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A new species of stygobiontic interstitial annelid, *Diurodrilus kunii* sp. nov., is described based on material collected from medium sand sediment ($\varphi$ = 1.2–1.7) at groundwater level (40–100 cm in depth; 5–15 m inland from splash zone) in the intertidal beach slope on Ishikari Beach, facing the Sea of Japan, Hokkaido, Japan. The new species differs from six known congeners in the arrangement of the anterior-head ventral ciliophores, the degree of development in the primary and secondary toes, and the shape of the spermatozoa. We inferred the phylogenetic position of the new species among other congeners for which 18S rRNA, 28S rRNA, and COI gene sequences were available in public databases. This is the first representative of the genus from the Northwest Pacific.

**Key words:** archiannelid, marine invertebrates, mesopsammon, meiofauna, meiobenthos, Polychaeta, polychaete

## INTRODUCTION

Annelids in the genus *Diurodrilus* Remane, 1925 are tiny, marine, exclusively mesopsammic worms (250–500 µm in body length), lacking fundamental annelid characteristics, such as chaetae, head appendages, parapodia, nuchal organs, and obvious segmentation (reviewed by Villora-Moreno, 1996; Worsaae and Rouse, 2008). They have large, ventral epidermal cells called ciliophores (Kristensen and Niilonen, 1982), which are "compound multiciliated cells with a regular organization of more than ten rows of basal bodies and cilia with extremely long rootlets that beat in unison" (Worsaae and Rouse, 2008), found otherwise only in the micrognathozoan *Limnognathia maerski* Kristensen and Funch, 2000 and the annelid *Neotenotrocha sterreri* Eibye-Jacobsen and Kristensen, 1994. The simple body plan in *Diurodrilus*, along with the unique ciliophores, made its systematic position quite controversial. Based on some similarities to Micrognathozoa, Worsaae and Rouse (2008) alluded even to non-annelid affinity, drawing the attention of higher-level metazoan phylogeneticists (e.g., Jenner and Littlewood, 2009; Edgecombe et al., 2011). Subsequent mitogenomic (Golombek et al., 2013) and phylogenomic (Laumer et al., 2015; Struck et al., 2015) studies, however, firmly placed *Diurodrilus* in Annelida, although its within-phylum phylogenetic position remains uncertain. *Diurodrilus* has thus been classified in the monotypic family Diurodrilidae in Annelida (Kristensen and Niilonen, 1982; Read, 2014).

Diurodrilids live in interstices between medium to coarse sand grains. Most species show strict zonation pattern, ranging from subtidal (down to 60 m depth; Rieger and Rieger, 1976) to upper intertidal moist sand substratum. Some species occur in coastal groundwater or phreatic system (Remane, 1934; Delamare-Deboutteville, 1960; Fize, 1963; Schmidt, 1969, 1972). Two different species may occur in the same beach, segregating their habitats along the land–marine axis in beach slope, with one species found in the upper intertidal deep layer while the other inhabits the sublittoral surface layer (reviewed by Villora-Moreno, 1996). All known *Diurodrilus* are dioecious. Although no direct observation has been made, males may transfer sperm into females' body via copulation, possibly using a projection near the anus (called anal cone) or a pair of club-shaped cuticular structures near genital openings as a penis function (Kristensen and Eibye-Jacobsen, 1995). Internal fertilization is likely because clusters of sperm have been found within females' bodies (Kristensen and Niilonen, 1982). Diurodrilids are probably direct developers, as juveniles are similar to adults in body form and may even be smaller than large oocytes (Worsaae and Rouse, 2008). The tiny and simple body structure in *Diurodrilus* is the result of progenesis (e.g., Worsaae and Kristensen, 2005), which has likely emerged several times independently within the lineage of Orbiniida, leading to other interstitial representatives such as *Apharyngtus*, *Dinophilus*, *Parergodrilus*, and *Trilobodrilus* (Struck et al., 2015).

*Diurodrilus* currently comprises six species (Read, 2014). Compared to rich documentation of four species — viz., *D. benazzii* Gerlach, 1952; *D. dohrni* Gerlach, 1953; *D. minimus* Remane, 1925; and *D. subterraneus* Remane, 1934 — that occur in European waters (Remane, 1925, 1934; Gerlach, 1952, 1953; Delamare-Deboutteville, 1953; Mock, 1981; Villora-Moreno, 1996 [and other references therein]; Worsaae and Kristensen, 2005; Worsaae and Rouse, 2008), records from other parts of the world are scarce and fragmentary. *Diurodrilus minimus* and *D. benazzii* have been recorded from India (Rao, 1969, 1972; Rao and Ganapati, 1968a, b); *D. subterraneus* and *D. westheidei* Kristensen and Niilonen, 1982 are known from Greenland (Kristensen and Niilonen, 1982); and *D. ankeli* Ax, 1967 from the Pacific coast of USA (Ax, 1967). In addition, unidentified forms have been reported from the Atlantic coast of USA (Rieger and Rieger, 1976), the Galapagos Islands (Schmidt and Westheide, 1977), and Australia (Paxton, 2000; Worsaae and Rouse, 2008); two undescribed species are known from New Zealand (Riser, 1984).

