

Article

Hypodontolaimus minus sp. nov. and *Bolbolaimus distalamphidus* sp. nov., Nematodes from the Yellow Sea, Including Phylogenetic Analyses Within Chromadoridae and Microlaimoidea

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Abstract: Two new species from the Yellow Sea, *Hypodontolaimus minus* sp. nov. and *Bolbolaimus distalamphidus* sp. nov., are described in this study. *Hypodontolaimus minus* sp. nov. is characterized by a relatively small body length, a cuticle with two longitudinal lateral differentiations connected with transverse bars, four files of sublateral somatic setae, a pharynx with an anterior and posterior bulb, L-shaped spicules, a slightly swollen proximal end, a distal end tapered with a posterior pointed hook, and a gubernaculum with dorsal caudal apophysis. *Bolbolaimus distalamphidus* sp. nov. is characterized by a relatively small body size, a strongly annulated cuticle, six short outer labial sensilla and four long cephalic setae, an amphideal fovea unispiral oval that is far from the anterior end, slightly curved spicules, gubernaculum with anterior-pointed apophysis, and a conical tail. Phylogenetic analyses within the family Chromadoridae and the superfamily Microlaimoidea based on combined rDNA sequences confirmed the placement of *Hypodontolaimus minus* sp. nov. and *Bolbolaimus distalamphidus* sp. nov. The subfamily of Chromadorinae is shown as a monophyletic clade, the genera of subfamily Hypodontolaiminae are shown as a paraphyletic group, and the genus of *Ptycholaimellus* shows high intraspecific diversity. The placement of genera *Aponema* and *Molgolaimus* within the superfamily Microlaimoidea is discussed based on combined rDNA sequences.

Keywords: free living nematode; Changdao Island; SSU; LSU



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1. Introduction

The phylum Nematoda exhibits remarkable species diversity and represents one of the least described mega-diverse animal phyla [1]. To date, fewer than 7000 marine nematodes have been formally described and accepted as valid [2], with classifications predominantly based on morphological characters. Phylogenetic relationships among marine nematodes remain challenging to resolve due to the convergent evolution of relatively limited set of morphological traits and inconsistencies in taxonomically significant characters such as cuticle punctations [3]. Molecular sequences of the mitochondrial cytochrome oxidase c subunit 1 (COI) gene have been utilized to elucidate cryptic species [4]. However, this approach has limited application due to the scarcity of available sequences in GenBank. Ribosome DNA markers are more commonly employed to analyze relationships, with small subunit (SSU) sequences typically used to resolve deep relationships, while LSU are preferred for resolving relationships at within- family level [3,5]. Nevertheless, our

understanding of relationships among marine nematodes based on molecular sequences data remains incomplete due to the limited number of SSU and LSU sequences currently available in the GenBank.

The family Chromadoridae represents one of the largest and most taxonomically complex families within nematodes, predominantly inhabiting marine environments [6]. This family is distinguished by two key synapomorphies: males possess a monorchic reproductive system with anterior testis, while females exhibit two antidromously reflexed ovaries, with the anterior gonad to the right of the intestine and the posterior one to the left of the intestine [7,8]. Currently, Chromadoridae is currently divided into five subfamilies: Chromadorinae, Euchromadorinae, Harpagonchinae, Hypodontolaiminae, and Spilipherinae [8], and reviewed systematically by Lorenzen [7] and Venekey et al. [9]. Phylogenetic relationships within Chromadoridae based on SSU have been examined by Venekey et al. [9] and Guo et al. [10], while Liang et al. [11] investigated phylogenetic relationships within the subfamily Hypodontolaiminae using both SSU and LSU sequences. However, comprehensive molecular phylogenetic analyses utilizing concatenated SSU and LSU sequences have not been previously conducted.

The taxonomic placement of superfamily Microlaimoidea is problematic due to the absence of clear synapomorphies [3,12]. Historically, Microlaimoidea was classified within Desmodorida alongside Desmodoroidea, primarily based on SSU reported by previous studies [13,14]. However, recent molecular phylogenetic investigations suggest that Microlaimoidea shares a closer evolutionary relationship with Chromadorida than with Desmodorida [15,16]. Similarly, Leduc et al. established a new order, Microlaimida Leduc, Verdon & Zhao, 2017 to accommodate Microlaimoidea based on molecular sequences [3]. In addition, they reinstated the family Molgolaimidae and transferred it from Desmodoridae to Microlaimoidea [3]. Consequently, Microlaimoidea now encompasses four families, Microlaimidae, Aponchiidae, Monoposthiidae, and Molgolaimidae.

By conducting a survey on the diversity and distribution of marine nematodes in Changdao Island, undisturbed sediment samples have been acquired since August 2023. This investigation has resulted in the isolation and identification of more than 200 nematode species [10,11]. In the present study, we describe two new species, *Hypodontolaimus minus* sp. nov. and *Bolbolaimus distalamphidus* sp. nov., provide their SSU and LSU rRNA gene sequences, and analyze the phylogenetic relationships within the family Chromadoridae and superfamily Microlaimoidea.

2. Materials and Methods

2.1. Specimen Collection and Laboratory Isolation

Undisturbed sediments were obtained from 16 sampling sites along the Changdao Island coast (37°54' N, 120°44' E) in August and December 2023. Samples were taken using a syringe with an internal inner diameter of 29 mm to a depth of 8 cm, vertically sliced into 0–2 cm and 2–8 cm sediment depth layers, and preserved in 10% formalin and in 95% ethanol, respectively, until laboratory processing. In the laboratory, sediment samples in formalin were stained with Rose Bengal overnight, and sorted through two sieves with sizes of 500 µm and 45 µm to remove macroinfauna and retain meiofauna. Meiofauna were washed out of sediment particles with Ludox-TM (Sigma-Aldrich Co., St. Louis, MO, USA) and transferred into Petri dishes [17]. Nematodes were picked out with a stereoscopic microscope and transferred into a mixture of 95% ethanol, glycerin, and distilled water in an embryo dish. After ethanol had totally evaporated, nematodes were then mounted in glycerin on permanent slides [18]. Observation and measurement were carried out with an Axiscope-5 differential interference contrast microscope (Zeiss, Jena, Germany), line drawings were created with ZEN 3.7 Labscope software with the aid of an iPad (Apple,

Cupertino, CA, USA), and photographs were taken with the aid of ZEN 3.7 software. The type specimens were deposited at the Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China.

Sediment samples fixed with ethanol were used for DNA extraction and treated as the formalin-fixed samples with Ludox. Nematodes were sorted and washed three times with sterile water before being transferred to temporary slides. Five male specimens of *Hypodontolaimus minus* sp. nov. and one male of *Bolbolaimus distalamphidus* sp. nov. were ascertained primarily based on buccal cavity, cuticle differentiation, pharynx bulb, spicules and gubernaculum shape, and tail shape on the temporary slides.

