°C for 7 min. Sequences were determined with the primers listed in Table 1 by using a Big-Dye Terminator Kit ver. 3.1 (Life Technologies, USA) and a 3730 DNA analyzer (Life Technologies, USA).

For the new species, we also obtained longer sequences by next-generation sequencing (NGS). DNA concentration was measured with a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, USA). DNA (100 ng) was fragmented using an Ion Xpress™ Plus Fragment qDNA Library Kit (Ion Torrent, USA). Fragments were ligated to barcode adapters using an Ion Xpress™ Barcode Adapters 33–48 Kit (Ion Torrent, USA). Using 2% E-Gelee SizeSelect™ Agarose Gels (Invitrogen, USA), the resultant barcode library was subjected to size selection, targeting fragments 480 nt long. After eight cycles of PCR amplification, the size-selected library was purified twice with AMPureXP (Beckman Coulter, USA), and the quantity of the library was determined with a High Sensitivity DNA Kit (Agilent Technologies, USA). Based on this library, emulsion-PCR-based beads were prepared by using an Ion PGM™ Template OT2 400 Kit (Ion Torrent, USA). Sequencing was carried out with an Ion Torrent PGM™ sequencer, using an Ion 314™ Chip ver. 2 (Ion Torrent, USA)/Ion PGM™ Sequencing 400 Kit (Ion Torrent, USA). Base calling was done with an Ion Torrent Server, with the output for each barcode in the form of binary sequence alignment/map (BAM)-format files. The adapter sequences in the BAM files were eliminated by using CLC Genomic Workbench ver. 7 (CLC Bio, USA); in addition, *de novo* assembly was performed with CLC Genomic Workbench ver. 7, yielding 3155 contigs; these were exported in FASTA format and subjected to similarity searches using BLAST+ ver. 2.2.29.

**Molecular phylogenetic analyses**

To infer phylogenetic relationships among species in *Trilobodrilus*, a maximum-likelihood (ML) analysis and Bayesian inference (BI) were carried out that included sequences from our two species and four species of dinophilids for which sequences were available in the public databases (Table 2). Because the sister taxon to Dinophilidae is uncertain (e.g., Rouset et al., 2007), outgroups were chosen to include taxa in seven polychaete families, with special emphasis on Dorvilleidae (cf. Eybly-Jacobsen and Kristensen, 1994). The sipunculan *Spiculosus nudus* Linnaeus, 1766 was used to root the trees (Kvist and Siddall, 2013; Weigert et al., 2014). Sequences were aligned gene by gene by using MUSCLE (Edgar, 2004) implemented in MEGA ver. 5.2 (Tamura et al., 2011) with the following settings: Gap Open = −400; Gap Extend = 0; Max Iterations = 8; Clustering Method (Iteration 1, 2) = neighbor joining; Clustering Method (Other Iterations) = neighbor joining; Min Diag Lengt

<p>| Table 1. List of primers used for amplification and sequencing, with original references. |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th><strong>Gene</strong></th>
<th><strong>Primer name</strong></th>
<th><strong>Reaction</strong></th>
<th><strong>Primer sequence (5′–3′ direction)</strong></th>
<th><strong>Direction</strong></th>
<th><strong>Source</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>K18SF</td>
<td>A/S</td>
<td>GTGATATGCTTGCTCTTAAAGATTAACG</td>
<td>Forward</td>
<td>Present study</td>
</tr>
<tr>
<td>28Sb</td>
<td>28S-01</td>
<td>S</td>
<td>GACTACCCCTGAAATTTGACGAG</td>
<td>Forward</td>
<td>Kim et al. (2000)</td>
</tr>
<tr>
<td>28Sr</td>
<td>28SF</td>
<td>S</td>
<td>AGTACATCTTCTACGG</td>
<td>Forward</td>
<td>Luan et al. (2005)</td>
</tr>
<tr>
<td>28S-2K'</td>
<td>28S-n05R</td>
<td>S</td>
<td>CTCACTCTCGTTGCTGCAT</td>
<td>Reverse</td>
<td>Palumbi (1996)</td>
</tr>
<tr>
<td>28S-01R</td>
<td>28S-15R</td>
<td>S</td>
<td>CAATTCGTTCGGCCCAAATTC</td>
<td>Reverse</td>
<td>Yamaguchi and Endo (2013)</td>
</tr>
<tr>
<td>28S-3K'</td>
<td>28S-3KF</td>
<td>S</td>
<td>AGGTAGCCCAATGCTCAG</td>
<td>Reverse</td>
<td>Yamaguchi and Endo (2013)</td>
</tr>
<tr>
<td>COI</td>
<td>LCO1490</td>
<td>A/S</td>
<td>GGTCAAAACATATAAAGATATTTG</td>
<td>Forward</td>
<td>Folmer et al. (1994)</td>
</tr>
<tr>
<td>HCO2198</td>
<td>A/S</td>
<td>TAAACTTCAAGGGTACCAAAAATCTA</td>
<td>Reverse</td>
<td>Folmer et al. (1994)</td>
<td></td>
</tr>
</tbody>
</table>