Ishikari Beach is an about 25-km stretch of sandy coastline along Ishikari Bay of Hokkaido facing the Sea of Japan, formed by deposits from the Ishikari River (Fig. 1). Meiofaunal studies so far carried out based on material from Ishikari Beach include Itô (1968, 1972) on harpacticoids, Tajika (1981, 1983) on turbellarians, Tsurusaki (1980) describing a marine tardigrade, Kajihara et al. (2015) on dinophilid polychaetes, and Yamauchi and Kajihara (2018) about macrodasyidans. Itô (1984, 1985) examined spatial distribution patterns and abundance of meiofauna in Ishikari Beach along a 20-m transect perpendicular to the shoreline with 5-cm depth interval down to the groundwater level with a focus on selected animal taxa, including nematodes, oligochaetes, the tardigrade *Hypsibius itoi* Tsurusaki, 1980 (now *Halobiotus itoi*), turbellarians, the hesionid polychaete *Hesionides arenaria* Friedrich, 1937, and three species of harpacticoids, *Leptastacus japonicus* Itô, 1968, *Arenopontia ishikariana* Itô, 1968, and *Paraleptastacus unisetosus* Itô, 1972. However, there has been no record of *Diurodrilus* from Ishikari Beach prior to the present study.

In this paper, we describe a new species of *Diurodrilus*



**Fig. 1.** Map showing sampling sites around Hokkaido, Japan. *Diurodrilus kuii* sp. nov. was found in Ishikari Beach and Otobe. *Diurodrilus* was sought, but not found, in another 21 sites indicated by crosses.

as the first representative of the genus from the Northwest Pacific. We also infer the phylogenetic position of the new species using 18S rRNA, 28S rRNA, and COI gene sequences.

## MATERIALS AND METHODS

The first specimen of the species dealt with in this study was brought to us on 29 October 2011 by Mr. Kunihiko Watanabe, who had conducted a systematic research of dinoflagellates as part of his Master's course studies under the supervision of Professor Takeo Horiguchi (Hokkaido University). It was collected in Ishikari Beach at about 2 km southwest from the mouth of the Ishikari River (Fig. 1). The sediment is medium sand ($\varphi$ = 1.2–1.7), containing little organic debris. In 2012, we surveyed at 23 localities around Hokkaido (Fig. 1), but *Diurodrilus* samples were found only at Otobe apart from Ishikari Beach. The Otobe material, collected on 23 September 2012 at 41°59′7.88″N, 140°07′03.40″E, could not be studied in detail due to the limited number of specimens obtained. We conducted monthly sampling from April 2012 to March 2013 in Ishikari Beach. For sampling, we followed the Horiguchi–Watanabe method, in which sediment samples were dug with an iron ladle for Chinese cooking (11 cm in diameter) attached at an end of a bar (we used the shaft of a broken mop for floor cleaning), at 0 m, 5 m, 10 m, and occasionally 15 m inland from the splash zone, about 40 cm to 1 m depth from the ground surface. Samples were collected at the same level of groundwater, of which temperature and refractive index (or 'salinity') were measured; an IS/Mill-E fractometer (AS ONE, Japan) was used for the latter. Worms were extracted by either decantation/sieving method using a mesh size of 63 μm, or by sea-ice method using 100-μm mesh. Specimens were sorted under dissecting microscopes (Nikon SMZ10 and SMZ1500) or inverted microscopes (OLYMPUS CK40 and CKX41). They were relaxed in a MgCl$_2$ solution isotonic to seawater. More than 100 specimens were observed in life, mounted on glass slides under coverslips, with a compound microscope OLYMPUS BX51 with Nomarski differential interference contrast (DIC); 91 specimens were fixed in either 10% formalin seawater or Bouin's fluid. For light microscopy, specimens were embedded in glycerol, mounted on glass slides or between two coverslips and positioned on an H-S slide (Westheide and Purschke, 1988; Shirayama et al., 1993), and sealed with Entellan New (Merck). For electron microscopy, seven specimens were dehydrated in an ethanol series, dried in a critical-point drier (Hitachi HCP-2), mounted on stubs, coated with gold in an ion sputter coater (JEOL JFC-1100), and observed by a scanning electron microscope (SEM) (Hitachi S-3000N) at 10 KV accelerating voltage. Type material has been deposited in the Invertebrate Collection at the Hokkaido University Museum (ICHUM), Sapporo, Japan.