2.2. DNA Extraction, PCR and Phylogenetic Inference

Nematode genomic DNA was extracted following the instructions of the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and used as amplification template. Ribosome DNA of SSU and LSU were amplified and primers G18S4F (5'-GCT TGT CTC AAA GAT TAA GCC-3')/18PR (5'-TGA TCC WMC RGC AGG TTC AC-3') for nearly full-length SSU rDNA [19], primers D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-3')/D3B (5'-TCG GAA GGA ACC AGC TAC TA-3') for the D2–D3 fragment of LSU [20]. The PCR procedure was conducted as described by Zhao et al. [21], and the PCR product was sequenced by Genewiz (China) and assembled in Genious v. 6.1.2. The newly obtained SSU sequences (accession number *H. minus* sp. nov., PQ249718; *B. distalamphidus* sp. nov., PQ435316) and LSU sequences (accession number *H. minus* sp. nov., PQ283889; *B. distalamphidus* sp. nov., PQ358938) were deposited in GenBank.

SSU and LSU sequences of family Chromadoridae and superfamily Microlaimoidea based on the same specimen were retrieved from Genbank, and only the SSU sequences longer than 600 bp were used. A total of 25 species from 9 genera in the family Chromadoridae and 18 species from 6 genera in the superfamily Microlaimoidea were selected for phylogenetic analysis (Table 1), with *Sphaerolaimus obesus* Meng, Guo & Wang, 2025 [22] as outgroup. A dataset of concatenated LSU + SSU sequences was constructed using PhyloSuite v. 1.2.3, and alignment was processed with Muscle for the family Chromadoridae and with Mafft for the superfamily Microlaimoidea. Substitution models of (GTR (general time-reversible) + G (gamma distribution)) were selected for maximum-likelihood (ML) analysis for Chromadoridae and (TN93 (Tamura-Nei) + G (gamma distribution) + I (proportion of invariable sites)) for Microlaimoidea. Phylogenetic analyses for ML were performed using Mega v. 10.2.6 with 1000 bootstrap replicates. Substitution models (GTR (general time-reversible) + G (gamma distribution) + I (proportion of invariable sites)) were selected for both of the Bayesian inference (BI) analyses. Phylogenetic analyses for Bayesian inference were constructed with CIPRES (<http://www.phylo.org>) and processed using MrBayes v. 3.2.7a on CIPRES with a chain length of 10,000,000 and a burn-in fraction of 0.25. The tree topology was viewed using FigTree v. 1.4.3 and refined with PowerPoint.

Table 1. Sequences information of SSU and LSU used in this study.

Species	Accession Number of SSU	Accession Number of LSU	Specimen Voucher	Geographical Location
<i>Aponema pseudotorosum</i>	MK446237	MK446242	TAN1708-NNCNZ3324	New Zealand
<i>Bolbolaimus distalamphidus</i> sp. nov.	PQ435316	PQ358938	1–8	China
<i>Bolbolaimus major</i>	OQ835637	OQ835636	7–21	China
<i>Chromadorita spinicauda</i>	OK317201	OK317226	NNCNZ3379	New Zealand
<i>Chromadorita humila</i>	OQ396742	OQ396736	23a	China
<i>Chromadora</i> cf. <i>nudicapitata</i>	OK317199	OK317224	NNCNZ3377	New Zealand
<i>Chromadorina germanica</i>	OK317200	OK317225	NNCNZ3378	New Zealand

Table 1. Cont.

Species	Accession Number of SSU	Accession Number of LSU	Specimen Voucher	Geographical Location
<i>Chromadorina germanica</i>	OQ396743	OQ396737	25a	China
<i>Chromadorina communis</i>	PQ396187	PQ412514	4–2	China
<i>Dichromadora sinica</i>	OR479916	OR479914	8–2	China
<i>Hypodontolaimus plurisetus</i> *	OR479915	OR479917	8–3	China
<i>Dichromadora major</i>	OR479911	OR479912	3–33	China
<i>Dichromadora media</i>	OR479913	OR479918	8–1	China
<i>Euchromadora ezoensis</i>	OQ396717	OQ417519	2c 2a	China
<i>Hypodontolaimus minus</i> sp. nov.	PQ249718	PQ283889	2–3	China
<i>Hypodontolaimus</i> sp.	OQ076315	OQ054305	A72	-
<i>Hypodontolaimus</i> sp.	OQ065249	OQ054304	A55	-
<i>Ixonema powelli</i>	MW078512	OR589721	S178_23	Sweden
<i>Ixonema powelli</i>	OL388482	OR589722	S178_55	-
<i>Microlaimus robustidens</i>	OL388480	OR589723	S178_33	-
<i>Microlaimus korari</i>	OK317206	OK317228	NNCNZ3384	New Zealand
<i>Microlaimus papillatus</i>	PQ432931	PQ433003	LJW–21–13	China
<i>Monoposthia costata</i>	MW078511	OR589729	S208_1	Sweden
<i>Monoposthia costata</i>	MW078510	OR589733	S226_15	Sweden
<i>Monoposthia costata</i>	MW078509	OR589731	S212_5	Sweden
<i>Monoposthia costata</i>	MW078508	OR589730	S212_3	Sweden
<i>Monoposthia costata</i>	MW078507	OR589727	S207_5	Sweden
<i>Monoposthia costata</i>	MW078506	OR589732	S212_7	Sweden
<i>Monoposthia costata</i>	MW078505	OR589728	S207_8	Sweden
<i>Monoposthia mirabilis</i>	MW078504	OR589735	S226_16	Sweden
<i>Monoposthia mirabilis</i>	MT846161	OR589736	S228_10	Sweden
<i>Molgolaimus kaikouraensis</i>	MK446235	MK446240	TAN1708– NNCNZ3322	New Zealand
<i>Neochromadora poecilosomoides</i>	OQ396720	OQ417520	8a 8c	China
<i>Neochromadora parabilineata</i>	OR126985	OR135360	11–18	China
<i>Ptycholaimellus</i> sp.	OQ091430	OQ054303	F94	-
<i>Ptycholaimellus</i> sp.	OQ076313	OQ054302	F93	-
<i>Ptycholaimellus</i> sp.	MZ779197	MZ779190	PIFJ01	-
<i>Ptycholaimellus</i> sp.	OQ065190	OQ054300	F24	-
<i>Ptycholaimellus</i> sp.	OQ065191	OQ054301	F25	-
<i>Ptycholaimellus ocellatus</i>	OQ248097	OQ241889	oF116 dF116	-
<i>Ptycholaimellus spiculuncus</i>	OK317202	OK317227	NNCNZ3380	New Zealand
<i>Ptycholaimellus ocellatus</i>	OQ538290	OQ466609	12a 12c	China
<i>Spilophorella</i> sp.	OQ065318	OQ054306	F78	-
<i>Sphaerolaimus</i> sp.	PP146615	PP146616	94–3	China

Note: * was formerly labeled as *Dichromadora multisetosa*.

