

Article

A New Species and Four New Recorded Species of Naididae (Annelida: Oligochaeta) from Korea [†]

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Abstract: This taxonomic study investigates marine tubificids, with a focus on the genera *Smithsonidrilus*, *Heterodrilus*, *Limnodriloides*, and *Tubificoides* and presenting a new species, *Heterodrilus koreanus* n. sp., discovered in the around Ulleungdo and Dokdo Islands in the East Sea of the Republic of Korea. The new species, *Heterodrilus koreanus* n. sp., is characterized by a very long and slender atrium with prostate glands attached to the ventral side, with indistinct ducts and large round ampullae in the spermathecae. The four recorded species are: *H. pentcheffi* Erséus, 1981, *Limnodriloides anxius* Erséus, 1990, *Smithsonidrilus expectatus* Erséus, 1993, and *Tubificoides heterochaetus* Michaelsen, 1926. This study not only enhances our comprehension of the intricate morphology within these genera but also contributes to the broader understanding of marine oligochaetes, particularly in the Korean marine ecosystem.

Keywords: *Heterodrilus*; *Limnodriloides*; *Smithsonidrilus*; *Tubificoides*; taxonomy; phylogeny

1. Introduction

Marine oligochaetes are found worldwide, with approximately 800 identified species inhabiting a variety of environments, including saline pools, rejectamenta, intertidal and subtidal sands, filamentous or macroalgal anchorages, and brackish water and deep-sea sediments [1–9]. Most marine species belong to the Naididae family. They are known to be particularly diverse and abundant in shallow subtropical and tropical waters [1,3,8–46]. Marine oligochaetes from Asia have been recorded in several studies, with approximately 100 species recorded in China over the past 30 years [3,20,37,40,47–52]. During the same period, 16 species (including brackish species) have been identified in Japan [53–58]. In comparison, taxonomic research on marine annelids in Korea has been focused on uncharacterized polychaetes [59–67]. There is no research on marine oligochaetes. Only two species, *Marionina coatesae* Erséus, 1990 and *Pontodrilus litoralis* (Grube, 1855), of marine oligochaetes have been recorded to date [68,69].

The present study considered the naidid fauna around the Ulleungdo and Dokdo Islands in the East Sea of Republic of Korea. Ulleungdo and Dokdo in the East Sea are located on the eastern side of the Korean Peninsula. They are part of an archipelago consisting of two large volcanic and 89 small islands. This location serves as a water mass where the East Korean Warm Current and the North Korean Warm Current intersect and mix [70–72]. The seafloor in this area is devoid of terrestrial sediments. It is characterized by a heterogeneous benthic zone with rocky areas and sediments composed of gravel, sand, and biogenic material. It is 217 km from the mainland of the Korean Peninsula. It is accessible, depending on the weather, from the nearest island, Ulleungdo. Only some areas of the island are accessible to the public. The investigation of the island and nearby waters is permitted only for research purposes. Research on the biodiversity of Ulleungdo



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and Dokdo Island and nearby waters has been conducted continuously since 1960. It has been confirmed that invertebrates living in waters of this area reveal a specialized species composition and regional distribution depending on the topography and habitat. To date, studies of marine invertebrate diversity have been conducted in relatively shallow water depths (<20 m). This study confirmed the biodiversity of benthic animals through scuba diving in waters of 20–50 m in depth, which had been little studied until now. Through this research, the habitat of marine oligochaetes was identified on a bottom of coarse sand and a medium to coarse bottom of gravel and rubble.

This study provides descriptions and photographs of one new species and four recorded species. Our findings contribute to the understanding of marine oligochaete diversity in Korean waters, highlighting the need for continued exploration and research in this region.

2. Materials and Methods

Specimen Sampling and Collection of New Species

Marine oligochaetes were collected from sediment samples obtained through scuba diving in subtidal zones (>20 m) east of Ulleungdo Island and Dokdo Island (Figure 1).

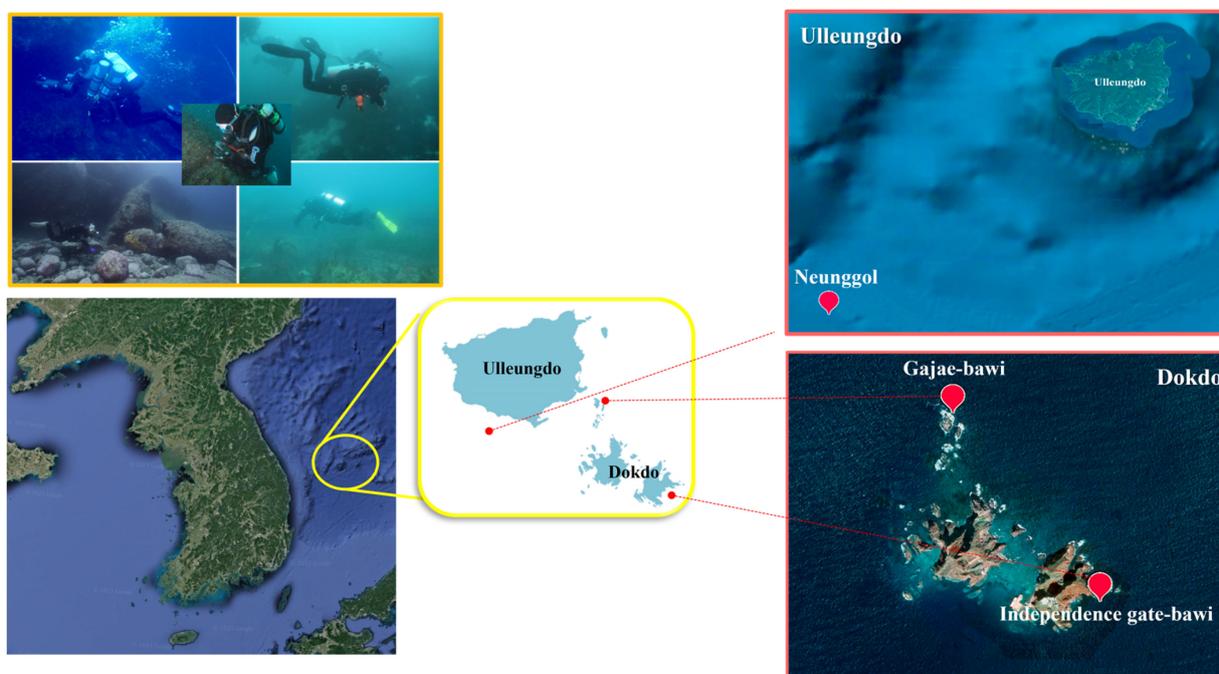


Figure 1. Collection Sites and Scuba Diving in Progress. Geographic locations of collection sites (red dot) and a visual representation of the scuba diving process during sample collection in the study. The scuba diving image provides insight into the fieldwork conducted to obtain specimens for taxonomic analysis.

- Neunggol is a huge underwater rock located in the south of Ulleungdo Island. It is famous for its strong currents. The bottom is coarse and made of sand and gravel.
- Independence gate-bawi is a sea arch located in the easternmost part of Dongdo. It is home to a large amount of seaweed, *Undaria*, and *Saccharina*, and *Eisenia*. The bottom is medium to coarse and made of gravel and rubble.
- Gajae Rock is located in the north of Seodo Island. It has good currents with many rocks and valleys. The large Crayfish Rock in the northernmost part has two valleys. The northern valley is made up of torn vertical walls. The bottom is coarse and made of sand. Specimens were kept alive and transported to the laboratory. Anesthesia and washing were carried out as previously described [73]. Specimens were sorted from

sieved mixtures using a stereomicroscope (SMZ-168, Motic, Hong Kong). They were then fixed in an 8% formalin solution or 80% ethanol. Fixed worms were mounted on microscope slides with Canada balsam following the protocol of Erséus, 1994 [74]. Taxonomic identifications were carried out using a BX41 research microscope fitted with an Olympus DP22 camera system (Olympus, Tokyo, Japan). InnerView™-i series software (Innerview Co. Ltd., Seongnam, Republic of Korea) was used to measure and edit photographs. Voucher specimens of species described here have been deposited in the Marine Biological Resource Institute, Sahmyook University, Seoul, Republic of Korea, and the National Institute of Biological Resources in Korea.

3. Molecular Analysis

3.1. Extraction, PCR Amplification, and Sequencing

DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. All PCR primers are described in Table 1. All have been used previously [46]. For 18S rDNA, about 1800 bp were amplified as two overlapping segments, ca 1000 bp each. The entire fragment was first amplified with primer set 1 (Tim A and Tim B). Two fragments were subsequently amplified using primer set 2 (Tim A and 110R) and primer set 3 (660F and Tim B). PCRs were performed in a total volume of 25 µL, containing 0.5 Units of Taq polymerase (KAPA HiFi PCR Kit, Kapa Biosystems, Wilmington, DE, USA), 5 µL of the 10× buffer, 1.8 µL of 20 mM of MgCl₂, 1 µL of each primer (10 mM each), 1 µL of a mix containing 10 mM of each dNTP, and 4 µL of template DNA. All PCRs for 16S and 18S were performed with an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 48–52 °C for 1 min, and elongation at 72 °C for 1 min 30 s, with a final elongation step at 72 °C for 7 min. To amplify the COI, the thermal profile was as follows: 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 44 °C for 1 min, 72 °C for 1 min 30 s, and 72 °C for 7 min. PCR products were verified using a 2% agarose gel electrophoresis. They were sequenced by Cosmo Genetech (Seoul, Republic of Korea). Sequences were deposited in GenBank.