Total genomic DNA was extracted from three specimens using DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's protocol. Amplification of 18S rDNA (18S) was carried out using three primer sets: 1F/4R (Giribet et al., 1996), 3F/18sbi (Giribet et al., 1996), and 18Sa2.0/9R (Whiting et al., 1997). The partial 28S rDNA (28S) fragment was amplified using the primer set LSU5/LSU3 (Littlewood, 1994), 28S-01/28Sr (Kim et al., 2000; Luan et al., 2005), or 28S-2KF/28jj-3′ (Palumbi, 1996; Yamasaki et al., 2013). The COI and Histone H3 fragments were amplified with the primer pair LCO1490/HCO2198 (Folmer et al., 1994) and H3aF/H3aR (Colgan et al., 1998), respectively. PCR reactions were performed using Ex*Taq* (TaKaRa). Hot-start thermal cycling was initiated with 2 min of denaturation at 94°C followed by 35 cycles of 45 sec at 94°C, 45 sec at 50°C, and 30 sec (COI and Histone H3) or 90 sec (18S and 28S) at 72°C; the reaction was completed with a final extension at 72°C at 7 min. The PCR products were visualized in a 1% agarose gel and purified according to Boom et al.'s (1990) method with some modifications (Kobayashi et al., 2009). Cycle
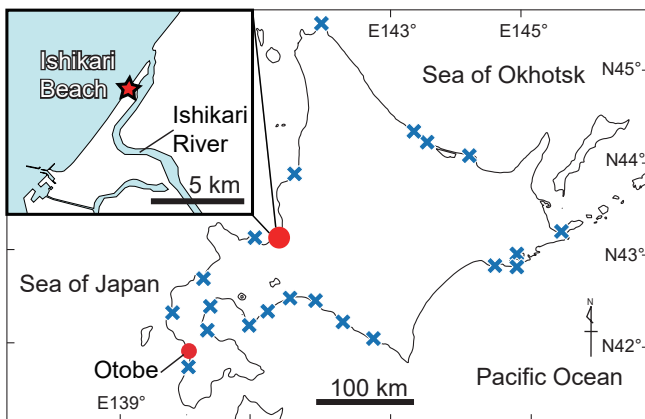
sequencing was performed with BigDye Terminator v.3.1 (Life Technologies). The PCR primers were used for sequencing reactions, together with eight additional 28S primers: a truncated version of 28z (Hillis and Dixon, 1991) by Thollesson and Norenburg (2003), 28SR-01 (Kim et al., 2000), 28Sf (Luan et al., 2005), 28v-5′ (Palumbi, 1996), 28S-15R, 28S-n05R, 28S-3KR, and 28S-42F (Yamasaki et al., 2013). The products were sequenced on ABI 3130 Genetic Analyzer (Life Technologies) for both strands. Chromatograms were edited and overlapping sequence fragments were assembled using ATGC ver. 4.0.6 (GENETYX). Due to poor chromatogram quality, the 28S region from one of the specimens was incompletely determined, resulting in two fragments (5′ region, LC342284; 3′ region, LC342285). The sequences have been deposited in DDBJ/EMBL/GenBank (Table 1).

To address the phylogenetic affinities of the new species, our analyses included two species of *Diurodrilus*, and *Parergodrilus heideri* Reisinger, 1925 and *Stygocapitella subterranea* Knöllner, 1934 as outgroups (Table 2). Of the three specimens from which total DNA was extracted, only one specimen yielded all three gene markers for the analysis (specimen #2, Table 1). Therefore, these sequences were included in the analysis (Table 2). Because the 28S sequence from specimen #2 was short, its 3′ region was complemented by a longer sequence from specimen #1. Histone H3 was determined from specimen #1, but was not included in the analysis as there were no comparable sequences in GenBank. For 18S and 28S, alignment of the sequences was performed by using MAFFT ver. 7 (Katoh and Standley, 2013), employing the *E-INS-i* strategy. For COI, sequences were aligned by MUSCLE (Edgar, 2004) implemented in MEGA ver. 6.0 (Tamura et al., 2013), using the *Align Codons* option. Alignment-ambiguous areas were removed by using Gblocks ver. 0.91b (Castresana, 2000), allowing smaller final blocks, gap positions within the final blocks, and less strict flanking positions, but not allowing many contiguous nonconserved positions. After eliminating ambiguous sites, each data set was 1772 bp (18S), 2106 bp (28S), and 658 bp (COI) long, respectively. These were concatenated by using MEGA ver. 6.0 (Tamura et al., 2013). To determine the best partition scheme for maximum-likelihood (ML) analysis and Bayesian inference (BI), PartitionFinder ver. 2.1.1 (Lanfear et al., 2017) was used employing the *greedy* algorithm. For BI, the most suitable substitution model for each partition selected by PartitionFinder ver. 2.1.1 (Lanfear et

al., 2017) was K80 + I for 18S, GTR + G for 28S and COI (1st codon), HKY for COI (3rd codon), and F81 for COI (2nd codon). ML analysis was performed by using RAxML ver. 8.0.0 (Stamatakis, 2014) with GTR + G model of nucleotide substitution for all partitions consisting of 1000 rapid bootstraps. BI was carried out using MrBayes ver. 3.2.3 (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004) with two independent Metropolis-coupled analyses (four Markov chains of 10,000,000 generations for each analysis). Trees were sampled every 100 generations. Values of run convergence indicated that sufficient amounts of trees and parameters were sampled (average standard deviation of split frequencies = 0; minimum estimated sample size of tree lengths = 171.7236; potential scale reduction factor of tree lengths = 1.026940). Run convergence was also assessed with Tracer ver. 1.6 (Rambaut et al., 2014). ML analyses were also performed for individual 18S and 28S datasets using MEGA ver. 6.0 (Tamura et al., 2013).