* A, PCR amplification; S, cycle sequencing
Table 2. List of species included in the phylogenetic analysis, with public database accession numbers.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Markers</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Outgroup (Dorvilleidae)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorvillea eruciformis (Malmgren, 1865)</td>
<td>AY176285</td>
<td>Worsaae et al. (2005);</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Struck et al. (2006)</td>
</tr>
<tr>
<td>Euloplocus jumarsi Blake, 1985</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Iphitime hartmanae Kirkegaard, 1977</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Microdorvillea sp.</td>
<td>AY527051</td>
<td>—</td>
</tr>
<tr>
<td>Ophryotrocha labronica La Greca and Bacci, 1962</td>
<td>DQ790047</td>
<td>Struck et al. (2006, 2007);</td>
</tr>
<tr>
<td>Parougia eliasi (Oug, 1978)</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Petotia onciensis Campoy and San Martin, 1980</td>
<td>AF412801</td>
<td>—</td>
</tr>
<tr>
<td>Protodorvillea kellersteini (McIntosh, 1869)</td>
<td>AF412799</td>
<td>—</td>
</tr>
<tr>
<td>Schistomegania longicornis (Ehlers, 1901)</td>
<td></td>
<td>HM473268 HM473662 Carr (unpubl.)</td>
</tr>
</tbody>
</table>

| **Outgroup (others)**                     |              |                          |
| Cirratulus spectabilis (Kinberg, 1866)    | AY708536     | Burnette et al. (2005);  |
| Diurodrilus subterraneus Remane, 1934     | KC790349     | Struck et al. (2007)     |
| Eunice sp.                                | AF412791     | —                        |
| Eurythoe complanata (Pallas, 1766)        | AY364851     | —                        |
| Nereis vexillosa Grube, 1851              | DQ790083     | —                        |
| Pectinaria gouldii (Verrill, 1873)        | DQ790091     | —                        |
| Spinulus nudus Linnaeus, 1766             | DQ300006     | —                        |

**RESULTS**

**Taxonomy**

Trilobodrilus itoi sp. nov. (Fig. 3)

Material examined. Syntypes, ZIHU 4885, 17 specimens, Au-coated and mounted on SEM stub, collected by M. Ikoma and H. Kajihara, 15 January 2012, Ishikari Beach (43°15′26″N, 141°21′26″E; type locality), about 2 km south-southwest from the mouth of the Ishikari River, Hokkaido, northern Japan; from surface layer of sediment (medium sand) to about 10 cm depth at water’s edge. Eleven specimens, same locality as for syntypes, collected by M. I., 30 September 2013, observed alive by light microscopy, destroyed after observation except for two non-type specimens: ZIHU 4886 (fixed in 10% formalin-seawater, mounted in glycerin on a glass slide, with the edge of the coverslip sealed with Canada balsam) and ZIHU 4887 (fixed in Bouin’s fluid, mounted in the same manner as ZIHU 4886). More than 50 specimens collected sporadically at the type locality from 29 October 2012 to 30 September 2013, all destroyed after observation by light microscopy and DNA extraction.