Table 1. Primers used for PCR and sequencing in this study.

Primer Name	Primer Sequence	Reference
18S Primer		
TimA	5'-AMCTGGTTGATCCTGCCAG-3'	Tim Littlewood (pers. comm. Norén and Jondelius 1999) [75] Erséus et al., 2002 [76] Norén and Jondelius, 1999 [77]
TimB	5'-TGATCCATCTGCAGGTTACCT-3'	
660F	5'-GATCTCGGGTCCAGGCT-3'	
1100R	5'-GATCGTCTTCGAACCTCTG-3'	
16S Primer		
16S ar-L	5'-CGC CTG TTT ATC AAA AAC AT-3'	Palumbi et al., 1999 [78]
16S br-H	5'-CGC CTG TTT ATC AAA AAC AT-3'	
COI Primer		
LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	Folmer et al., 1994 [79]
HCO2198yy	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	

3.2. GenBank Data

We obtained sequence datasets for three locations (16S, 18S, and COI) from the new *Heterodrilus* species, 15 other *Heterodrilus* species, 9 related oligochaetes species, and a polychaete species. Sequences of 30 species of ingroup and out-group taxa were accessed from GenBank (Table 2). New DNA sequences were deposited into GenBank (Accession no. 16S: MW794267–MW794271, 18S: MW795716–MW795720, COI: MT250545–MT250549).

Table 2. Species used, places of origin, and GenBank accession numbers for 16S, 18S, and COI sequences (new sequences are indicated by bold lettering).

Species	Locality	16S	18S	COI
INGROUP: Clitellata, Naididae				
<i>Heterodrilus bulbiporus</i> Erséus 1981	Fort Pierce, Florida, USA	KJ753872	KJ753886	KJ753850
<i>Heterodrilus</i> cf. <i>virilis</i> Erséus 1992a	Lizard Island, Australia	KJ753880	KJ753892	KJ753853
<i>Heterodrilus chenianus</i> Wang and Erséus 2003	Hainan, China	AY885601	AY885574	KJ753856
<i>Heterodrilus decipiens</i> Erséus 1997a	Rottneest Island, W. Australia	AY885603	AF209455	-
<i>Heterodrilus devexus</i> Erséus 1997a	Dampier, W. Australia	AY885602	AY885575	-
<i>Heterodrilus ersei</i> (Giere 1979)	Lee Stocking Island, Bahamas	AY885606	AY885576	KJ753857
<i>Heterodrilus flexuosus</i> Erséus 1990	Carrie Bow Cay, Belize	AY885600	AY885573	-
<i>Heterodrilus keenani</i> Erséus, 1981	Heron Island, Australia	-	AY040688	AY040703
<i>Heterodrilus minisetosus</i> Erséus 1981	Lee Stocking Island, Bahamas	AY885599	AF411885	KJ753859
<i>Heterodrilus modestus</i> Erséus 1990	Lee Stocking Island, Bahamas	KJ753876	KJ753888	KJ753855
<i>Heterodrilus koreanus</i> n. sp.1	Dokdo, the East Sea, Korea	MW795716	MW794267	MT250545
<i>Heterodrilus koreanus</i> n. sp.2	Dokdo, the East Sea, Korea	MW795717	MW794268	MT250546
<i>Heterodrilus koreanus</i> n. sp.3	Dokdo, the East Sea, Korea	MW795718	MW794269	MT250547
<i>Heterodrilus koreanus</i> n. sp.4	Dokdo, the East Sea, Korea	MW795719	MW794270	MT250548
<i>Heterodrilus koreanus</i> n. sp.5	Dokdo, the East Sea, Korea	MW795720	MW794271	MT250549
<i>Heterodrilus occidentalis</i> Erseus 1981	Fort Pierce, Florida, USA	KJ753877	KJ753889	-
<i>Heterodrilus paucifascis</i> Milligan 1987	Carrie Bow Cay, Belize	AY885605	AF411865	KJ753858
<i>Heterodrilus pentcheffi</i> Erséus 1981	Lee Stocking Island, Bahamas	KJ753878	KJ753890	KJ753854
<i>Heterodrilus perkinsi</i> Erséus 1986	Fort Pierce, Florida, USA	KJ753879	KJ753891	KJ753849
<i>Heterodrilus queenslandicus</i> (Jamieson, 1977)	Heron Island, Australia	AY885604	AF411881	-
OUTGROUPS: Clitellata, Naididae				
<i>Heronidrilus heronae</i> (Erséus and Jamieson, 1981)	Heron Island, Australia	AY885616	AF209454	KJ753861
<i>Adelodrilus pusillus</i> Erséus, 1978	Koster area, SW Sweden	KJ753881	KJ753894	KJ753867
<i>Aktedrilus arcticus</i> (Erséus, 1978)	Koster area, SW Sweden	AY885591	AF209451	AF064042
<i>Pectinodrilus molestus</i> (Erséus, 1988)	Carrie Bow Cay, Belize	AY885598	AF209462	KJ753864

Table 2. Cont.

Species	Locality	16S	18S	COI
<i>Thalassodrilus prostatus</i> (Knöllner, 1935)	Göteborg, SW Sweden	KJ753883	KJ753896	KJ753871
<i>Tubificoides pseudogaster</i> (Dahl, 1960)	Kysing Fjord, Denmark	AY885609	AF411873	HM460209
<i>Heronidrilus gravidus</i> Erséus, 1990	Carrie Bow Cay, Belize	AY885617	AF411887	-
Clitellata, Phreodrilidae				
<i>Insulodrilus bifidus</i> Pinder and Brinkhurst, 1997	Bow River, W. Australia	AY885593	AF411906	KJ753862
Clitellata, Enchytraeidae				
<i>Buchholzia fallax</i> Michaelsen, 1887	Toscana (soil), Italy	AY885581	AF411895	KJ753848
Polychaeta				
<i>Ophelina acuminata</i> Örsted, 1843	Øresund Strait, Denmark	HM746716	AY340439	MN138411

4. Phylogenetic Analysis

For sequence alignment, MAFFT ver. 7.450 [80] within Geneious Prime (Biomatters Ltd., Auckland, New Zealand) was used, applying the L-INS-I setting (Scoring matrix: 200PAM/k = 2) for the three loci (16S rDNA, 18S rDNA, and COI of 623 bp, 1830 bp, and 658 bp, respectively). Furthermore, we individually concentrated on consensus sequences (2961 bp) in the three loci of the datasets. All parameters except topology were unlinked between partitions. An additional matrix was also created by concentrating on mitochondrial data, with data obtained from the nuclear 18S locus and partitioning alignments both by locus and by codon position.

Evolution models of best fit were chosen using the Akaike information criterion (AIC) implemented by ModelFinder (Kalyaanamoorthy et al., 2017) [81] within MEGA X [82]. Pairwise sequence distances were calculated using MEGA X [82]. The tree was inferred by using the Maximum Likelihood (ML) method and General Time Reversible (GTR) model. The GTR + G + I model was identified as the best-fit model for all loci (16S, 18S, and COI) using ModelFinder with 1000 bootstrap replicates [81]. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Joining (NJ) algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) method. Trees were drawn to scale, with branch lengths measured in terms of the number of substitutions per site.

Bayesian inference (BI) analyses were performed for mtDNA (two regions), ntDNA (one locus), and consensus sequence data (mtDNA and three loci) separately (16S: 450 bp, 18S: 1613 bp, COI: 442 bp, mtDNA: 1838 bp, and consensus: 2925 bp). Bayesian analyses were carried out in Geneious using MrBayes 3.2 and the GTR substitution model [83,84]. Analyses of alignments were carried out in two independent parallel runs of four Metropolis-coupled Monte Carlo Markov Chains (MCMCs) implemented in MrBayes. Four chains were run twice in parallel for 2.1×10^6 generations, and trees were sampled every 1000 generations. Stationarity of the analyses was inferred when the average standard deviation of split frequencies was less than 0.01. All trees were rooted with *Ophelina acuminata*.

5. Results

Taxonomy account
 Phylum Annelida
 Class Clitellata
 Subclass Oligochaeta
 Order Tubificida
 Family Naididae Ehrenberg, 1831
 Subfamily Limnodriloidinae Erséus, 1982

Genus *Limnodriloides* Pierantoni, 1903

Hair chaetae absent. Chaetae all bifid, generally more than two per bundle anteriorly. Modified spermathecal chaetae in X. Clitellum extending over XI–XII. Body wall without papillae and adherent foreign particles. Male and spermathecal pores paired or unpaired in XI and X, respectively. Esophagus in IX modified, either dilated and glandular or with a pair of anteriorly directed diverticular. Male genitalia paired. Pseudopenes or true penes present, lacking cuticular sheaths. Spermathecae paired or unpaired, dorsal or ventral-lateral. Sperm arranged in loose bundles or as slender spermatozeugmata.

Type species. *Limnodriloides appendiculatus* Pierantoni, 1903.