## Taxonomy

### *Diurodrilus kunii* sp. nov.
(Figs. 2–5)

**Material examined.** Holotype: ICHUM 5830, mature female, fixed in 10% formalin–seawater, mounted on glass slide in glycerol, collected on 14 April 2012 at 43°15′31″N, 141°21′32″E. Paratypes: ICHUM 5831 (four specimens), mounted on SEM stubs, 18 November 2011, 43°15′29″N, 141°21′30″E; ICHUM 5832 (two specimens), mounted on SEM stubs, collection data same as the holotype; ICHUM 5833 (1 specimen), mounted on SEM stub, 29 June 2012, 43°15′42″N, 141°21′43″E. Additional materials: more than 100 specimens in life, mounted on glass slide; 90 specimens, fixed in either 10% formalin seawater or Bouin's fluid, mounted in glycerol on either glass slide or H-S slide.

**Etymology.** The species is named after Kunihiko Watanabe, the first finder of the species during his Master's course on interstitial dinoflagellates under Professor Takeo Horiguchi, Hokkaido University, Japan. The new specific name is a noun in the genitive case.

**Diagnosis.** A *Diurodrilus* with three anterior-most pairs of ciliophores on anterior head arranged at nodes of opposed angle brackets < >; protonephridia present in 1st and 3rd trunk segments; primary and secondary toes unequal in size; basis of primary toe not widen as in *D. dohrni*; anal cone absent; acrosome/middle piece length ratio being 0.35.

**Description.** *General morphology.* Body transparent and colorless, 240–350 μm in length including toe (holotype 305 μm, n = 11), comprised of anterior head (prostomium), posterior head (peristomium or metastomium), six trunk seg-

**Table 1.** GenBank accession numbers used for sequences newly generated in this study for *Diurodrilus kunii* sp. nov.

| Specimen number | Collection date | 18S | 28S | COI | Histone H3 |
|---|---|---|---|---|---|
| #1 | 18 November 2011 | LC342280 | LC342284, LC342285 | – | LC342286 |
| #2 | 14 April 2012 | LC342281 | LC342282 | LC342279 | – |
| #3 | 14 April 2012 | – | LC342283 | – | – |

**Table 2.** List of annelid taxa used in the molecular analysis with GenBank accession numbers.

| Species | 18S | 28S | COI | References |
|---|---|---|---|---|
| *Diurodrilus kunii* | LC342281 | LC342282 (complemented by LC342285) | LC342279 | this study |
| *Diurodrilus subterraneus* | KC790349 | KC790349 | KC790350 | Golombek et al. (2013) |
| *Diurodrilus* sp. | EF552204 | EF552205 | – | Worsaae and Rouse (2008) |
| *Parergodrilus heideri* | AJ310504 | AY366514 | – | Rota et al. (2001) for 18S; Joerdens et al. (unpublished) for 28S |
| *Stygocapitella subterranea* | AF412810 | AY366516 | AY364852 | Struck et al. (2002) for 18S; Joerdens et al. (unpublished) for 28S and COI |
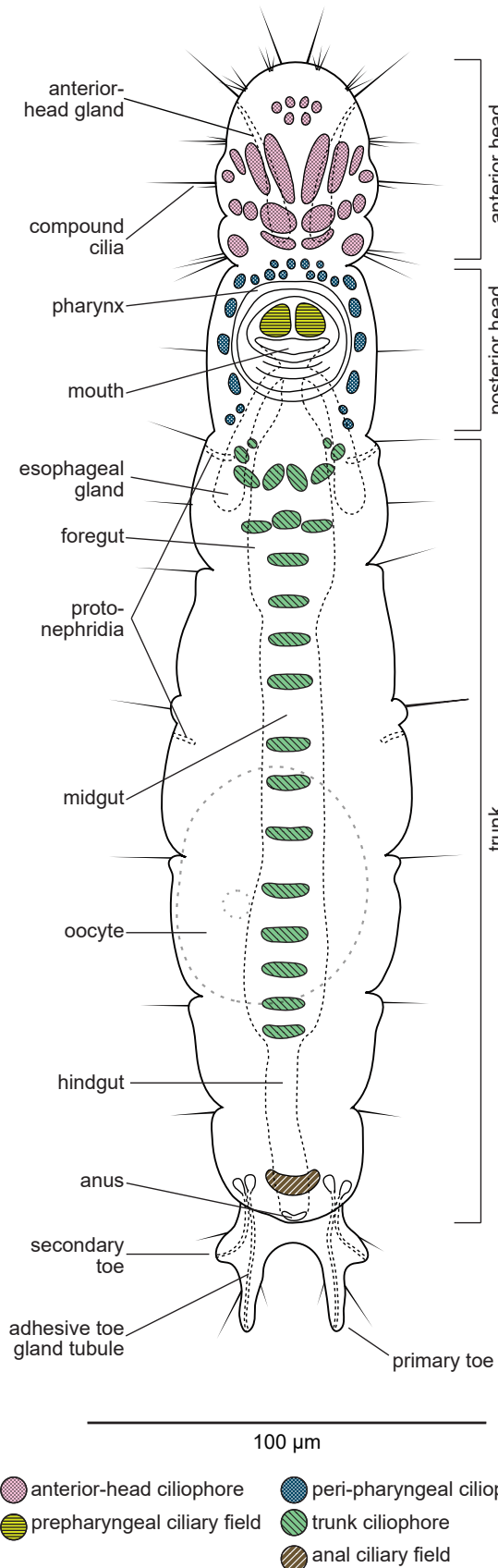
Fig. 2. *Diurodrilus kunii* sp. nov., diagrammatic representation (ventral view) based on DIC microscopy and SEM observation of multiple individuals.