Sequences of rDNA within the family Chromadoridae were aligned, and p-distances of interspecific and intrageneric sequences were calculated in Mega v. 10.2.6 based on the Kimura 2-parameter model with 1000 bootstrap replicates.

3. Results and Discussion

3.1. Systematic of *Hypodontolaimus minus* sp. nov.

Order Chromadorida Filipjev, 1929

Family Chromadoridae Filipjev, 1917

Subfamily Hypodontolaiminae De Coninck, 1965

Genus *Hypodontolaimus* de Man, 1886

3.1.1. Diagnosis (Based on Pinto & Neres 2020 [23])

Body cuticle with punctuated ornamentation of two longitudinal rows of larger dots. Inner labial sensilla inconspicuous in most species. Six outer labial papillae and four cephalic setae in separate circles. Several somatic setae could be present. Amphideal fovea

transverse slit-like to bean-like. Buccal cavity with large S-shaped hollow dorsal tooth. Pharynx swollen in buccal region to accommodate the dorsal tooth, single posterior end bulb. Precloacal supplements cup-shaped when present. Gubernaculum present or absent.

3.1.2. Remarks

The genus *Hypodontolaimus* de Man, 1886 was erected to accommodate *Spilifera inaequalis* Bastian, 1865, and the genus character was characterized by having a large S-shaped hollow dorsal tooth, pharynx with a large muscular buccal bulb and a terminal bulb, amphideal fovea oval or oval loop shaped, and homogeneous cuticular ornamentation with two to four longitudinal rows of larger dots in a lateral position [24]. Later, Filipjev (1918) transferred *Chromadora baltica* Schneider, 1906 to *Hypodontolaimus* and synonymized *H. sbuetschlii* Filipjev, 1918 as *H. balticus* [25]. Micoletzky (1922) transferred *H. arabicus* Cobb, 1890 to genus *Prochromadorella* Micoletzky, 1924 [26,27], and Kreis (1929) transferred *H. geophilus* (de Man, 1876) to genus *Dichromadora* Kreis, 1929 [28]. Later, *H. abyssalis* Allgén, 1933, *H. mediterraneus* Brunetti, 1949, *H. schuurmansstekhoveni* Gerlach, 1951, *H. dimorphus* Wieser, 1954, and *H. steineri* Wieser, 1954 were described. Wieser (1954) transferred *Dichromadora longiseta* Allgén, 1933 and *Spiliphora setosa* Bütschli, 1874 to *Hypodontolaimus*, and transferred *H. minor* Allgén, 1927 and *H. norvegicus* Allgén, 1946 to *Chromadorita* Filipjev, 1922 based on absence of lateral differentiation [29]. After Wieser, *H. pumilio* Gerlach, 1956, *H. angelae* Inglis, 1961, *H. colesi* Inglis, 1962, *H. solivagus* Hopper, 1963, *H. interruptus* Wieser & Hopper, 1967, *H. pilosus* Hopper & Meyers, 1967, *H. reversus* Hopper, 1968, *H. golikovi* Platonova, 1971, *H. setosoides* Blome, 1982, *H. galapagensis* Blome, 1985, *H. kiseloevi* Baranova & Dashchenko, 1992, *H. plurisetus* Baranova & Dashchenko, 1992, *H. marleenae* Muthumbi & Vincx, 1998, *H. antarcticus* Andrassy & Gibson, 2007, *H. ventrapophyses* Huang & Gao, 2016, and *H. portuguese* Pinto & Neres, 2020 were described. As genus *Ptycholaimellus* was considered valid by Inglis [30], *H. ponticus* Filipjev, 1922, *H. macrodentatus* Timm, 1961, *H. pandispiculatus* Hopper, 1961, *H. monodon* Schuurmans Stekhoven, 1942, and *H. slacksmithi* Inglis, 1969 were transferred to *Ptycholaimellus* and Gerlach (1955) synonymized *H. zosteræ* Allgén, 1929 with *Ptycholaimellu sponticus* (Filipjev, 1922) Gerlach, 1955 [31]. Allgén (1927) synonymized *H. striatus* Ditlevsen, 1918 with *H. balticus* [32], and Filipjev (1934) transferred *Iotadorus punctulatus* Cobb, 1920 to *Hypodontolaimus* [33].

Hypodontolaimus heymonsi (Steiner, 1921) was considered as *species inquirendum* for the description based on female specimen [34], and *H. sivertseni* Allgén, 1951 was considered as *species inquirendum* for lack of detailed cuticle description [9]. *Hypodontolaimus patagiatus* Wieser, 1960 was considered as *nomen nudum* by Gerlach & Riemann [35]. Up to now, 27 species are considered valid in *Hypodontolaimus*.

The list of valid species in *Hypodontolaimus* is as follows:

- Hypodontolaimus abyssalis* Allgén, 1933.
- Hypodontolaimus angelae* Inglis, 1961.
- Hypodontolaimus antarcticus* Andrassy & Gibson 2007.
- Hypodontolaimus balticus* (G. Schneider, 1906) Filipjev, 1918.
- Hypodontolaimus colesi* Inglis, 1962.
- Hypodontolaimus dimorphus* Wieser, 1954.
- Hypodontolaimus galapagensis* Blome, 1985.
- Hypodontolaimus golikovi* Platonova, 1971.
- Hypodontolaimus inaequalis* (Bastian, 1865) de Man, 1886.
- Hypodontolaimus interruptus* Wieser & Hopper, 1967.
- Hypodontolaimus kiseloevi* Baranova & Dashchenko, 1992.
- Hypodontolaimus longiseta* (Allgén, 1933) Wieser, 1954.
- Hypodontolaimus marleenae* Muthumbi & Vincx, 1998.

Hypodontolaimus minus sp. nov.
Hypodontolaimus mediterraneus Brunetti, 1949.
Hypodontolaimus obtusicaudatus Allgén, 1947.
Hypodontolaimus pilosus Hopper & Meyers, 1967.
Hypodontolaimus plurisetus Baranova & Dashchenko, 1992.
Hypodontolaimus portuguese Pinto & Neres, 2020.
Hypodontolaimus pumilio Gerlach, 1956.
Hypodontolaimus punctulatus (Cobb, 1920) Filipjev, 1934.
Hypodontolaimus reversus Hopper, 1968.
Hypodontolaimus schuurmansstekhoveni Gerlach, 1951.
Hypodontolaimus setosoides Blome, 1982.
Hypodontolaimus setosus (Bütschli, 1874) Wieser, 1954.
Hypodontolaimus solivagus Hopper, 1963.
Hypodontolaimus steineri Wieser, 1954.
Hypodontolaimus ventrapophys Huang & Gao, 2016.
List of species inquerenda in *Hypodontolaimus*:
Hypodontolaimus heymonsi (Steiner, 1921) Filipjev, 1930.
Hypodontolaimus sivertseni Allgén, 1951.
List of nomen nudum in *Hypodontolaimus*:
Hypodontolaimus patagiatus Wieser, 1960.