Limnodriloides anxius Erséus, 1990 [27] (Figure 2A–E)

Limnodriloides monotheucus (partim); Erséus 1982 [17] (pp. 250–253) (nec figs. 28–29); 1986 [23] (p. 309); nec sensu Cook, 1974 [85].

Limnodriloides monotheucus: Erséus 1987: 274.

Limnodriloides anxius: Erséus, 1990 [3] (pp. 243–303), Figs. 26I–O, 27A–D.

Material examined. NIBRIV0000901382, NIBRIV0000901503, NIBRIV0000901504. Independence gate-bawi at Dokdo-ri, Ulleung-eup, Ulleung-gun, Gyeongsangbuk-do, Republic of Korea, (37.239363° N 131.871777° E), 1 May 2022, in medium-coarse sediment of 20 m in depth at 14 °C through scuba diving.

Description. Length 4.7–6.9 mm long (three complete specimens)

Prostomium small conical shape (Figure 2A). Clitellum poorly developed. Chaetae bifid, upper tooth thinner and shorter than lower (Figure 2B,C). Chaetae 30–64 µm long, 2(1)–4 per bundle anteriorly, absent from (X)XI. Pharyngeal glands in IV–V. Esophageal diverticula present in IX. Male genitalia paired, vas deferens shorter than atrium, entering atrium subapically. Atrial ampulla oblong, prostatic pad elongate, ventral in ampulla (Figure 2E). Prostate gland lobed, large. Atrial duct slender, 56–71 µm long, convoluted. Ental part atrial duct thick inner epithelium. Duct terminating in pseudopenial papilla inside copulatory sac; sac 20–38 µm long (Figure 2E). Spermathecal pore unpaired, mid-dorsal in middle of X (Figure 2D). Spermatheca elongate pear-shaped, 95–150 µm long, inner epithelium thicker in ectal than in ental part. Sperm arranged as 1–2 spindle-shaped spermatozeugmata in spermatheca. Spermatozeugmata 80–95 µm long; length about 6–8 × width (Figure 2D).

Distribution. West Atlantic (Belize, Bonaire, Venezuela, Bravados, Florida through Virginia) and Korea.

Habitat. Lower intertidal and subtidal, generally muddy sand to at least 20 m in depth.

Remarks. According to Erséus (1990) [27], both the chaetal arrangement and male genitalia exhibit significant variability in *Limnodriloides monotheucus* and *L. anxius*. As a result, previous literature often reported this species as *L. monotheucus*. However, a key distinguishing feature between these two species is the shape of the spermatozeugmata within sperm, with *L. monotheucus* having cylindrical sperm, setting it apart from *L. anxius*. This character offers reliable means for accurate distinguishing the two species. Our specimens (4.7–6.9 mm) were smaller than the those in the original description (7.8–8.8 mm). Despite the size difference, the atrial ampullae in our specimens closely resembled those of the type series. Additionally, while the shape of the spermatheca was not precisely an enlarged pear-shape, it bore a strong resemblance. Furthermore, the sperm within the spermatheca was organized as 1–2 spindle-shaped spermatozeugmata. Therefore, our samples were confirmed to share key characteristics with type species. This species was reported for the first time in Korea key characteristics with type species.

Genus *Smithsonidrilus* Brinkhurst, 1966

Body wall smooth without papillae or adherent foreign particles. Chaetae all bifid. Hair chaetae and modified genital chaetae absent. Pharyngeal glands generally well developed in IV or V. A pair of esophageal diverticula present anteriorly in IX. Coelomocytes, small and sparse, not of the ‘Rhyacodriline-type’. Male and spermathecal pores unpaired,

midventral in XI. Male ducts complex, filling most of coelom in XI. A pair of ciliated vasa deferentia sub-apically joining paired, slender atria. Each atrium consisting of (1) an ental ampulla, which is partly ciliated, (2) an ectal duct, which is not ciliated but granulated, and (3) a heavily granulated, elongate diverticulum attached at junction between ampulla and duct, and which ventrally bears a very large, lobed, and broadly attached prostate gland. Pseudopenes present, paired or unpaired. Spermatheca paired or unpaired, ampulla with sperm arranged as loose s, ring, bundles, or slender spermatozeugmata in X. Sperm as longitudinal bundles in spermatheca.

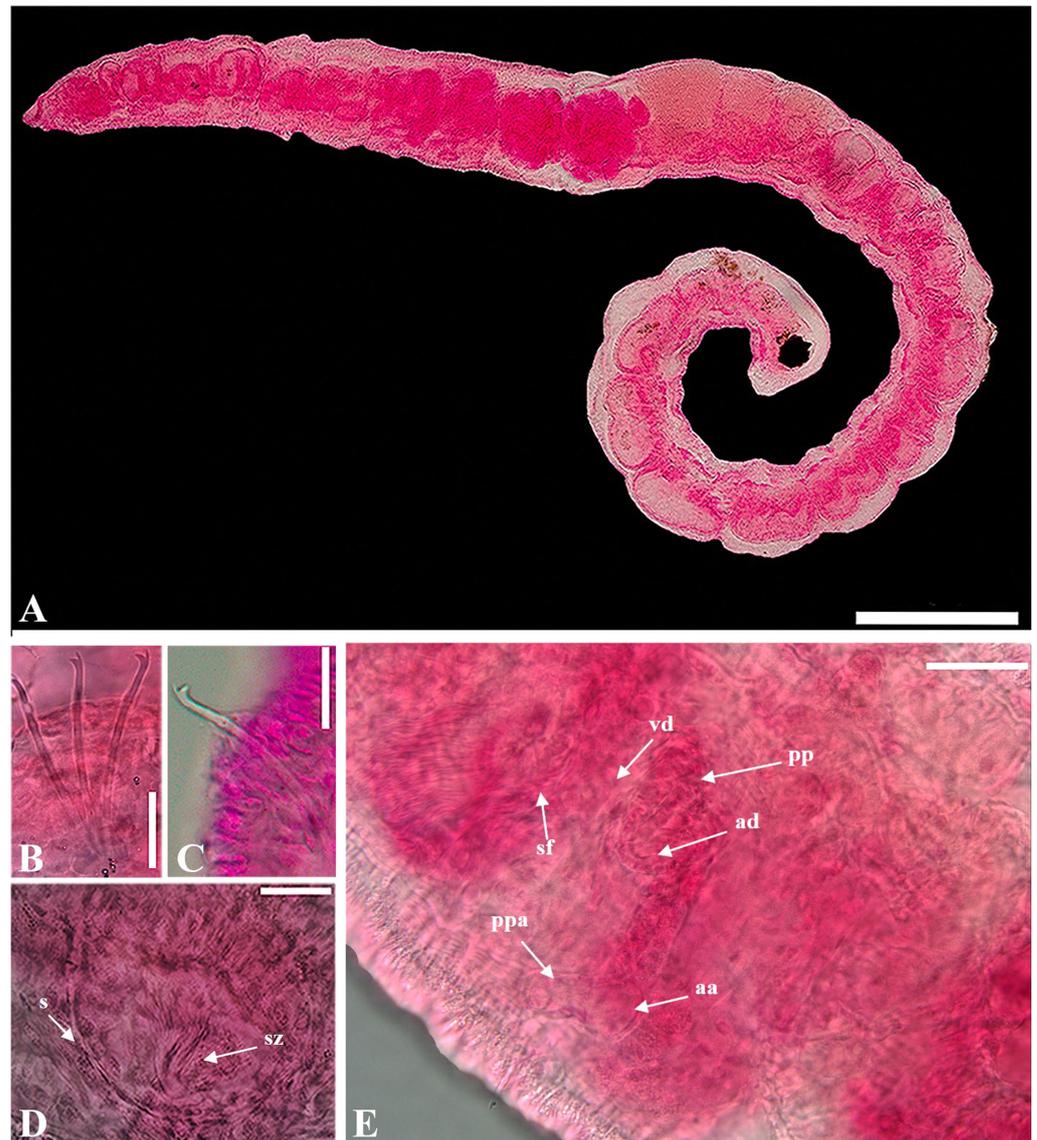


Figure 2. *Limnodriloides anxius*. (A) Total body, (B) anterior chaetae, (C) posterior chaeta, (D) spermatheca with spermatozoa, (E) lateral view of male genitalia in segment of XI. Scale bars: 400 μm in (A), 20 μm in (B,C), 40 μm in (D,E). aa: atrial ampulla, ad: atrial duct, pp: pseudopenis, ppa: prostatic pad, sf: sperm fennel, s: spermathecae, sz: spermatozoa, vd: vas deferens.

Type species. *Smithsonidrilus marinus* Brinkhurst, 1966.

Smithsonidrilus expectatus Erséus, 1993 (Figure 3A–D)

Smithsonidrilus expectatus Erséus, 1993 [31] (pp. 587–590), Figure 1.

Material examined. MABIKNA00157827, Neunggeol at Ulleung-eup, Ulleung-gun, Gyeongsangbuk-do, Republic of Korea, (37.26586° N, 130.52299° E), 5 June 2022, in medium-coarse sediment from 52 m in depth at 11 °C, obtained through scuba diving. Specimens were deposited at the National Marine Biodiversity Institute of Korea (MABIK).