**Fig. 3.** *Diurodrilus kunii* sp. nov., DIC micrographs taken in life. **(A)** Holotype (ICHUM 5830), female with two oocytes, collected on 14 April 2012. **(B)** Male with mature spermatozoans (arrowheads), collected on 12 November 2011; the specimen was destroyed after observation.

ments, and a pair of toes; third trunk segment widest (Figs. 2, 3A, B). Anterior head three-lobed, divided by non-ciliated indistinct constrictions. Cuticular reinforcements or plates not found. Toes comprised of primary and secondary toes, unequally developed (Figs. 2, 3A, 4A). Alimentary canal divided into pharynx and fore-, mid-, and hindgut (Figs. 2, 3B), each internally densely ciliated. Anus ventrally, without forming anal cone.

*Sensory pattern and ciliophores.* Anterior head with two pairs of long anterolateral compound cilia on first lobe, pair of short compound cilia between first and second lobes, and pair of compound cilia each on second and third lobe (Figs. 2, 3A). Anterior-head ciliophores in 12 pairs, of which the anterior-most three were arranged at nodes of opposed angle brackets < > (Figs. 2, 4C).

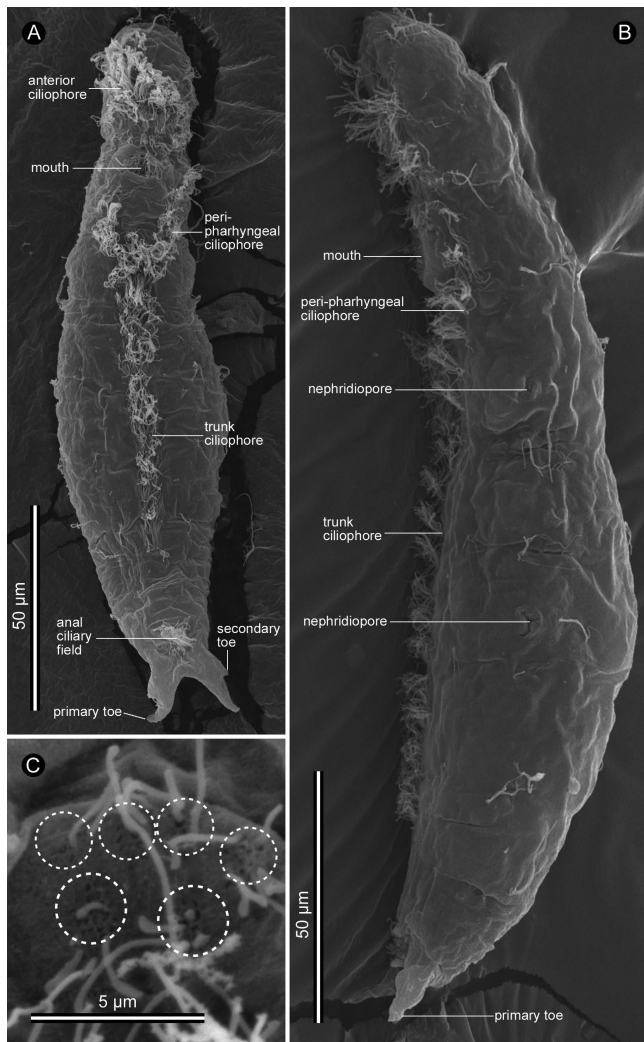Posterior head with pair of compound cilia, ventrally provided with numerous (~11 pairs) ciliophores (Fig. 2). Pair of

**Fig. 4.** *Diurodrilus kunii* sp. nov., SEM images. **(A)** Paratype, ICHUM 5831, whole body, ventral view. **(B)** Paratype, ICHUM 5833 whole body, lateral view. **(C)** Paratype, ICHUM 5832, anterior-most three pairs of anterior-head ciliophores, ventral view.
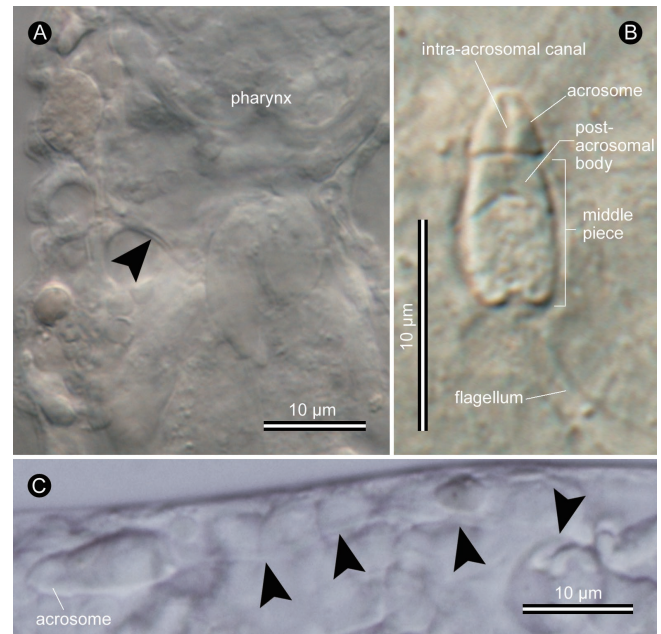