3.1.3. Description of New Species

Hypodontolaimus minus sp. nov. (Figures 1–3).

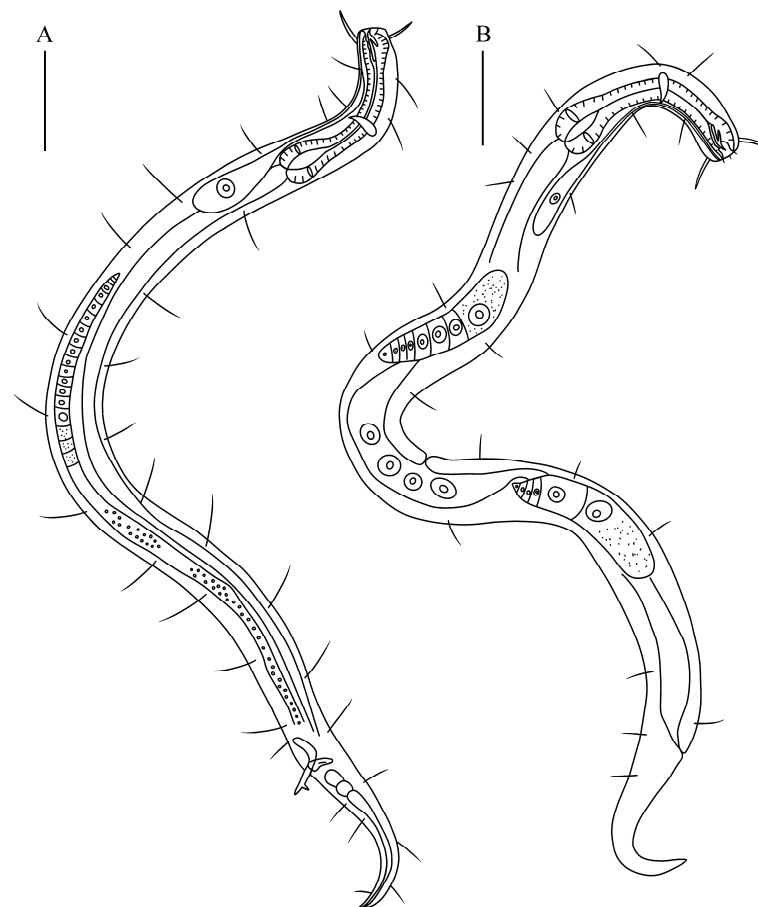


Figure 1. *Hypodontolaimus minus* sp. nov. (A) Lateral view of holotype male entire body. (B) Lateral view of female entire body. Scale bars: 50 µm.

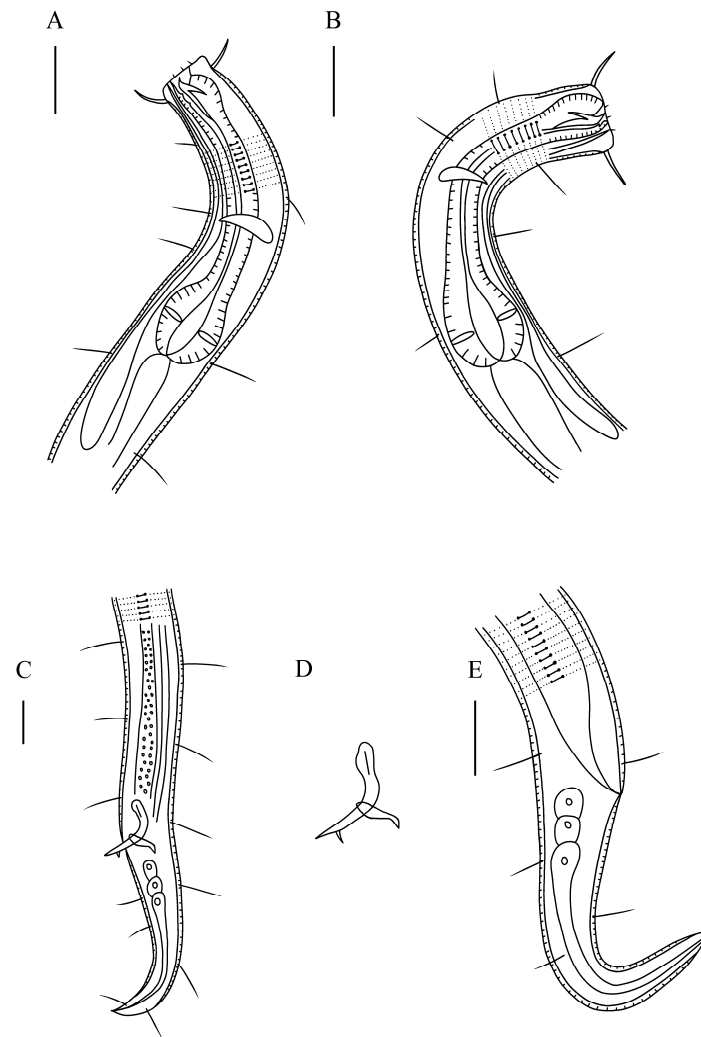


Figure 2. *Hypodontolaimus minus* sp. nov. (A) Lateral view of holotype male anterior region, showing buccal cavity and cuticle. (B) Lateral view of female anterior region, showing buccal cavity and cuticle. (C) Lateral view of holotype male posterior body, showing tail region. (D) Spicules and gubernaculum. (E) Lateral view of female posterior body, showing tail region. Scale bars: (A–C,E) = 20 μ m.

Type Material

Four males and three females were measured and studied. Holotype: m#1 on slide number 23DKBCD-2-2-38, paratypes: m#2 on slide 23DKBCD-2-2-7, m#3 on 23DKBCD-2-2-12, m#4 on 23 DKBCD-2-2-9, f#1 on 23DKBCD-2-2-26, f#2 on 23DKBCD-2-2-17, and f#3 on 23DKBCD-2-2-47.

Type Locality and Habitat

All specimens were collected from the sublittoral region of Changdao Island, Shandong Province, China, in December 2023. Geographic coordinates: 37°54' N, 120°44' E. Collection depth: 2–8 cm sediment depth, sandy sediments.

Etymology

The species epithet refers to the small body length.