Description. Prostomium pointed triangular (Figure 3A). Clitellum extending over 2/3X–2/3XII. All chaetae bifid, upper tooth thinner and shorter than lower (Figure 3B). Chaetae 42–86 µm long, 2–3 per bundle anteriorly, 1(2) per bundle posteriorly, absent from XI, two per bundle thereafter. Male pore unpaired, located mid-ventrally, posterior to middle of XI. Spermathecal pore unpaired, mid-ventral, near middle of X. Male genitalia complex, with ejaculatory duct and pseudopenis unpaired. Spermathecal ampulla unpaired with oval-shape (Figure 3C), Male and spermathecal pores unpaired, located mid-ventrally posterior of XI and middle of X, respectively. Sperm funnel conspicuous and deep (Figure 3D). Vas deferens thin-walled, ciliated, as long as atrial ampulla, not clearly set off from latter. Atrial ampulla 100–170 µm long, ectally dilated, without cilia inside. Ectal part of atrial ampulla narrow. Prostate gland large and lobed, attached to diverticulum of atrial duct (Figure 3D). Atrial ampulla ectally terminating and slender, with non-granulated atrial duct. Atrial ducts ectally transiting into conspicuous, unpaired ejaculatory duct. Ejaculatory duct heavily muscular, often folded. Spermatheca unpaired with inconspicuous vestibula, a large, thin-walled, oval ampulla, and a filiform diverticulum attached to inner end of ampulla (Figure 3C). Sperm in random mass, denser in diverticulum than in ampulla throughout spermatheca.

Distribution. Florida Keys (Atlantic coast of southern Florida), and the East Sea of Korea Habitat. Barely subtidal (known from 0.1–0.6 m in depth), medium to coarse sand.

Remarks. According to the original description [31], *Smithsonidrilus expectatus*, belonging to the *S. marinus* complex, is characterized by unpaired male and spermathecal pores. However, it distinguishes itself from other species within this group in terms of spermathecal structure and copulatory apparatus. In contrast to other members of the complex, this species lacks a distinct filiform diverticulum at the inner end of the spermathecal ampulla. While maintaining a complex copulatory apparatus, it possesses a much simpler configuration than other species in the *S. marinus* complex. These differences in spermathecal structure and copulatory apparatus provide clear distinctions between this species and its counterparts within the *S. marinus* complex.

Subfamily Tubificidae d’Udekem, 1855

Genus *Tubificoides* Lastočkin, 1937

Body wall naked or with fine particle adhering to cuticle, often forming distinct papillae. Prostomium retractable or not. Hair chaetae absent or present in dorsal bundles; dorsal chaetae simple-pointed, bifid, or pectinated. Ventral chaetae all bifid and modified genital chaetae absent. Male and spermathecal pores paired in posterior of XI and middle of X, respectively. Male genitalia paired in XI. Vas deferens ciliated, thin-walled, entering atrium subapically, generally opposite to large stalked prostate gland. Main body of atria somewhat cylindrical, terminal section again histologically distinct, ending in penial structure bearing a penis sheath of varying form.

Type species. *Tubificoides heterochaetus* Lastočkin, 1937 = *T. swirencowi* Jaroschenko, 1948 (non *T. heterochaetus* (Michaelsen, 1926)).

Tubificoides pollex Milligan, 1991 (Figure 4A–D)

Tubificoides pollex Milligan, 1991 [86] (pp. 340–341), Figure 1A–E.

Material examined. 1 May 2022 NIBRIV0000901383, NIBRIV0000901505, NIBRIV0000901506. Independence gate-bawi at Dokdo-ri, Ulleung-eup, Ulleung-gun, Gyeongsangbuk-do, Republic of Korea, (37.239363° N, 131.871777° E), 1 May 2022, in medium-coarse sediment of 20 m in depth at 14 °C through scuba diving. Specimen has been deposited at the National Institute of Biological Resources (NIBR).

Description. Prostomium not retractable, short, bluntly triangular. Anterior segments distinctly wider than postclitellar segments (Figure 4A). Body wall slightly papillate posteriorly with foreign material adhering. Clitellum weak, extending over X–XII. Ventral chaetae bifid, upper tooth slightly thinner than lower, 3–4 per bundle (Figure 4B). Ventral chaetae absent in XI; thereafter, two per bundle, upper tooth shorter and much thinner than lower. Posterior segments with one chaeta in both dorsal and ventral bundles. Anterior dorsal bundles of II–VIII with 1–2 bifid needle, upper thinner and shorter than lower, and 1(2) hair chaetae, short, 1(2) per bundle (Figure 4C). Pharyngeal glands in IV and V. Esophagus appearing slightly enlarged in IX. Male genitalia paired. Sperm funnel large. Vas deferens thin-walled, about twice as long as atrium, internal ciliation fine and dense. Prostate gland large, attached to atrium opposite entrance of vas deferens. Penis cuticularized, thumb-shaped, a large, oval lateral opening bearing a flap-like projection (Figure 4D). Spermathecae with narrow, elongate, and large oval ampullae. Sperm traps not well developed.

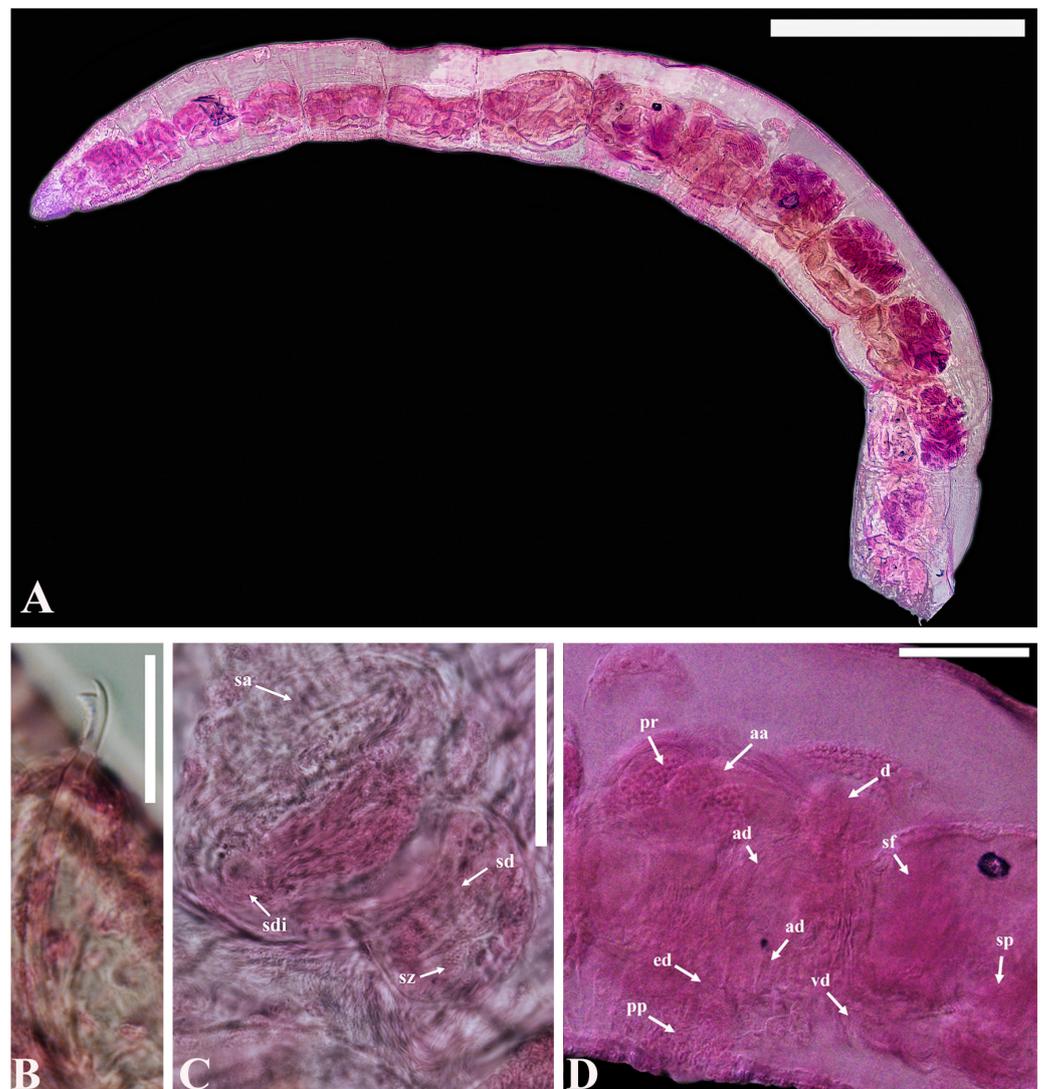


Figure 3. *Smithsonidrillus exspectatus*. (A) Total body, (B) chaeta, (C) spermatheca with spermatozoa, (D) lateral view of male genitalia. Scale bars: 400 μ m in (A), 20 μ m in (B,C), 100 μ m in (D), aa: atrial ampulla, ad: atrial duct, d: duct, ed: ejaculatory duct, pp: pseudopenis, pr: prostate glands, sf: sperm funnel, sa: spermathecal ampulla, sd: spermathecal duct, sdi: spermathecal diverticulum, sp: spermatheca, sz: spermatozoa, sf: sperm funnel, vd: vas deferens.