**Fig. 5.** *Diurodrilus kunii* sp. nov., DIC micrographs taken in life; specimens were destroyed after observation. **(A)** Protonephridium in 1st trunk segment, indicated by arrowhead. **(B, C)** Fully developed spermatozoa in body cavity; arrowhead in C indicate flagellum.
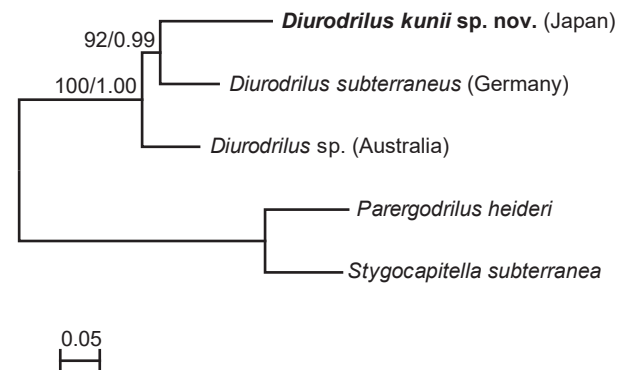


**Fig. 6.** Phylogenetic tree resulting from maximum-likelihood analysis based on combined 18S rRNA, 28S rRNA, and COI gene sequences. Numbers near nodes indicate maximum-likelihood bootstrap support values in percent and posterior probability from Bayesian inference, respectively.

pre-pharyngeal ciliary fields present just anterior to mouth opening (Figs. 2, 4A, B).

Each trunk segment possessing small anterolateral swelling on each side, from which compound cilia project (Figs. 2, 4A). Ventral ciliophores on trunk segments not observable with certainty (Fig. 4A, B); 1st trunk segment seemingly possessing 12 ciliophores, of which anterior four pairs arranged transversely, followed by a row of 3 ciliophores arranged transversely, then single mid-ventral one; 2nd to 4th trunk segments each seemingly possessing 3 ciliophores arranged mid-ventrally in longitudinal row; ciliophores on 5th trunk segment not observable; 6th trunk segment with pre-anal ciliary field (Figs. 2, 4A).

*Excretory system.* Pair of protonephridia present on 1st and 3rd trunk segments (Figs. 2, 4B, 5A).

*Reproductive system.* Mature female containing one or two oocytes, up to about 45 × 75 μm in size (Fig. 3A). Spermatozoa in body cavity 63.5 μm long: acrosome 3.5 μm long, 4.0 μm wide, with intra-acrosomal canal; middle piece 10 μm long, 6 μm wide, containing two-partite post-

acrosomal bodies (3.5 μm long); flagellum 50 μm long, 1 μm wide (Figs. 3B, 5B, C).

*Movement.* The gliding motion is not continuous, but rather sporadic, alternating a quick motion and pause (Supplementary Movie).

**Phylogeny and genetic distance.** The resulting ML and BI trees were identical in topology (Fig. 6), with *D. kunii* sp. nov. being sister to *D. subterraneus*, rather than to an undetermined species of *Diurodrilus* from Australia. The individual 18S and 28S trees also showed the same topology (Supplementary Figures S1, S2). The 658-bp COI sequence of *D. kunii* differed from that of *D. subterraneus* by 0.407 in terms of K2P, and 0.261 in terms of uncorrected *p*-distance.

**Table 3.** Summary of the results of monthly samplings from April 2012 to March 2013 at Ishikari Beach. Density data are only available until 21 August 2012. Macrodasyidans were presented by *Cephalodasys mahoae* and/or *Turbanella lobata*.