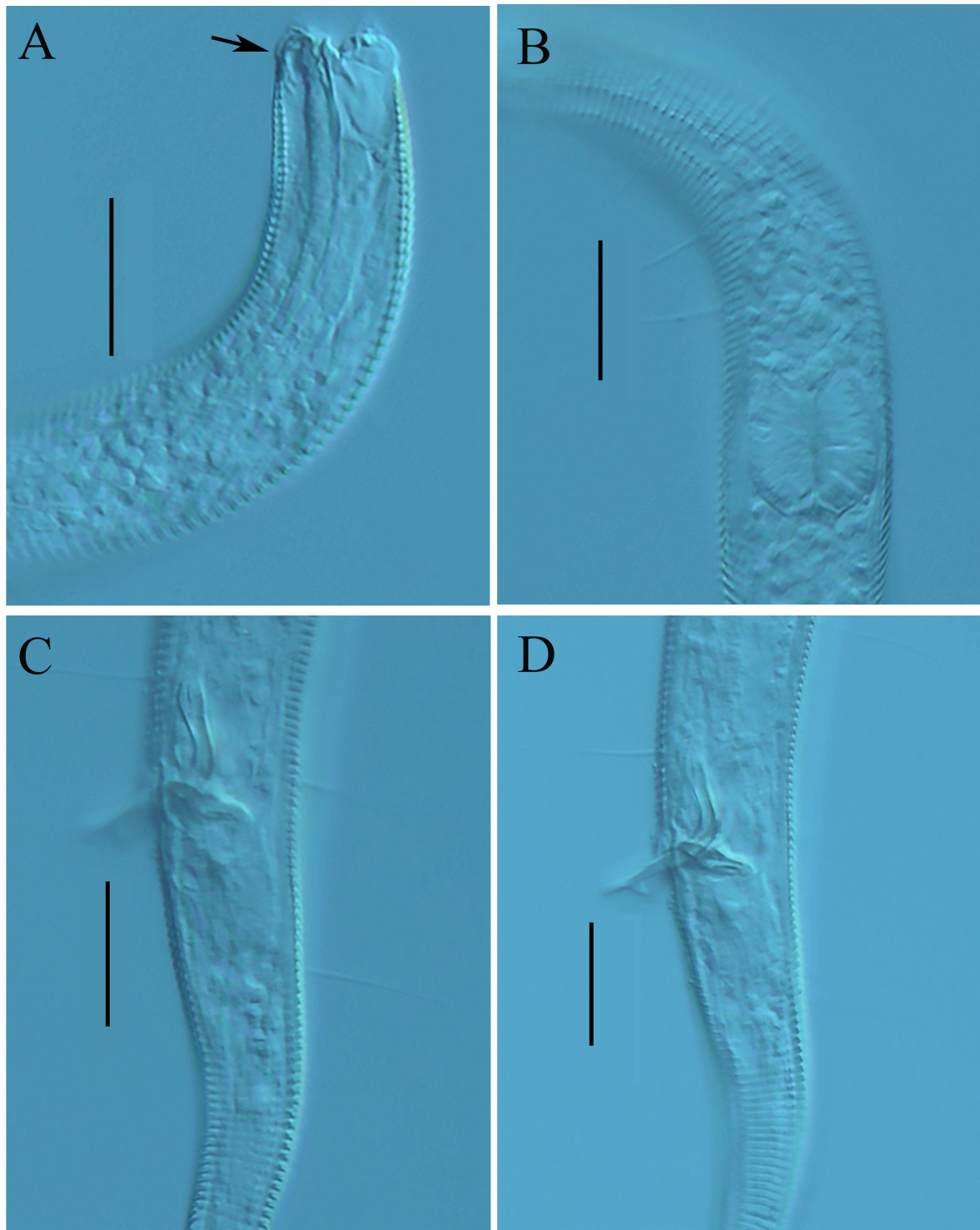


Figure 3. *Hypodontolaimus minus* sp. nov. (A) Lateral view of holotype male anterior region showing buccal cavity, dorsal tooth, and ventral secretory pore (arrow). (B) Lateral view of holotype male anterior region showing cuticle and posterior pharynx bulb. (C) Lateral view of holotype male posterior region showing proximal end of spicules. (D) Lateral view of holotype male posterior body, showing distal end of spicules and gubernaculum. Scale bars: (A–D) = 20 μ m.

Measurement

All measurement data can be found in Table 2.

Table 2. Individual measurements of *Hypodontolaimus minus* sp. nov. (in μm , except ratio).

Characters	Holotype	Paratypes	Paratypes
	Male	Males (n = 3)	Females (n = 3)
Total body length	592	510–573	570–656
Maximum body diameter	25	24–25	25–33
Head diameter	17	14–17	17–18
Cephalic setae length	10	12–15	14–15
Buccal cavity depth	6	6–8	7–9
Nerve ring from anterior end	50	43–46	40–51
Body diameter at nerve ring	22	22–23	23–23
Pharynx length	104	90–103	100–107
Pharynx bulb length	24	19–22	22–26
Body diameter at base of pharynx	24	24–25	25–28
Cloacal/anal body diameters	23	22–23	22–23
Spicules length along arc	31	33–37	-
Gubernaculum length	14	12–14	-
Vulva from anterior end	-	-	255–320
Body diameter at vulva	-	-	25–33
V%	-	-	44.7–50
Tail length	88	89–98	93–103
a	23.7	21.3–22.9	18.2–22.8
b	5.7	5.6–6.0	5.7–6.1
c	6.7	5.5–6.4	6.1–6.4
c'	3.8	4.0–4.3	4.1–4.7

Description of *Hypodontolaimus minus* sp. nov.

Males: Body characterized by relatively small body length, with a blunt anterior end and attenuated posterior end. Cuticle homogeneous with transverse rows of punctations. Lateral differentiation consisted of two longitudinal rows of larger dots connected with transverse bars, extending from the anterior end to the posterior end. Somatic setae along the body, up to 23 μm in length. Six inner labial sensilla and six outer labial sensilla papilliform, four cephalic sensilla setiform, 10–15 μm in length. Amphideal fovea unobservable. Buccal cavity with a large S-shaped hollow dorsal tooth. Pharynx with anterior dorsal bulb and rounded posterior bulb, 19–24 μm in diameter at the posterior bulb. Nerve ring situated at the middle of the pharynx. The secretory-excretory system is situated posterior to the pharyngo-intestinal junction and the ventral gland opening at the anterior end. Cardia not observed.

Reproductive system monorchic with outstretched testis. Spicules paired and L-shaped, 1.3–1.7 cloacal body diameters in length, proximal end slightly widened, distal end tapering with posterior pointed hook. Gubernaculum with dorso-caudal apophysis, about 12–14 μm in length. Precloacal supplements absent. Tail conical and tapering gradually with a small spinneret, 3.8–4.3 cloacal body diameters in length. Three caudal glands are present with a common outlet.

Females: Similar to males. Reproductive system amphidelphic with reflexed ovaries, anterior branch located to the right of the intestine and posterior one to the left of the intestine. Vulva at the middle of the total body, 50% of body length from the anterior end.

3.1.4. Differential Diagnosis and Discussion

Hypodontolaimus minus sp. nov. is characterized by relatively small body length, cuticle with two longitudinal lateral differentiations connected by transverse bars, four files of sublateral somatic setae, spicules L-shaped, proximal end slightly swollen, distal end tapered with posterior pointed hook, and gubernaculum with dorsal caudal apophysis.