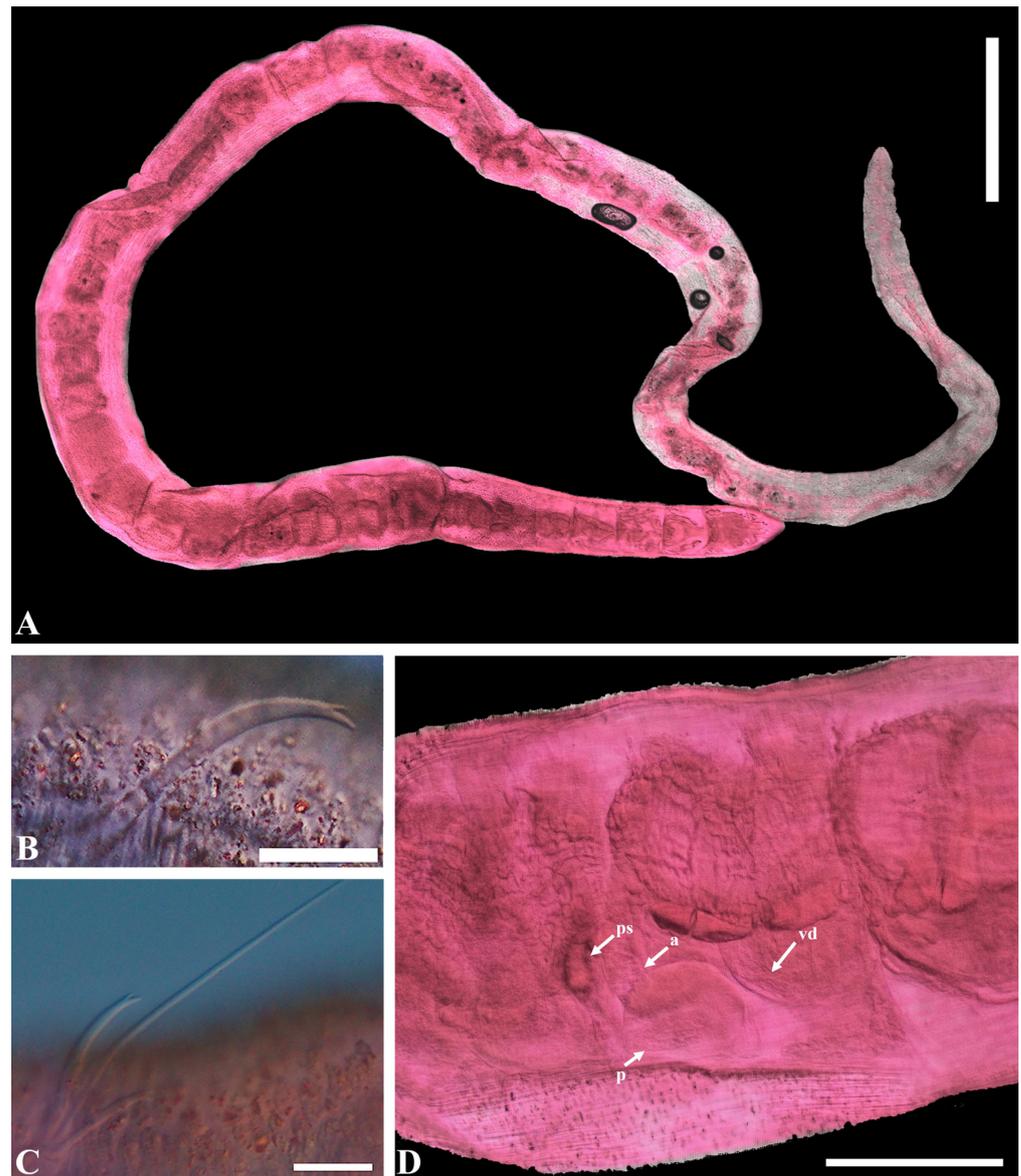


Figure 4. *Tubificoides pollex*. (A) Total body, (B) ventral chaeta, (C) dorsal bundle, (D) lateral view of male genitalia in segment of XI. Scale bars: 800 μm in (A), 20 μm in (B,C), 200 μm in (D), a: atrium, p: prostate gland, ps: penis sheath, vd: vas deferens.

Distribution. Europe (North Sea, Danube estuary), North America (Virginia, North Carolina, Louisiana, Florida), Northern Gulf of Mexico, and the East Sea of Korea.

Habitat. Silt mixed with calcareous sand, subtidal to 25 m in depth.

Remarks. *Tubificoides pollex* exhibits several distinctive morphological features that set it apart from other species within the genus *Tubificoides*. The most prominent feature is the conical shape of the cuticularized penis sheath, a characteristic not observed in any other *Tubificoides* species. Additionally, *T. pollex* features a lateral opening on the penis sheath, which is marked by a distinctive flap-like projection along its proximal margin. This unique combination of morphological characteristics distinguishes *T. pollex* from its closest relatives within the genus *Tubificoides*, including *T. diazi* Brinkhurst & Baker, 1979, *T. crenicoleus* Baker, 1983, *T. longipenis* (Brinkhurst, 1965), *T. uncinatus* Helgason & Erskus, 1987, and *T. lunatus* Milligan, 1991. Furthermore, none of the other species in the *Tubificoides* genus exhibit both a conical shape of the penis sheath and the presence of lateral openings with flap-like

projections. These distinctive characteristics make *T. pollex* a unique and easily identifiable member of the *Tubificoides* genus.

Subfamily Rhyancodrilinae Hrabe, 1963

Genus *Heterodrilus*, 1902

Body wall naked. Hair chaetae absent. Anterior chaetae trifid. Posterior chaetae bifid or simple-pointed. Chaetae two per bundle anterior to clitellum, only one chaeta each bundle posterior to clitellum. Penial chaetae generally present in XI, Spermathecal chaetae absent. Male pores paired, located in line with ventral chaetae in posterior pair on XI. Spermathecal pores paired in line with ventral chaetae in posterior part of XI. Granulated coelomocytes generally abundant. Male efferent ducts paired in XI. Protrusible pseudopenes, without cuticularized lining. Prostate glands lobed, not pedunculate, broadly attached to walls of atria.

Type species. *Heterodrilus arenicolus* Pierantoni, 1902.

Heterodrilus pentcheffi Erséus, 1981 [16], (Figure 5A–D)

Clitellio arenicolus (partim) Giere, 1979 [12] (p. 304).

Heterodrilus pentcheffi Erséus, 1981 [16] (pp. 121–123), Figures 1 and 2, Table 1; 1984 [87] (p. 196); 1986 [23] (p. 289–290), Figure 1.

Heterodrilus sp. Erséus, 1981 [16] (pp. 123–124), Figures 1–4.

Material examined. NIBRIV0000910381, Gajae-bawi at Dokdo-ri, Ulleung-eup, Ulleung-gun, Gyeongsangbuk-do, Republic of Korea, (37.247542° N 131.862955° E), 21 June 2022, in medium-coarse sediment from 24.1 m in depth at 16 °C, obtained through scuba diving. Specimens were deposited at the National Institute of Biological Resources (NIBR).

Description. Prostomium round (Figure 5A). Clitellum extending over 1/2X–XII. Chaetae trifid, two per bundle, with upper tooth thin and short, middle tooth long and basally wide, 40–110 µm long, two per bundle, one per bundle thereafter. Posterior chaetae lower tooth generally becomes reduced and straight, single-pointed (Figure 5C). Penial chaetae simple-pointed, erect expanded, with two per bundle (Figure 5D). Male pore paired in posterior of XI. Spermathecal pore paired in anterior of X, with small epidermal pad located mid-ventrally between spermathecal pores. Coelomocytes granulated, very abundant. Male genitalia paired, vas deferens very long and tightly coiled in a spiral, narrowing entally. Atrium very long and slender, M-shaped, with slender outer muscular layer and densely granulated inner epithelium, bulbous pseudopenis. Prostate gland with large lobes broadly attached to most of the length of the atrium. Spermathecae paired, each with a narrow distinct duct and voluminous sacciform ampulla with an irregular thick wall containing large granules of secretion. Sperm arranged as large bundles.

Distribution. Caribbean (Belize, Panama), East coast of North America (Florida, North Carolina, New Jersey), Bermuda, Galapagos Islands, and the East Sea of Korea.

Habitat. Coarse sand, subtidal from 0.5 m to 39 m in depth.

Remarks. *Heterodrilus pentcheffi* has chaetae similar to *H. minisetosus*, *H. bulbiporus*, *H. perkinsi*, and *H. koreanus* n. sp. Among these, *H. minisetosus*, *H. bulbiporus*, *H. perkinsi* are common in Florida waters [32]. Most *H. minisetosus* do not have penial chaetae. If they do, they are very small. This worm has distinctly bifid chaetae in posterior segments and vas deferens that are uncoiled. Other species, such as *H. bulbiporus* and *H. perkinsi*, have distinctly bifid posterior chaeta, two per bundle. *H. pentcheffi* differs from *H. koreanus* n. sp. in terms of the shape of spermathecae and prostate gland attachment part of atrium.