| Collection date | Air temperature (°C) | Distance from the splash zone (m) | Water temperature (°C) | Salinity (psu) | Groundwater level (cm) | Occurrence of *Diurodrilus kunii* (individuals/100 mL) | Co-occurred taxa (not exhaustive) |
|---|---|---|---|---|---|---|---|
| 9 April 2012 | — | 5 | — | 20 | 38 | 5.6 | Macrodasyida |
|  |  | 10 | — | 15 | 75 | + | — |
| 14 April 2012 | 8.5 | 5 | 6 | 20 | 65 | 0.08 | — |
|  |  | 10 | 2 | 13 | 92 | 0.76 | — |
| 31 May 2012 | 16 | 5 | 16 | 3 | 60 | + | — |
|  |  | 10 | 15 | 5 | 80 | + | — |
| 29 June 2012 | 30.5 | 0 | 22 | 35 | 0 | — | — |
|  |  | 5 | 18 | 2 | 63 | 0.11 | — |
|  |  | 10 | 19 | 2 | 85 | 0.2 | — |
| 26 July 2012 | 22.5 | 5 | 22.5 | 4 | 60 | + | — |
|  |  | 10 | 22 | 3 | 102 | 5.33 | — |
| 21 August 2012 | 31 | 5 | 24 | 12 | 60 | + | — |
|  |  | 10 | 21 | 9 | 85 | — | — |
|  |  | 15 | 21 | 3 | 100 | 0.33 | — |
| 30 August 2012 | 28 | 0 | — | 34 | 0 | — | — |
|  | 29 | 5 | 24 | 4 | 60 | — | — |
|  | 32 | 10 | — | 2 | 80 | + | Macrodasyida; Platyhelminthes |
|  |  | 15 | 21 | — | 90 | — | — |
| 31 August 2012 | — | 0 | — | — | 0 | — | — |
|  |  | 2.5 | 22 | 33 | 48 | — | — |
|  |  | 5 | 21 | 34 | 54 | — | Macrodasyida |
|  |  | 7.5 | 25 | 33 | 68 | — | — |
|  |  | 10 | 26 | 34 | 73 | + | Macrodasyida |
| 28 September 2012 | 21.5 | 5 | 21 | 31 | 52 | — | — |
|  |  | 10 | 21 | 10 | 100 | — | — |
| 29 October 2012 | 17.5 | 5 | 12.5 | 16 | 42 | — | Annelida (*Trilobodrilus itoi*); Nematoda; Platyhelminthes |
|  |  | 10 | 12 | 10 | 60 | — | Nematoda; Ostracoda |
|  |  | 15 | 12 | 12 | 75 | + | Macrodasyida |
| 20 November 2012 | 4 | 5 | 5.5 | 23 | 55 | — | Macrodasyida |
|  |  | 10 | 6 | 10 | 110 | — | Harpacticoida; Macrodasyida; Nematoda; Platyhelminthes |
| 17 December 2012 | 0 | 0 | — | 30 | 0 | — | Annelida (Oligochaeta; *Trilobodrilus itoi*); Macrodasyida; Nematoda |
|  |  | 5 | 4 | 30 | 20 | — | — |
|  |  | 10 | 2 | 30 | 55 | + | Harpacticoida; Macrodasyida; Ostracoda; Platyhelminthes |
| 15 January 2013 | −4 | 0 | — | 30 | 0 | — | Annelida (*Trilobodrilus itoi*) |
|  |  | 5 | −2 | 30 | 40 | + | Macrodasyida |
|  |  | 10 | — | 5 | 90 | + | Macrodasyida |
| 20 February 2013 | −4 to −7 | 0 | — | 31 | 0 | — | Annelida (Oligochaeta); Nematoda; Platyhelminthes |
|  |  | 5 | — | 31 | 55 | — | Annelida (Oligochaeta); Macrodasyida; Nematoda; Platyhelminthes |
|  |  | 10 | — | 20 | 90 | — | Annelida (Oligochaeta); Macrodasyida; Nematoda |
| 22 March 2013 | 2 | 0 | — | 30 | 0 | — | — |
|  |  | 2 | — | — | 50 | — | — |
|  |  | 5 | — | 28 | 85 | — | — |
|  |  | 10 | — | 25 | 137 | — | — |

**Taxonomic remarks.** Among the six named species of *Diurodrilus*, *D. kunii* is most similar to *D. ankeli* and *D. dohrni* in having unequally sized primary and secondary toes; it differs from *D. benazzii*, which lacks secondary toes; from *D. minimus* and *D. subterraneus*, which have equally sized primary and secondary toes; and from *D. westheidei*, which has unequally sized primary and secondary toes, but their glandular tubules do not join each other within the 6th trunk segment (pygidium) before these are connected to the glandular cells. The arrangement of ciliophores on the ventral side of the anterior head is known for *D. ankeli*, *D. benazzi*, *D. subterraneus*, and *D. westheidei*. While the ciliophore pattern appears to be species-specific, there are always 6–10 small ciliophores comprising an anterior-most group ('anterior ciliophore' in Villora-Moreno, 1996). In *D. kunii*, this anterior-most group contains six ciliophores that are arranged at nodes of opposed angle brackets < > (or double V V, each slightly tilted outward) (Figs. 2, 4C). This pattern of ciliophores in *D. kunii* differs from that in *D. ankeli*, which has four pairs of lateral, and single mid-ventral, ciliophores. The shape of spermatozoa in *D. kunii* is also unique among congeners in that the posterior part of the acrosome has almost the same width as the succeeding middle piece. It is thus different from *D. dohrni*, in which the posterior part of the acrosome is obviously narrower than the middle piece.