**Sequences.** The following sequences were determined by standard sequencing from a single, non-type specimen collected on 17 December 2012, for which no morphological voucher remains: COI, AB924371 (658 nt, coding 219 aa); 18S rRNA, AB924372 (1714 nt); 28S rRNA, AB924373 (1120 nt). In addition, 75,340 reads (equivalent to 18.6 M bases) by new-generation sequencing from eight specimens.
Two species of *Trilobodrilus* from Japan

in all, collected on 30 September 2013, have been deposited in the DDBJ Sequence Read Archive under accession number DRA001682. The complete 18S sequence with parts of the ETS and ITS regions (3463 nt), as well as partial 28S sequences including part of the ITS region (4620 nt), both assembled from DRA001682, are available as Supple-


Documentable Files S1 and S2, respectively.

**Diagnosis.** *Trilobodrilus* with second ciliary band incomplete dorsally, where single mid-dorsal ciliary tuft is present; third ciliary band dorsally discontinuous, with large gap.

**Description.** Body 0.9–1.3 mm long, 0.08–0.12 mm wide (n = 11); colorless, translucent; stomach light yellow; salivary glands opaque white, sometimes tinged with green (Fig. 3A); oocytes cream white. Head with four apical ciliary tufts on anterior end, arranged dorsally and ventrally on both sides (Fig. 3B), as in congeners. Three ciliary bands present anteriorly (Fig. 3B–D); first band completely encircling head near tip (Fig. 3B, C); second band situated just anterior to mouth, dorsally discontinuous (Fig. 3C); third band post-oral on segment 1, dorsally discontinuous, with wide gap (Fig. 3C). Single, long, mid-dorsal ciliary tuft in gapped portion of second band, oriented slightly anteriorly (Fig. 3B, C). Pair of nuchal organs in segment 1, one on each side, just anterior to third ciliary band (Fig. 3D). Small, short ciliary tufts distributed sporadically on sides of body (Fig. 3E, H). Pair of salivary glands (Fig. 3A, J) between segments 2 and 3. Width of central ciliary tract (Fig. 3E) about 40% (35–44%, n = 4) of body diameter in middle of body. Epidermal inclusions highly variable in shape, mostly elliptical, up to 7 × 12 μm in size (Fig. 3F, G); spherules 3–22 (mostly 7–8) in number (n = 86, five specimens) (see below “Epidermal inclusions”). Spindle glands not found (Fig. 3F). No mature male found. Females contained up to two oocytes, each up to 150 μm in diameter, arranged in tandem beneath intestine (Fig. 3J); “storage cells” or “coelenchyme” of Fransen (1980, fig. 4A), up to 30 μm in diameter, packed within coelom in mature females (Fig. 3J). Mature individuals with oocytes found in September and October.

**Remarks.** *Trilobodrilus itoi* differs from congeners, except for *T. nipponicus*, in having a mid-dorsal ciliary tuft in the gapped portion of the second ciliary band on the head (Table 3). *Trilobodrilus itoi* differs from *T. nipponicus* in having the third ciliary band (on the first body segment) discontinuous dorsally (vs continuous in *T. nipponicus*), and the ventral ciliary tract is narrow, about 40% of body width (vs wide, about 45% in *T. nipponicus*).

**Ecology.** At Ishikari Beach, *T. itoi* appeared to be narrowly restricted to the swash zone; none were found landward in subsurface water. Densities were locally variable; in calm sea conditions, up to 20 individuals were collected per 300 ml of sand. The salinity during the sampling period was generally 30; the water temperature was 12°C on 29 October 2012 and 4°C on 17 December 2012. *Itō* (1985) found the sediment grain size at the site (φ scale; Krumbein, 1937) to be 1.2–1.7. Animals that co-occurred in the habitat included prosobranch gastropods (*Architrochogaster yamadai*, Tajika, 1993); harpacticoid copepods (*Arenionota ishikariensis* Itō, 1968 and *Leptastacus japonicus* Itō, 1968); nematodes (genera *Ancylostoma*, *Bathyfilum*, *Enoploderma*, *Enoploides*, *Epacanthion*, *Epsilonema*, *Mesacanthion*, *Metoncholaimus*, *Neochromadora*, *Oncholaimus*, *Parasaveljevia*, *Rhyzohenna*, *Theristus*, and *Trissonchulus*) (D. Shimada, pers. comm.); the non-interstitial gammarid amphipod *Anisogammarus pugettensis* (Dana, 1853) (cf. Hoshide, 1971); and the mysid *Archaeomysis articulata* Hanamura, 1997 (cf. Hanamura, 1999). With sporadic water changes, individuals of *T. itoi* were able to survive for more than a month without feeding in a Petri dish placed in an incubator at 12°C.