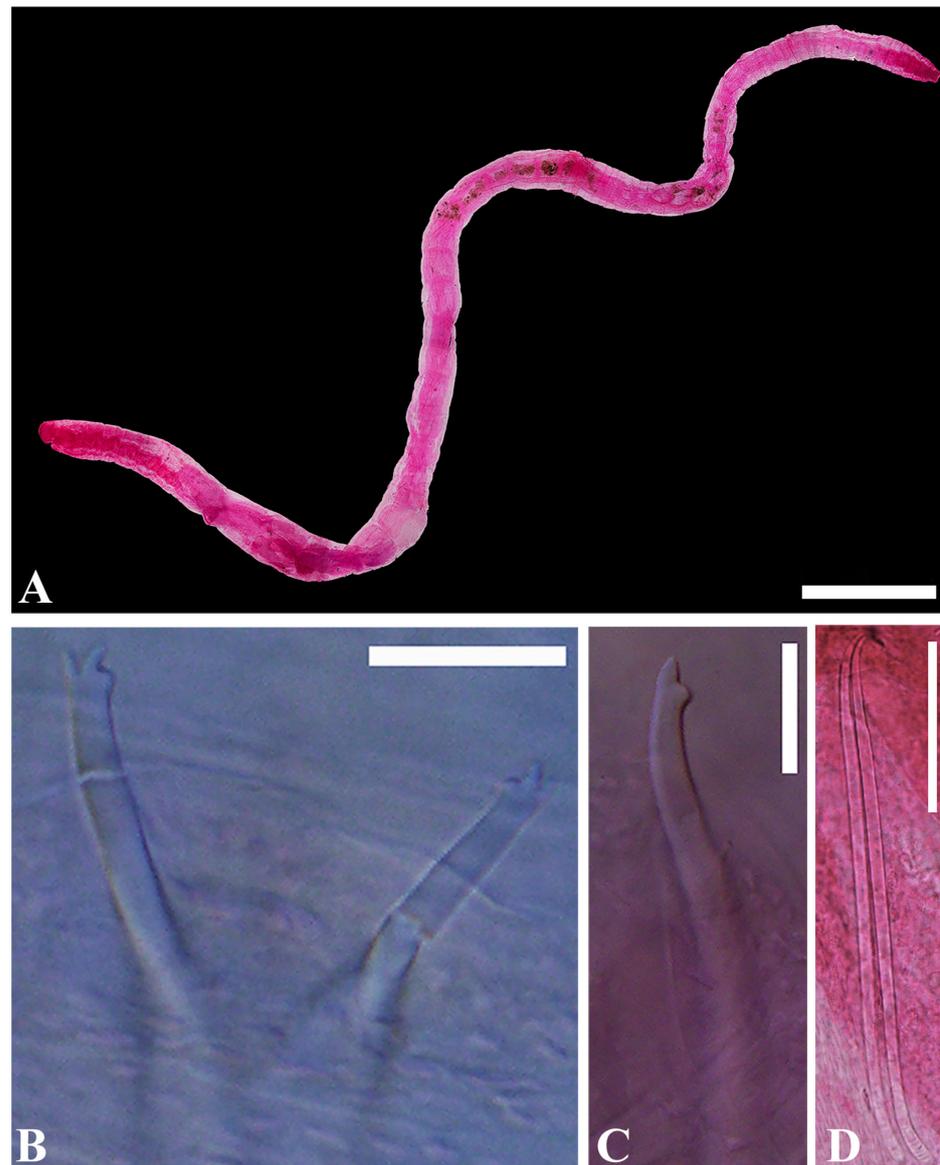


Figure 5. *Heterodrilus pentcheffi*. (A) Total body, (B) anterior chaetae, (C) posterior chaeta, (D) penial chaetae. Scale bar: 1 mm in (A), 40 μ m in (B–D).

Heterodrilus koreanus n. sp. (Figure 6A–G, Table 3)

Holotype. NIBRIV0000901381. Mature, complete individual, stained in alcoholic paracarmine and whole-mounted in Canada Balsam. This specimen has been deposited at the National Institute of Biological Resources (NIBR)

Paratype. NIBRIV0000901501, NIBRIV00009015012. Mature, complete individuals, stained in alcoholic paracarmine and whole-mounted in Canada Balsam. Specimens have been deposited at the National Institute of Biological Resources (NIBR).

Other Material. Collected from the type locality. Ten specimens were fixed on 80% ethanol. Specimens have been deposited in the Marine Biological Resource Institute, Sahmyook University, Seoul, Republic of Korea.

Type Locality. Independence gate-bawi at Dokdo-ri, Ulleung-eup, Ulleung-gun, Gyeongsangbuk-do, Republic of Korea (37.239363° N 131.871777° E), 1 May 2022, in medium-coarse sediment from 20 m in depth at 14 °C, obtained through scuba diving.

Table 3. Morphological comparison of *Heterodrilus koreanus* n. sp. with allied species.

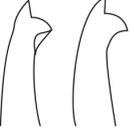
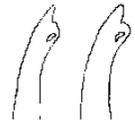
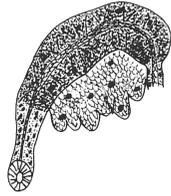
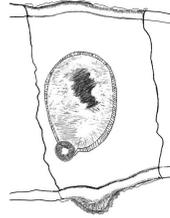
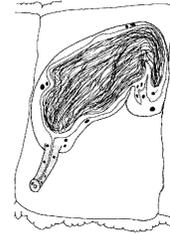
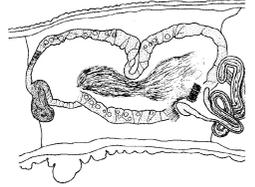
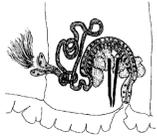
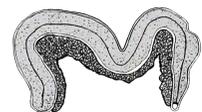
Species		<i>H. keenani</i>	<i>H. chenianus</i>	<i>H. koreanus</i> n. sp.	<i>H. claviatriatus</i>	<i>H. mediopapillosus</i>
Chaetae	Anterior chaeta (with subdental ligament)	trifid 	trifid 	trifid 	trifid 	trifid 
	Posterior chaeta	bifid with subdental ligament 	bifid without subdental ligament 	bifid with subdental ligament 	bifid with subdental ligament 	bifid with subdental ligament 
		Penial chaeta	relatively small, straight, single-point, ectal tips slightly curved, inconspicuous ectal node, ectal tips slightly curved	absent	stout, simple-pointed, relatively straight with blunt tip, slightly curved at the end	straight, single-point, ectal node followed by tapered, slightly curved tip
Spermathecae		oblong ampulla with distinct narrow duct 	absent 	large oval ampulla with indistinct duct 	very large, sacciform ampulla with distinct narrow duct 	large ampullae of varying shape with narrow lumen 

Table 3. Cont.

Species	<i>H. keenani</i>	<i>H. chenianus</i>	<i>H. koreanus</i> n. sp.	<i>H. claviatriatus</i>	<i>H. mediopapillosus</i>	
Male genitalia	Atrium	C-shaped with slender 	Club-shaped with more or less curved 	M-shaped with cylindrical, slender, very long 	Club-shaped with slender 	M-shaped with tubular 
	Prostate glands	ventral surface of atrium	broadly attached ventral surface of ental part of atrium	ventral surface of atrium	ventral surface of atrium	ventral surface of atrium
	Pseudopenis	very in inconspicuous	not mention	small	Small, sometimes protruded	pear-shaped
	Vas deferens	very long, coiled	longer than atrium with irregularly coiled	long and tightly coiled, not spiral	some tight coiled, not spiral	long and irregularly coiled
	locality	Heron Island in Great Barrier Reef of S. Australia	Wuzhi Island in South Hainan of China	Dokdo in the East Sea of Korea	Heron Island in Great Barrier, Reef of S. Australia	Wakayama Prefecture in S. Japan
reference	Erséus, 1981 [16]	Wang and Erséus, 2003 [43]	This study	Erséus, 1981 [16]	Takashima & Mawatari, 1997 [88]	

Description. Length 1.3–1.9 mm (holotype 1.3 mm), width at X 4.33–4.85 μm (holotype 4.85 μm). Hair chaetae absent. Prostomium short, conical shape (Figure 1A). Chaetae with thin subdental ligament, 53.98–97.56 μm long, two per bundle in segments anterior to clitellum, only one chaeta representing each bundle posterior to clitellum, reversed, large, and stout. Anterior chaetae trifold with upper and middle teeth same length, pointed and lower tooth shaft present, posterior chaetae bifid with wide diverging broad teeth, upper tooth longer and thicker than lower (Figure 6B,C). Clitellum extending over 1/2X–1/2XII, without ventral chaetae in XI. Pharyngeal glands present in IV–V. Coelomocyte abundant with granulated round shape (Figure 6E). Penial chaetae, two per bundle, 269.31–321.32 μm , stout, simple-pointed, relatively straight with blunt tip and slightly curved at the end in XI (Figure 6D). Atrium cylindrical, slender, very long, 636–721 μm long, 34–63 μm wide, M-shaped with thick muscular outer layer and granulated inner epithelium in XI, sometimes extending posterior to X or anterior to XII. Prostate glands multi-lobed, attached along most of the ventral surface of the atrium (Figure 6F). Male pores paired, located slightly ventral to line of ventral somatic chaetae, posteriorly in XI. Unpaired epidermal papilla present ventrally to line in X (Figure 6G). Pseudopenis small. Vas deferens long and tightly coiled, not forming a proper spiral in anterior part of XI. Sperm funnel small in X. Spermathecae paired in X, indistinct duct and large oval ampulla with thick wall containing large granules of secretion. Spermathecal chaetae absent. Sperm in spermathecal ampulla as thimble-shaped spermatozoa (Figure 6G).