This new species belongs to the group species without preloacal supplements, and is similar to *H. galapagensis*, *H. plurisetus*, *H. pumilio*, *H. punctulatus*, and *H. steineri* with long cephalic setae and gubernaculum with apophysis (Table 3). The new species differs from *H. galapagensis* in spicules shape (distal end tapered with posterior pointed hook vs. distal end tapering without posterior pointed hook) and gubernaculum apophysis shape (dorsal-caudal directed vs. caudal directed) [36]; it differs from *H. plurisetus* in spicules shape and length (distal end tapered with posterior pointed hook vs. distal end truncate without hook) [37]; it differs from *H. pumilio* in spicules shape and length (L-shaped, distal end with posterior pointed hook, 31–37 μm vs. curved in an arc, distal end without posterior pointed hook, 22 μm), gubernaculum apophysis shape (dorsal-caudal directed vs. caudal directed), and tail length (3.8–4.7 anal body diameters vs. 3 anal body diameters) [38]; it differs from *H. punctulatus* in spicules shape (L-shaped, distal end with posterior pointed hook vs. curved in an arc, distal end bifurcated) [33]; it differs from *H. steineri* in spicules shape and length (distal end tapered with posterior pointed hook, 31–37 μm vs. distal end truncate without hook, 23 μm), and gubernaculum apophysis shape (dorsal-caudal directed apophysis vs. gubernaculum with a strong hook-shaped apophysis) [29].

Table 3. Morphological differentiating data of *Hypodontolaimus* with long cephalic setae.

Species	Body Length	Spicule Length	a	b	c
<i>H. galapagensis</i>	456–472 μm	33 μm	16.3–18.9	5.2–5.4	6.1–6.9
<i>H. plurisetus</i>	546–1024 μm	20–27 μm	13.3–21.7	5.2–8	7.9–12
<i>H. pumilio</i>	436–444 μm	22 μm	17.4–20	5.1–5.4	6.8–7.8
<i>H. punctulatus</i>	400 μm	-	15.4	6	5
<i>H. steineri</i>	590–820 μm	23 μm	21.7–23.6	5.8–6.5	7.9–9.9
<i>H. minus</i>	510–573 μm	31–37 μm	21.3–23.7	5.6–6	3.8–4.3

Hypodontolaimus minus sp. nov. forms a well-supported clade with *H. plurisetus* in the phylogenetic tree based on rDNA, and it differs only by 2% (4 bp in 2443 bp). Nevertheless, considering that spicules shape is an important diagnostic character at the species level, and despite the high molecular similarity, *Hypodontolaimus minus* sp. nov. should be considered as a new species within the genus *Hypodontolaimus*.

3.2. Systematic of *Bolbolaimus distalamphidus* sp. nov.

Superfamily Microlaimoidea Micoletzky, 1922.

Family Microlaimidae Micoletzky, 1922.

Genus *Bolbolaimus* Cobb, 1920.

3.2.1. Diagnosis (Based on Guo et al. [39])

Cuticle roughly annulated, sometimes ornamented with dots. Head set off or not. Cephalic setae situated in the same circle as outer labial sensillae or in separate circle. Amphideal fovea unispiral, partially or completely surrounded by cuticle annulations. Buccal cavity cuticularized with dorsal tooth and two smaller subventral teeth. Pharyngeal with anterior bulb and well-developed posterior bulb. Spicules short, arcuate; gubernaculum with dorsally or dorso-caudally apophysis.

3.2.2. Remarks

The morphological difference between *Microlaimus* and *Bolbolaimus* remains subtle, primarily based on head and pharyngeal morphology [40]. The genus *Bolbolaimus* was reviewed, and molecular relationships within the family Microlaimidae were also primarily discussed. Species with head set off coupled with the absence of anterior pharynx bulb are assigned to *Microlaimus*, whereas those with head not set off or/and presence of

anterior pharynx bulb, and head slightly set off are classified as *Bolbolaimus* [39]. Currently, 11 species within the genus *Bolbolaimus* are recognized as valid taxa.

List of valid species in *Bolbolaimus* as follows:

Bolbolaimus brevis Gagarin & Nguyen vu Thanh, 2019.

Bolbolaimus chitwoodi (Gerlach, 1950) Jensen, 1978.

Bolbolaimus crassiceps (Gerlach, 1953) Jensen, 1978.

Bolbolaimus distalamphidus sp. nov.

Bolbolaimus major Guo, Liang, Lv & Wang, 2023.

Bolbolaimus obesus Phan Ke Long, Gagarin, Nguyen Dinh Tu, Nguyen Thixuan Phuong & Nguyen vu Thanh, 2017.

Bolbolaimus parvus Gagarin & Nguyen vu Thanh, 2019.

Bolbolaimus punctatus Cobb, 1920.

Bolbolaimus riemanni (Riemann, 1966) Jensen, 1978.

Bolbolaimus teutonicus (Riemann, 1966) Jensen, 1978.

Bolbolaimus tongaensis Leduc, 2016.

Bolbolaimus wieseri (Hopper, 1961) Jensen, 1978.

List of species *inquerenda* in *Bolbolaimus*:

Bolbolaimus abebai Muthumbi, 1999.

Bolbolaimus amabilis de Man, 1922.

Bolbolaimus bahari Muthumbi, 1999.

Bolbolaimus cobbi Chitwood, 1937.

3.2.3. Description of New Species

Bolbolaimus distalamphidus sp. nov. (Figures 4–6).