**Etymology.** The specific name, a noun in the genitive case, is in honor of Dr Tatsunori Itō (1945–1990), a Japanese meiobiologist and crustacean systematist, whose handbook for the general public, *Organisms in Sand Intertides* (Itō, 1985), influenced several successive generations in Japan. Ishikari Beach, the type locality, was one of Itō’s primary research sites early in his career (e.g., Itō, 1970).

**Trilobodrilus nipponicus** Uchida and Okuda, 1943 (Fig. 4)

**Trilobodrilus nipponicus** Uchida and Okuda, 1943, p. 301, figs. 1, 2.

?**Trilobodrilus nipponicus**: Wieser (1957), p. 284, fig. 4; Chandrasekhara Rao and Ganapati (1968), p. 28, fig. 5.

**Material examined.** ZIHU 4946, 22 specimens, Au-coated and mounted on three SEM stubs, collected on a narrow beach about 50 m long at the base of a steep cliff near Cape Akappu, Akkeshi, Hokkaido (43°00′58″N, 144°50′02″E) by M. Ikoma, S. F. Hiruta, and H. Kajihara, 23 September 2014. In addition, one specimen examined alive with light microscopy was destroyed after observation, and four other specimens were used for DNA extraction.

**Sequences.** The following sequences were determined

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**Table 3.** Morphological characters and their states in the six species in *Trilobodrilus*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Body length (mm)</th>
<th>Body width (mm)</th>
<th>Number of mid-dorsal tuft on 2nd ciliary band</th>
<th>3rd ciliary band continuous (+) or not (−)</th>
<th>Size of epidermal inclusions (μm)</th>
<th>Number of spherules per envelope</th>
<th>Mean ratio of length to width of epidermal inclusions</th>
<th>Spindle glands present (+) or absent (−)</th>
<th>Stomach color</th>
<th>Sex</th>
<th>Habitat</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. asi Westheide, 1967</em></td>
<td>1</td>
<td>0.1</td>
<td>0</td>
<td>−</td>
<td>10 × 15</td>
<td>5–10</td>
<td>1.81 (±)</td>
<td>+</td>
<td>?</td>
<td>dioecious</td>
<td>intertidal</td>
<td>Westheide (1967)</td>
</tr>
<tr>
<td><em>T. heideri Remane, 1925</em></td>
<td>1.5–1.9</td>
<td>0.1–0.2</td>
<td>0</td>
<td>−</td>
<td>7</td>
<td>3–10</td>
<td>1.41 (±)</td>
<td>+</td>
<td>brown</td>
<td>dioecious</td>
<td>subtidal</td>
<td>Remane (1925); Westheide (1967)</td>
</tr>
<tr>
<td><em>T. hermaproditus</em></td>
<td>1.7</td>
<td>0.2</td>
<td>2</td>
<td>6 × 9</td>
<td>3–7</td>
<td>1.42 (±)</td>
<td>+</td>
<td>greenish brown</td>
<td>hermaphroditic</td>
<td>intertidal</td>
<td>subtidal</td>
<td>Riser (1999)</td>
</tr>
<tr>
<td><em>T. indicus Chandrasekhara Rao, 1973</em></td>
<td>0.9–1.2</td>
<td>0.1–0.12</td>
<td>1</td>
<td>7 × 9</td>
<td>3–22</td>
<td>2.42 (±)</td>
<td>+</td>
<td>transparent?</td>
<td>dioecious</td>
<td>intertidal</td>
<td>Chandrasekhara Rao (1973)</td>
<td></td>
</tr>
<tr>
<td><em>T. itoi sp. nov.</em></td>
<td>0.9–1.3</td>
<td>0.08–0.12</td>
<td>1</td>
<td>5.5 × 16</td>
<td>9–13</td>
<td>2.28 (±)</td>
<td>+</td>
<td>yellowish or yellowish brown</td>
<td>dioecious</td>
<td>intertidal</td>
<td>intertidal</td>
<td>Present study Uchida and Okuda (1943); present study</td>
</tr>
<tr>
<td><em>T. nipponicus</em> Uchida and Okuda, 1943</td>
<td>0.7–1.4</td>
<td>0.09–0.16</td>
<td>1</td>
<td>5.5 × 16</td>
<td>9–13</td>
<td>2.28 (±)</td>
<td>+</td>
<td>yellowish or yellowish brown</td>
<td>dioecious</td>
<td>intertidal</td>
<td>intertidal</td>
<td>present study</td>
</tr>
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*a*Composed of three rows, the 3rd row present only on ventral side; *b*horse-shape shelled, not meeting ventral ciliary tract.
by standard sequencing from four specimens, for which no morphological vouchers remain: COI, LC009442–LC009445 (658 nt, coding 219 aa); 18S rRNA, LC009446 (from one specimen, 1784 nt); 28S rRNA, LC009447 (from one specimen, 3596 nt).