Etymology. The specific epithet '*koreanus*' refers to 'Korea', the type locality.

Remarks. The genus *Heterodrilus* is large and exclusively marine. It is distributed globally in tropical and subtropical zones [16,33,46,84]. Its species are common in sandy sediments. The morphology of *Heterodrilus koreanus* n. sp. appears similar to that of *H. claviatriatus* (Erséus 1981) or *H. mediopapillosus* (Takashima & Mawatari, 1997) (Table 1).

The atrium of the latter two species is very long and slender, with prostate glands attached to the ventral surface of the atrium and the vas deferens being long and irregularly coiled. Anterior chaetae are trifold, with the middle tooth being larger than the other two, containing a thin subdental ligament. The new species differs from *H. claviatriatus* in having spermathecae with distinct narrow ducts. The penial chaetae and atrium are shorter in our specimens. *H. koreanus* n. sp. more closely resembles the Japanese species *H. mediopapillosus* in that they share an M-shaped atrium and unpaired epidermal papilla (Table 1). However, the spermathecal ampulla of *H. mediopapillosus* is oval-shaped, with a faint outline of the duct (Figure 6F), while the penial chaetae of our species are shorter and slightly curved at the end (Figure 6E).

H. koreanus n. sp. has not only morphological but also ecological differences. *Heterodrilus* species have been found in tropical or subtropical seas in previous studies [2,16,43,84,87]. The Great Barrier Reef Sea is a tropical part of the ocean. The Sea of Wakayama is also subtropical. However, the East Sea of Korea is cooler since it lies in a more northern region. Considering that this species shows several differences compared to other species in the genus *Heterodrilus*, it appears to be a new species.

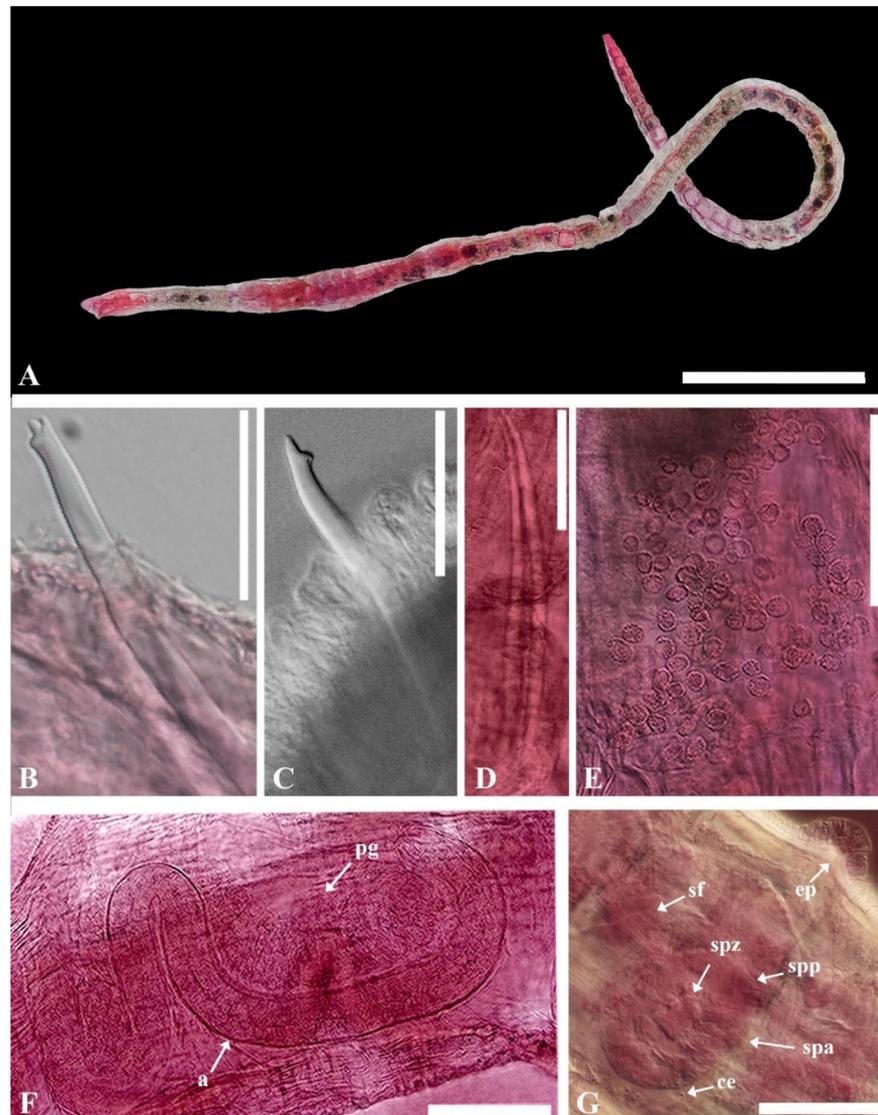


Figure 6. *Heterodrilus koreanus* n. sp. (A) total body, (B) anterior chaetae, (C) posterior chaetae, (D) penial chaetae, (E) coelomocytes, (F) segment of X, (G) spermathecae. Scale bars. (A) = 400 μ m, (B–D) = 60 μ m, (E) = 100 μ m, (F,G) = 80 μ m. a: atrium, ce: coelomocytes, ep: epidermal papilla, pg: prostate glands, sf: sperm funnel, spa: spermathecal ampulla, spp: spermathecal pore, spz: spermatozoa.

6. Molecular Analysis

Phylogenetic trees based on two loci (16S and COI) provided statistical support at different values, showing no topological conflicts. Most specimens of *Heterodrilus koreanus* n. sp. showed monophyly of *Heterodrilus* in Phalloporilinae. The 18S ML showed unsettled positions of *H. koreanus* n. sp. 4. In all trees, *H. ersei* was a sister group of all other *Heterodrilus* species except for *H. koreanus* n. sp. The bootstrap analysis also supported *H. koreanus* n. sp. and some relationships within this genus, although it could not resolve relationships with *Heterodrilus* (pp = 0.93).

H. ersei was identified as a sister group to all other *Heterodrilus* species, with the exception of *H. koreanus* n. sp. This makes it clear that *H. ersei* was identified as a sister group to all other *Heterodrilus* species, except for *H. koreanus* n. sp. found in three separate instances: *H. koreanus* n. sp. 5 in the 16S tree, *H. koreanus* n. sp. 1 in the COI tree, and *H. koreanus* n. sp. 4 in the 18S tree (Figure 7).

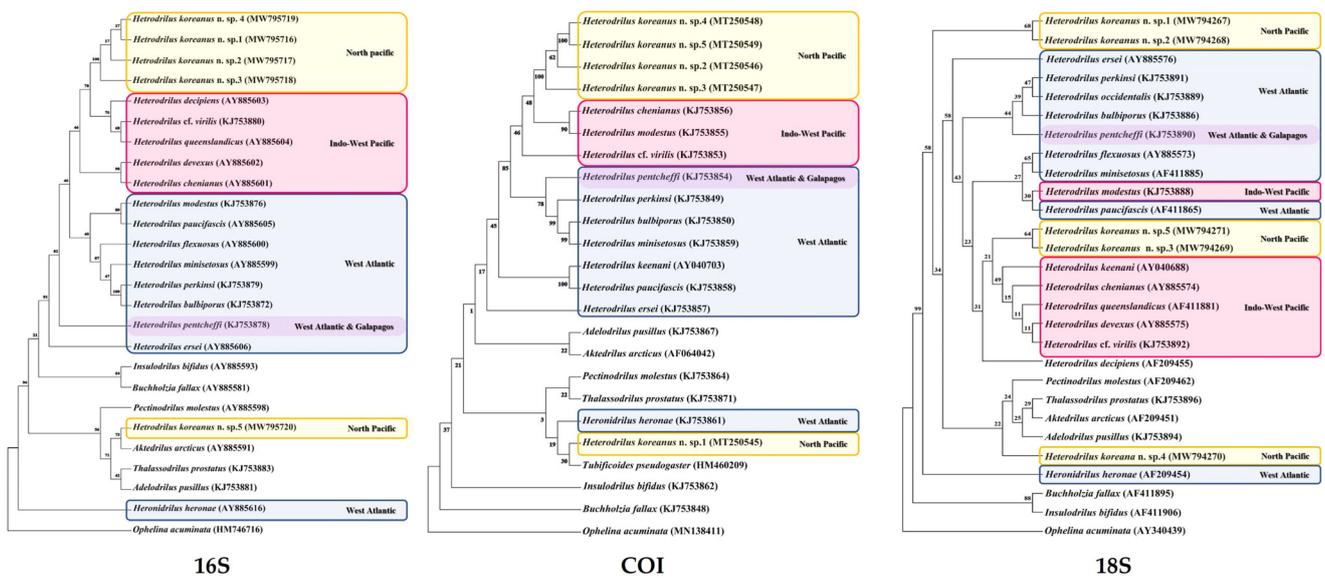


Figure 7. Phylogenetic trees of *Heterodrilus* based on 16S, 18S, and COI sequences. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. Percentages of replicate trees in which associated taxa were clustered together in the bootstrap test (1000 replicates) are shown next to branches, with branch lengths measured in terms of the number of bootstrap values.