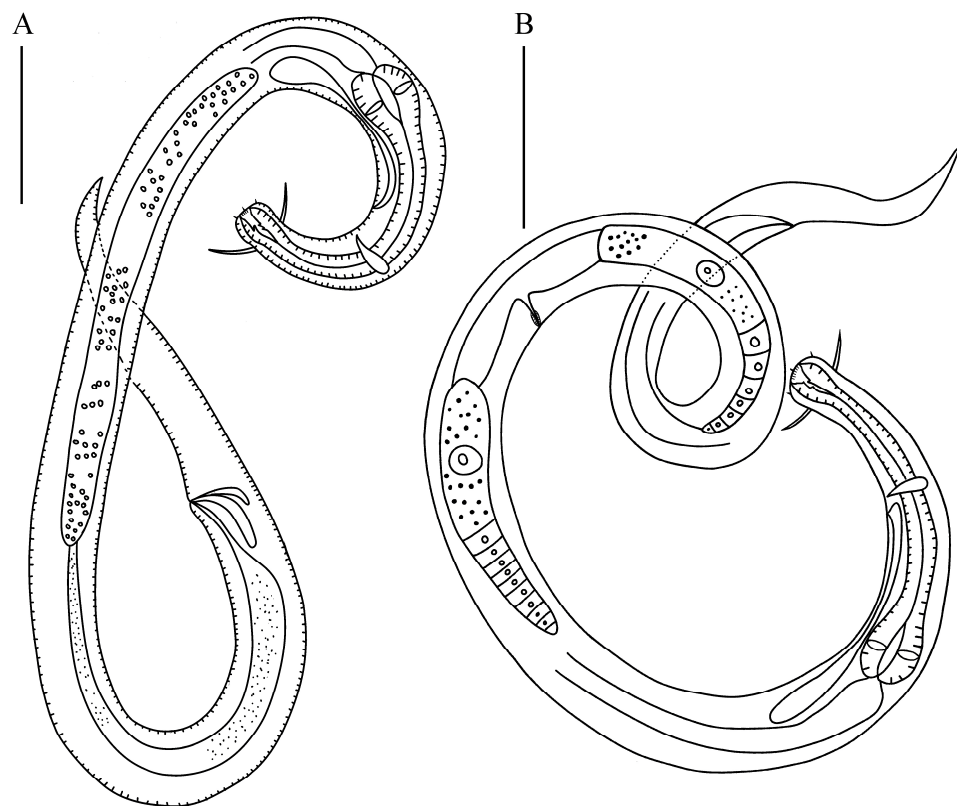


Figure 4. *Bolbolaimus distalamphidus* sp. nov. (A) Lateral view of holotype male entire body. (B) Lateral view of female posterior body. Scale bars: 50 µm.

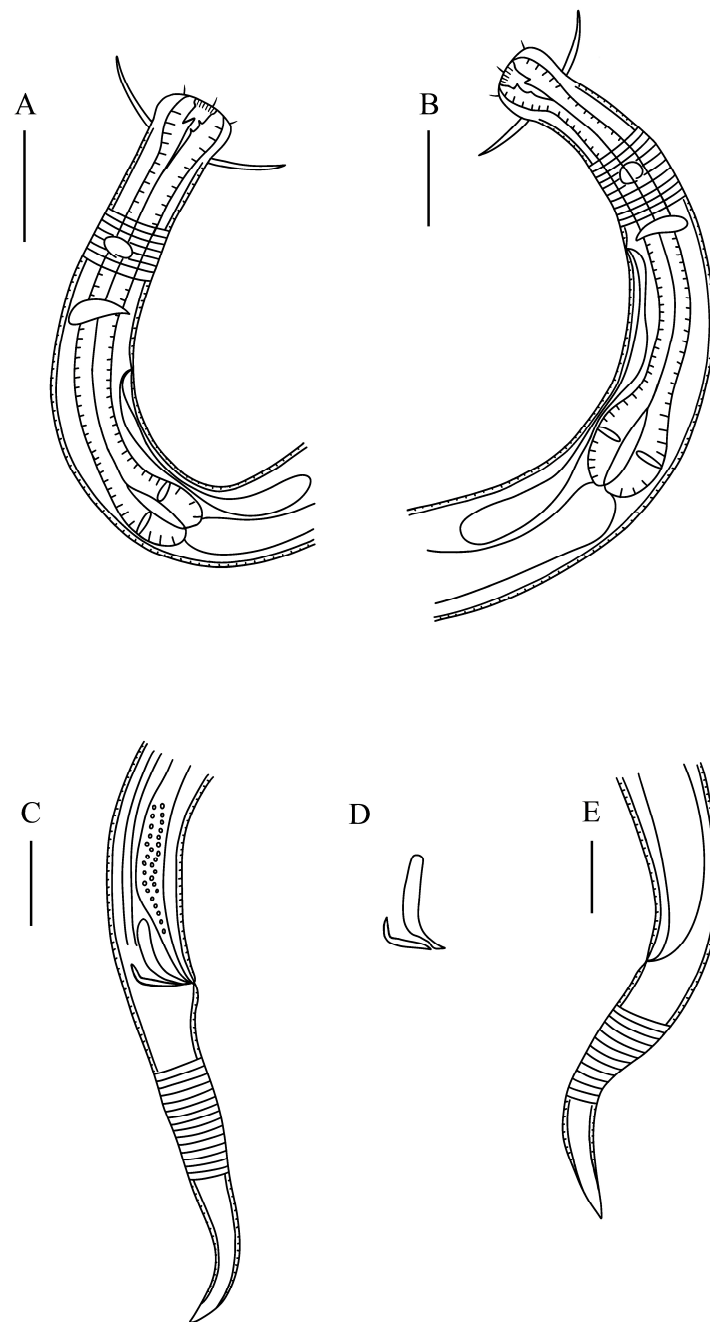


Figure 5. *Bolbolaimus distalamphidus* sp. nov. (A) Lateral view of holotype male anterior region, showing amphideal fovea, buccal cavity, and pharynx region. (B) Lateral view of female anterior region, showing amphideal fovea, buccal cavity, and pharynx region. (C) Lateral view of holotype male posterior region, showing tail. (D) Spicules and gubernaculum. (E) Lateral view of female posterior body. Scale bars: (A–C,E) = 20 μ m.

Type Material

Two males and one female were measured and studied. Holotype: m#1 on slide 23MZGC1-3-28; paratypes: m#2 on 23MZGC-X-23 and f#1 on 23MZGC1-3-12.

Type Locality and Habitat

All specimens were collected from the sublittoral region of Changdao Island, Shandong Province, China, in March, August 2023. Geographic coordinates: 37°54' N, 120°44' E. Collection depth: 2–8 cm sediment depth, sandy sediments.