**Diagnosis.** *Trilobodrilus* with second ciliary band dorsally incomplete, with single mid-dorsal tuft of cilia; third ciliary band nearly complete dorsally.

**Description.** Body 0.7–1.4 mm long, 0.09–0.16 mm wide (n = 23); colorless, translucent (Fig. 4A); stomach orange yellow; oocytes opaque white. Ciliary pattern same as for *T. itoi*, except: 1) third ciliary band continuous dorsally (n = 14), although sometimes sparse (n = 7) (Fig. 4B); and 2) width of ventral ciliary tract about 52% (46–55%, n = 4) of body width (Fig. 4D). Epidermal inclusions elongate, 11–16 μm long, 3.5–5.5 μm wide, with 9–13 spherules per envelope (Fig. 4F); size ratio of major spherule to other spherules in each envelope appearing to be greater than that in *T. itoi* (Figs. 3G, 4F). Spindle glands not found.

**Remarks.** Our topotypic material agrees completely with the original description of *T. nipponicus*, except that lateral ciliary tufts were sparse in our material (Fig. 4C, E), rather than regularly arranged on each segment as described and illustrated by Uchida and Okuda (1943, fig. 1). *Trilobodrilus nipponicus* has been distinguished morphologically from congeners in having a single, mid-dorsal ciliary tuft on the head. Chandrasekhara Rao and Ganapati (1968) identified specimens from India as *T. nipponicus*.
because they had this type of mid-dorsal tuft. Because *T. itoi* also has a mid-dorsal tuft, the Indian form can no longer be positively identified as *T. nipponicus*. Rieger and Rieger (1975) also depicted a mid-dorsal tuft in an unidentified form collected from subtidal sand off Beaufort, North Carolina, Atlantic coast, USA; its identity should be investigated in future studies. A form from the Pacific coast near Seattle, USA, identified by Wieser (1957) as *T. nipponicus* lacks a mid-dorsal tuft; because the second ciliary band is continuous dorsally (Wieser, 1957, fig. 4), this form probably represents a different species.

**Ecology.** Although sediment-particle sizes were not measured, the sediment on the beach at Akkeshi appeared to be coarser than that at Ishikari Beach. *Trilobodrilus nipponicus* was extremely patchily distributed. We collected sediment at ~20 randomly chosen points along the beach in a zone a few meters landward or seaward from the water’s edge, but detected *T. nipponicus* in only one sediment sample. We found 29 specimens in a 300 ml of sediment sample, in which unidentified acoels, harpacticoids, and nematodes predominated; fragments of cirratulid branchial filaments were also found. Uchida and Okuda (1943) reported that “the Archiannelid, *Saccocirrus major*, the Rhabdocoelid Turbellarian, *Thylacorhynchus* sp. and some nemerteans” co-occurred with *T. nipponicus*. While we did observe saccocirrids, they did not appear to utilize the same microhabitat as *T. nipponicus*. An unidentified species of *Proschizorhynchella* (Kalyptorhynchia: Schizorhynchia) co-occurred at low frequency (K. Tamura pers. comm.). Nematodes in the genus *Cephalothrix* were found at the beach, but no species in *Ototyphlonemertes*, an interstitial ribbon-worm genus often found intertidally. We examined sediment on the northern and western coasts of Akkeshi Bay, and on two other beaches on the eastern coast (south of the type locality), but did not find *Trilobodrilus*. Sediments were gravely on the northern coast, silty on the western coast, and composed of finer sand on the two southeastern beaches.