Consensus trees (mt DNA and all loci) from the Bayesian inference (BI) analysis are shown in Figure 8. These trees showed that *H. koreanus* n. sp. was a distinct lineage within *Heterodrilus*. The monophyly of *H. koreanus* n. sp. was strongly supported (pp = 1). It had a close relationship with *H. chenianus* (patristic distances: 0.44–0.84) (Figure 8). Monophyly of *heterodrilus* (pp = 0.93) was corroborated. This monophyly contained all Phallodrilinae.

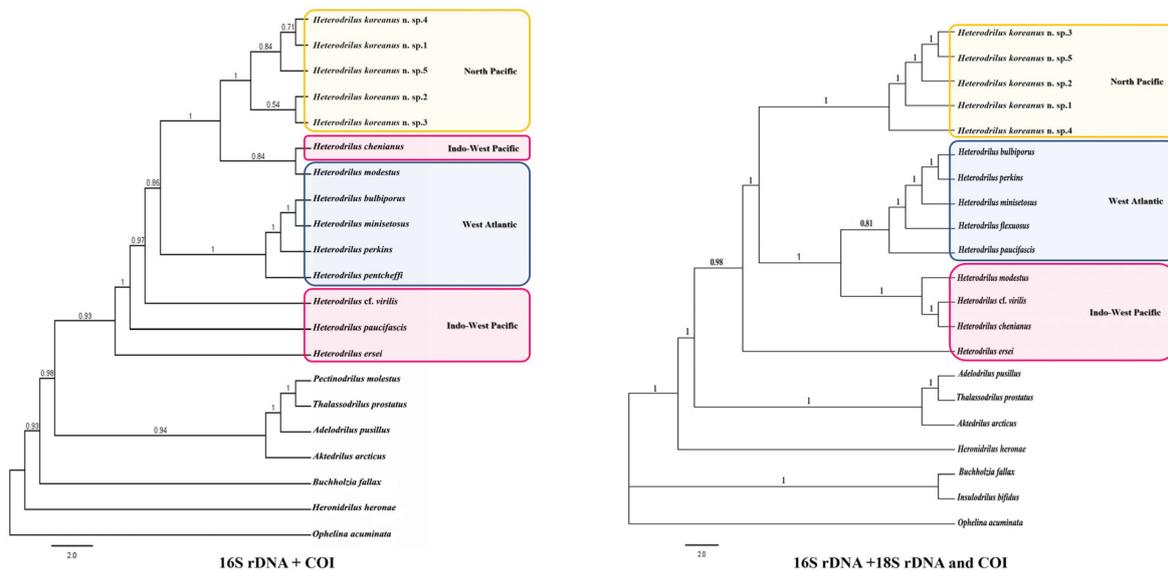


Figure 8. Consensus trees obtained from the Bayesian MCMC analysis of combined (16S rDNA + COI, 16S rDNA +18S rDNA and COI) datasets. Posterior probabilities >0.50 are indicated. Inset images depict that branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. Percentages of replicate trees in which associated taxa were clustered together in the bootstrap test (1000 replicates) are shown next to branches, with branch lengths measured in terms of number of substitutions per site.

Within the *Heterodrilus* clade, the *H. ersei* is the sister group of all remaining species ((pp = 0.96 (mtDNA) and 0.74 (all loci)). The latter formed four clades (see Figure 8): Clade 1, Clade 2, *H. cf. virilis*, and *H. paucifascis*. Clade 1+2 and *H. cf. virilis* were suggested as a sister group of *H. paucifascis*. Within Clade 1, three species (*H. koreanus*, *H. chenianus*, and *H. minisetosus*) were strongly supported (pp = 1). *H. koreanus* and *H. chenianus* + *H. minisetosus* were also supported by having posterior probabilities of 1. Otherwise, *H. chenianus* + *H. minisetosus* were resolved with low support (pp = 0.84). Clade 2 contained a strongly supported group (pp = 1) of four species (*H. bulbiporus*, *H. minisetosus*, *H. perkinsi* and *H. pentcheffi*), in which *H. bulbiporus* + *H. minisetosus* were proposed as closely related sister species, with pp = 1. *H. ersei*.

7. Discussion

This study identified a new species of marine oligochaete and provided their descriptions. Our data represent the first record of marine oligochaetes in Korea. One new species and four recorded species belong to genera *Heterodrilus*, *Tubificoides*, and *Smithsonidrilus*, based on morphological analysis. The new species, *Heterodrilus koreanus* n. sp., and four recorded species, *H. pentcheffi* Erséus, 1981, *Limnodriloides anxius* Erséus, 1990, *Smithsonidrilus exspectatus* Erséus, 1993, *Tubificoides heterochaetus* Michaelsen, 1926, could be distinguished from other similar species by comparing their morphological characteristics. For more accurate identification of the new species, molecular analysis was also performed.

Heterodrilus koreanus n. sp. has several morphological features that can distinguish it from its close relatives *H. claviatriatus* (Erséus 1981) and *H. mediopapillosus* (Takashima & Mawatari 1997). Phylogenetic analysis also showed that it was a close relative of *H. keenani* Erséus, 1981 and *H. chenianus* Wang & Erséus, 2003. The atrium of *H. koreanus* n. sp. is a long cylindrical tube. It has an M shape. In comparison, *H. claviatriatus*, *H. keenani*, and *H. chenianus* have a shorter atrium and are club-shaped or C-shaped. Although *H. mediopapillosus* is very similar, the shape of its spermathecae is different. These species also have two large penial chaetae per bundle. However, *H. chenianus*, which is closely related phylogenetically, does not have spermathecae or penial chaetae (see Table 3).

According to previous studies, the genus *Heterodrilus* is a group of largely tropical and subtropical marine tubificids [16,23,27,43,84,89]. These four species have been collected in warm seas. Notably, two morphologically similar species, *H. chenianus* in southern China and *H. mediopapillosus* in Japan, have been found in warmer areas than the survey region of this study. Since the habitats are very different, it is difficult to say whether they are the same species, even if some similar traits are shared. Regrettably, this phylogenetic analysis could not include the most similar species, *H. claviatriatus* or *H. mediopapillosus*, because sequences of these species are not yet available from the NCBI database. This analysis, however, contained some morphologically similar species available in NCBI, making it possible to compare sequences obtained from the new species and other related species within the corresponding genus. Thus, the results of this study are not fully comparable.

Our molecular analysis, based on three loci (16S, COI, and 18S), revealed a complex phylogenetic relationship within the genus *Heterodrilus*. The phylogenetic trees provided statistical support at different values, indicating no topological conflicts. Most specimens of *Heterodrilus koreanus* n. sp. showed monophyly within Phalloporilinae, but this clade did not encompass all Phalloporilinae members. Instead, it represented a distinct lineage within the broader taxonomic group. The 18S ML showed unsettled positions of *H. koreanus* n. sp., emphasizing the challenges of resolving relationships with this species using this particular locus. Remarkably, the sequences of separate individuals of *H. koreanus* (1, 4, and 5) appeared in different clades when considering individual genes (16S, COI, and 18S). Only in the combined tree (Figure 8) did all five specimens of *H. koreanus* form a single, compact clade. This observation underscores the imperfectness of relying on a single gene, such as COI, for species identification. The incongruence in individual gene trees highlights the need for a more holistic approach in molecular taxonomy, especially when dealing with closely related species. The position of *H. ersei* in our phylogenetic analysis is noteworthy.

It appears as a sister group to all other *Heterodrilus* species except for *H. koreanus* n. sp. This suggests a closer evolutionary relationship with the broader diversity within *Heterodrilus*, and its exclusion from the monophyletic group including *H. koreanus* n. sp. prompts further investigation into its evolutionary history and ecological significance.

To summarize, our study reveals two main groups within the genus *Heterodrilus*, characterized by distinct morphological features and geographical distributions [16]. This work significantly contributes to the knowledge of marine oligochaete diversity in Korea. Additionally, it highlights the challenges of molecular taxonomy within *Heterodrilus*, showcasing the imperfectness of relying on single-gene identification, particularly illustrated by the varied placement of *H. koreanus* n. sp. within Phallodrilinae in individual gene trees. The study underscores the necessity for comprehensive analyses involving multiple genes to unravel the intricate evolutionary relationships within this taxonomic group. Further investigations, incorporating additional species and molecular markers, are crucial to refine the phylogenetic framework and deepen our understanding of oligochaete evolution in marine environments.

Author Contributions: Conceptualization, J.L.; methodology, J.L.; software, J.L.; validation, J.L.; formal analysis, J.L.; investigation, T.L.; resources, J.L.; data curation, J.L.; writing—original draft preparation, J.L.; writing—review and editing, J.L. and T.L.; visualization, J.L.; supervision, T.L.; project administration, T.L.; funding acquisition, T.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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