**Ecological remarks.** The micro-distribution of *D. kunii* appears to be patchy in Ishikari Beach. They can be locally abundant, up to nine individuals per 100 mL of sand collected on 23 November 2011 (HK pers. obs). The number of individuals of other taxa from the same sample were: 56 nematodes, nine harpacticoids (mostly *Arenopontia ishikariana*), six turbellarians (acoels, kalyptorhynchs, and proseriates), five macrodasyidans (*Turbanella lobata* Yamauchi and Kajihara, 2018), one oligochaete, and one ostracod. Other meiofauna that co-occurred with *D. kunii* in different places and occasions were rhombognathid mites and the macrodasyidan *Cephalodasys mahoae* Yamauchi and Kajihara, 2018. *Diurodrilus kunii* occurs throughout the year in Ishikari Beach. In our monthly samplings from April 2012 to March 2013, specimens were collected except in February, March, September, and November (Table 3). This is likely due to the patchy distribution, rather than reflecting actual phenology. Whenever they occurred, sexually mature specimens were almost always found (HK and MI pers. obs.). In life, individuals of *D. kunii* are highly contractile and can speedily slide among sand particles by using the ventral ciliophores (Supplementary Movie), although their nimbleness is the same as or slightly greater than that of the co-occurring *Turbanella lobata*, but less than that of harpacticoids. During the sampling period, the salinity ranged 2–34 psu. We collected sediment material at a 5-m interval from the splash zone. In some cases, *D. kunii* was more abundant at the 10-m site where the salinity was lower than the 5-m site; it was never found at 0-m site (splash zone). Therefore, they seem to prefer lower salinity, while they are truly euryhaline.

## DISCUSSION

One of the unanswered questions about *Diurodrilus* evolution is whether adaptation to subterranean environment occurred only once or multiple times independently.

This question has been deepened by the discovery of *D. kunii*, which is the third representative of stygobiontic congeners along with *D. benazzi* and *D. subterraneus*. Dispersal potential in diurodrilids is expected to be low, given their assumed direct manner of development. This implies parallel evolution of adaptation to subterranean habitat is likely to have happened in lineages leading to those 'landlocked' stygobionts, resulting in non-monophyletic assemblage of stygobiontic/phreatic species. On the other hand, our molecular phylogeny shows the two stygobiontic species (*D. kunii* and *D. subterraneus*) form a clade that is sister to a non-stygobiontic, surface-layer dweller (*Diurodrilus* sp., subtidal, 1–2 m depth; Worsaae and Rouse, 2008). However, these 'landlocked' species would have become non-monophyletic if other surface-layer dwelling species were included in the analysis.

Villora-Moreno (1996) argued that the reduced toes in the stygobiontic *D. benazzi* and *D. subterraneus* might be the result of adaptation to less disturbing environment in subterranean habitat, because toes are associated with adhesion to substrates. However, the shape of the toes in *D. kunii* is rather similar to that in *D. ankeli* from San Juan Island, WA, USA, which is not a stygobiont (Ax, 1967). While the degree of toe development is difficult to measure quantitatively, it may not be as relevant to adhesive function as Villora-Moreno (1996) supposed.

The microhabitat of *D. kunii* appears to be segregated from that of the dinophilid *Trilobodrilus itoi* Kajihara et al., 2015. Ax (1967) observed an undescribed species of *Trilobodrilus* along with *D. ankeli* in the intertidal zone in San Juan Island. Whether or not *Trilobodrilus* sp. and *D. ankeli* shared exactly the same microhabitat is not apparent from Ax's (1967) description, while *D. kunii* and *T. itoi* are almost never found simultaneously in the same one-scoop-sand sample. *Diurodrilus kunii* is always found in the groundwater level (40–100 cm in depth) at places 5–15 m inland from splash zone on the intertidal beach slope, whereas *T. itoi* occurs almost exclusively in the surface layer of the splash zone (except in one occasion, in which *T. itoi* was collected from underground water 5 m inland from the splash zone on 29 October 2012). Similar niche separation of *Diurodrilus* species has been reported, where one species occupies upper to middle intertidal stygobiontic environment whereas the other occurs in lower intertidal to sublittoral surface layer in the same beach (Rao and Ganapati, 1968a; Schmidt, 1969; Westheide, 1972; Wolff et al., 1980). Because we have not paid much sampling effort in the subtidal zone in Ishikari Bay, the existence of a sublittoral congeneric counterpart of *D. kunii* cannot be ruled out.

## COMPETING INTERESTS

The authors have no competing interests to declare.

## AUTHOR CONTRIBUTIONS

HK conceived and designed the study. HK, MI, HY collected specimens. HK, MI, HY, SFH analyzed the data. HK, MI, HY, SFH prepare the figures and tables. HK wrote the manuscript.

## SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://bioone.org/journals/supplementalcontent/10.2108/zs180197/10.2108.zsj.36.250.s1.pdf, https://bioone.org/journals/supplementalcontent/10.2108/zs180197/10.2108.zsj.36.250.s1.wmv)

**Supplementary Figure S1.** Maximum-likelihood tree based on 18S dataset.

**Supplementary Figure S2.** Maximum-likelihood tree based on 28S dataset.

**Supplementary Movie.** Video footage of a living individual of *Diurodrilus kunii* taken under a binocular lens.

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