**Epidermal inclusions**

Spherules could be observed by light-microscopy when the animal was slightly squashed under a cover slip, but forceful squashing was necessary to count the number of spherules in a single envelope. After forceful squashing, however, the spherule(s) partly or entirely exuded from the envelope, especially near the edge of the body (Fig. 5). We eventually gave up counting the exact number of spherules in each envelope, because 1) smaller spherules below 0.2 μm in diameter (i.e., approaching the limit of the optical resolution for a 100 × oil-immersion lens) occurred between larger spherules, and 2) the spherules were not arranged at the same focal depth, with the deeper portion obscured.

The mean value in the ratio of length to width of the epidermal inclusions among the five specimens of *T. itoi* ranged from 1.35 to 1.55 (Fig. 6A), with no significant differences among the five in variation (*P* = 0.13: Bartlett’s test) or mean.

![Fig. 5. *Trilobodrilus itoi* sp. nov., photomicrograph of epidermal inclusions near edge of body; arrowheads indicate major spherules appearing to be squeezed out of epidermis.](image)

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Fig. 6. Standard box-and-whisker plots of the length–width ratio of epidermal inclusions: the box encompasses the first and third quartiles; the thick horizontal bar within the box depicts the median; and whiskers depict the true minimum and maximum values, except where outliers (circles) greater than 1.5 times the interquartile range were identified. (A) Intraspecific variation in *Trilobodrilus itoi* sp. nov. among five specimens (specimen No. 1 was a mature, but tiny, female 0.9 mm in body length; Nos. 2–5 were immature specimens, 1.1–1.3 mm in body length). (B) Interspecific variation among six congeners; means with the same lower-case letter at the top of the graph are not significantly different based on Tukey’s HSD test (*α* = 0.05). Data from the literature: *T. axi* and *T. heideri*, Westheide (1967); *T. indicus*, Chandrasekhara Rao (1973); *T. hermaphroditus*, Riser (1999).
Two species of *Trilobodrilus* from Japan

Our study corroborated that of Struck et al. (2002, 2005) in failing to support the morphology-based hypothesis (Eibye-Jacobsen and Kristensen, 1994) of a close relationship between Dinophilidae and Dorvilleidae. All the dorvilleids included in our analyses (except for *Pettiboneia urcienisis* Campoy and San Martin, 1980) formed a clade (91% BF, 1.00 PP) that was the sister group to *Eunice* sp. (64% BF, 0.99 PP). The interspecific K2P genetic distance for COI based on one specimen of *T. itoi* and four of *T. nipponicus* was 22.5–22.7%. These values are comparable with interspecific K2P distances for COI in other genera of interstitial polychaetes, e.g., 23.6–27.0% among six species in *Protodrilus* (Di Domenico et al., 2013; Martínes et al., 2013), and in genera of non-interstitial annelids, e.g., an average of 26.2% in *Hydroides* (Sun et al., 2012) and 19.2–26.2% among three species of *Glycera* (Schüller, 2011).

**DISCUSSION**

We discovered two species of *Trilobodrilus* along the shores of Hokkaido Island, one at Ishikari Beach on the west coast, and the other in Akkeshi Bay on the east, with the two localities 1050 km (northward course) and 1230 km (southward course) apart in along-shore distances (Fig. 1). While we did not record precise physical/geological data for the habitat such as granulometry, the two beaches do differ in physical properties. In beach morphodynamics (Wright and Short, 1984), Ishikari Beach is the intermediate to dissipative type, with finer sand, while the beach in Akkeshi Bay is more or less reflective, with coarser sand. Our sampling site on Ishikari Beach, which is a stretch of sandy shore 25 km long, is 2 km from a river mouth, while the small, unnamed beach in Akkeshi Bay, roughly 50 m long, appeared to receive a continuous input of organic detritus from the nearby rocky and particulate shores that are rich in macroalgae and sea grasses. In addition to differences in the physical properties between the two beaches, they are under the influence of different ocean currents: the warm Tsushima Current flowing north off Ishikari Beach on the Sea of Japan, and the cold Oyashio Current flowing south outside Akkeshi Bay on the Pacific Ocean (Fig. 1).

Overall, the distribution of the two species appeared to be quite restricted, differing widely in scales ranging from microhabitat to the geographical and oceanographic levels. In this context, the putative wide distribution of *T. nipponicus* across the Indo–Pacific (Wieser, 1957; Chandrasekhara Rao and Ganapati, 1968) is likely the result

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**Phylogeny and genetic distance**

In the best ML tree ($ln L = -23774.622593$) (Fig. 7) and BI tree (Supplementary Fig. S1), Dinophilidae is monophyletic, with 97% bootstrap frequency (BF) and 1.00 posterior probability (PP). Within Dinophilidae, *T. itoi* and *T. nipponicus* form a clade (97% BF, 1.00 PP), as do the two European species, *T. axi* and *T. heideri* (96% BF, 1.00 PP). These two clades together comprise a monophyletic group (67% BF, 0.98 PP).

*Dinophilus* sp. sensu Worsaae and Rouse (2008) is the sister group to *Trilobodrilus* spp. (97% BF, 1.00 PP), and *Dinophilus gyrociliatus* Schmidt, 1857, is the sister group to all other species in Dinophilidae (97% BF, 1.00 PP). Our results thus refute the suggestion (Westheide, 1967) that, based on morphological similarity, *T. heideri* might be more closely related to *T. nipponicus* than to *T. axi*.

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**Fig. 7.** Phylogenetic tree resulting from maximum-likelihood analysis based on combined COI, 18S, and 28S rRNA gene sequences ($ln L = -23774.622593$). Numbers near nodes indicate maximum-likelihood bootstrap support values in percent (> 60%) and posterior probability from Bayesian inference (> 0.90), respectively.
of misidentification (see Remarks above), as opponents of
the "everything is everywhere" hypothesis (for review, De
Wit and Bouvier, 2006; Fontaneto and Brodie, 2011; Williams,
2011) have put forward (e.g., Coleman, 2002; Foissner,
2006; Taylor et al., 2006). The species in Trilobodrilus so far
investigated are all direct developers lacking a planktonic
larval phase (Uchida and Okuda, 1943; Ax, 1968), which
suggests relatively low dispersal ability, which in turn may
be related to the restricted distributions observed in this
study. The phylogeographic pattern in our phylogeny, in
which the two Japanese species comprise the sister group
to a clade containing the two European forms, may also be
related to the poor dispersal potential in Trilobodrilus.

Species in Trilobodrilus were thought to be morphologi-
cally similar, distinguished by "the number of body seg-
ments, structure of head and pygidium, distribution of ciliary
rings, lateral tufts of cilia, tactile bristles, structure of epider-
mal glands [sic], etc." (Chandrasekhara Rao, 1973), until the
fourth congener (T. indicus) was described. While the fifth
species (T. hermaphroditus) differs from all of its congener
in being hermaphroditic, Riser (1999) pointed out that the
body segmentation can vary during specimen preparation
(pressure under the cover slip, anaesthetization, and fixa-
tion), and other characters, such as the four anterior tactile
bristles (ciliary tufts) on the tip of the head, are present in all
congeners and thus have little value in distinguishing
between species. With regard to the epidermal inclusions,
our observations indicated that 1) counting the number of
spherules per envelope is impractical because there can be
a number of extremely small (< 0.2 μm) spherules at differ-
ent focus depths, but 2) the mean length–width ratio (L/W)
may be of taxonomic utility, with three groups of species
having been detected: T. indicus and T. nipponicus (elongate
type, L/W > 2.0); T. axi (medium type, 1.5 ≤ L/W ≤ 2.0); and
T. heideri, T. hermaphroditus, and T. itoi (oval type, L/W <
1.5).

The effectiveness of incorporating DNA data in studies of
meiofaunal taxonomy has become apparent for many
taxa, including Nemertodermatida (Meyer-Wachsmuth et al.,
2011) have put forward (e.g., Coleman, 2002; Foissner,
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