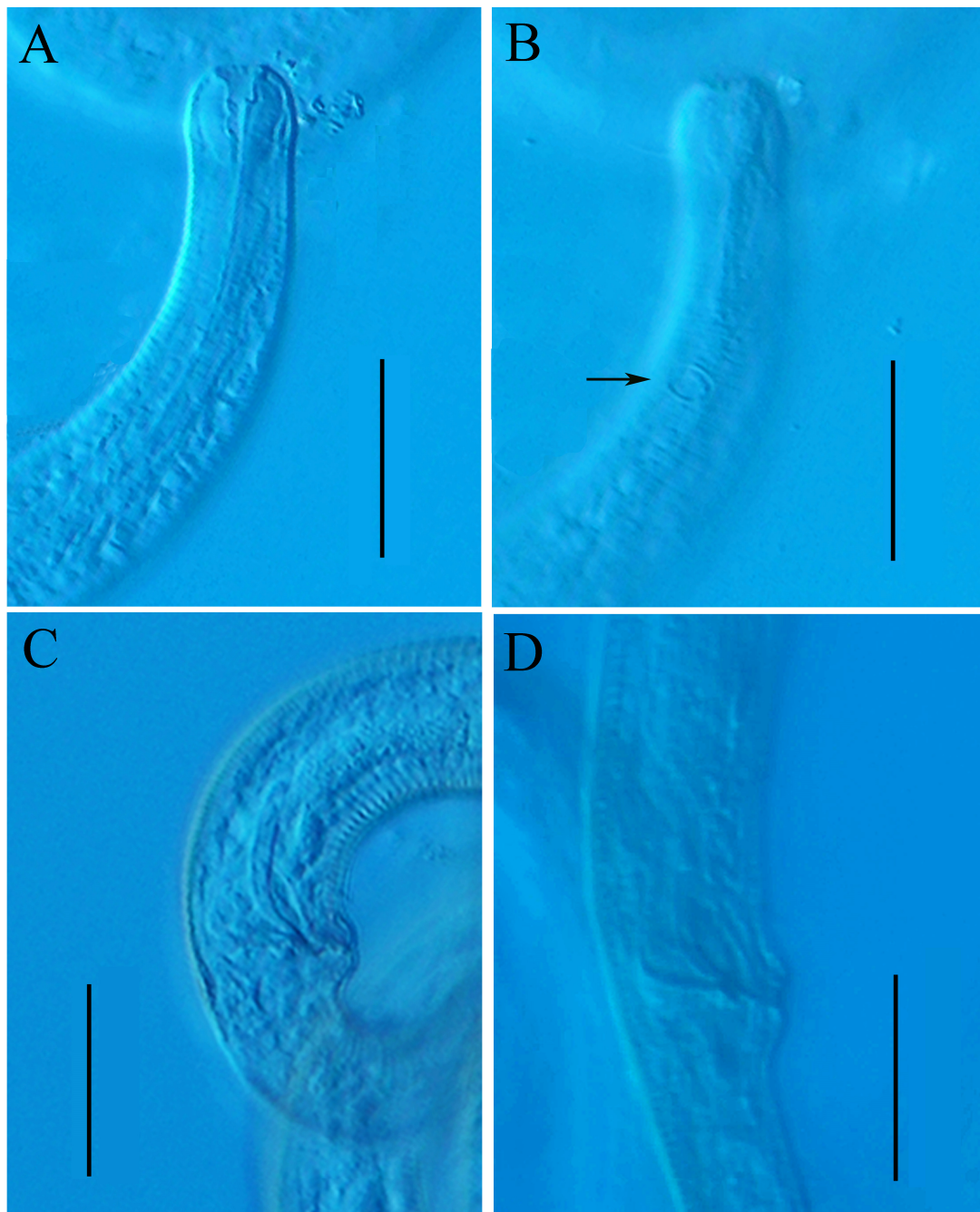


Figure 6. *Bolbolaimus distalamphidus* sp. nov. (A) Lateral view of female anterior region, showing buccal cavity. (B) Lateral view of female anterior region showing amphideal fovea (arrow). (C) Lateral view of holotype male posterior body, showing spicules. (D) Lateral view of holotype male posterior body, showing gubernaculum. Scale bars: (A–D) = 20 μm .

Etymology

The species name refers to the distinctive position of amphideal fovea, which is far from the anterior body end.

Measurement

All measurement data can be found in Table 4.

Table 4. Individual measurements of *Bolbolaimus distalamphidus* sp. nov. (in μm , except ratio).

Characters	Holotype Male	Paratype Male (n = 1)	Paratype Female (n = 1)
Total body length	556	546	600
Maximum body diameter	17	17	19
Head diameter	12	11	13
Outer labial sensilla length	2	2	2
Cephalic setae length	8	10	7
Buccal cavity depth	9	7	10
Amphideal fovea width	3	3	5
Amphideal fovea height	2	2	3
Amphideal fovea from anterior end	27	30	29
Body diameter at amphideal fovea	14	13	15
Nerve ring from anterior end	46	50	46
Body diameter at nerve ring	16	16	15
Pharynx length	99	104	100
Pharynx bulb length	18	14	17
Body diameter at the base of pharynx	17	16	18
Cloacal/anal body diameters	17	17	16
Spicules length along arc	20	22	-
Gubernaculum length	15	12	-
Vulva from anterior end	-	-	316
Body diameter at vulva	-	-	19
V%	-	-	52.7
Tail length	96	86	100
a	32.7	32.1	31.6
b	5.6	5.3	6.0
c	5.8	6.3	6.0
c'	5.6	5.1	6.3

Description of *Bolbolaimus distalamphidus* sp. nov.

Males. Body relatively small and thin, with faint brown color. Cuticle strongly annulated, starting immediately posterior to cephalic setae till tail tip. Head blunt, slightly set off. Inner labial sensilla papilliform and outer labial sensilla short, 2 μm in length (0.16–0.18 head diameters) in separate circles. Four cephalic setae 0.67–0.9 head diameters in length. Amphideal fovea small, unispiral, and oval shaped, situated far from anterior end (2.25–2.73 head diameters), 3 μm in width and 2 μm in height (21.4–23.1% corresponding body diameters) and completely surrounded by cuticle annulation. Buccal cavity relatively small, 7–9 μm in depth, obviously cuticularized with one large dorsal tooth and two small subventral teeth. Pharynx cylindrical, with an anterior pharynx bulb and a posterior oval-shaped bulb. Nerve ring situated slightly anterior to middle pharynx region (46.5–48.1% of pharynx length). Secretory-excretory system present, ventral gland situated posterior to pharynx bulb, secretory-excretory pore situated posterior to the nerve ring, 62–64 μm from anterior end. Cardia not observed.

Reproductive system diorchic, testes opposed and outstretched. Sperm cells maximum dimensions 7 $\times$ 6 μm . Spicules paired and slightly curved, 1.18–1.29 cloacal body diameters in length, proximal end not cephalated, and distal end tapered. Gubernaculum short with anterior pointed apophysis. Precloacal supplements not observed. Tail conical, 5.1–5.6 cloacal body diameters in length. Caudal glands invisible.

Female. Similar to males in most characteristics. Amphideal fovea slightly larger, 5 μm in width and 3 μm in length (33% corresponding body diameter). Reproductive system didelphic, with opposed and outstretched ovaries. Anterior ovary to the left of intestine and posterior ovary to the right of intestine. Vulva at the middle of the total body. Vagina short and surrounded by constrictor muscles.

3.2.4. Differential Diagnosis and Discussion

Bolbolaimus distalamphidus sp. nov. is characterized by the small body length, head slightly set off, buccal cavity with a large dorsal tooth and two small subventral teeth, six outer labial sensilla short and four cephalic setae long, amphideal fovea situated 2.25–2.73 head diameters from anterior end, pharynx with anterior bulb and posterior bulb, spicules slightly curved, gubernaculum with anterior pointed apophysis, and tail conical.

The new species differs from other species of the genus *Bolbolaimus* by amphideal fovea far from the anterior end. *Bolbolaimus distalamphidus* sp. nov. is similar to *B. crassiceps*, *B. parvus*, and *B. teutonicus* in gubernaculum with anterior pointed apophysis and tail shape (Table 5), but it differs from *B. crassiceps* in body length (546–600 µm vs. 1095–1120 µm), amphideal fovea size and position (2.25–2.73 head diameters from anterior end vs. at anterior body end), and spicules length (20–22 µm vs. 23–27 µm) [41]; it differs from *B. parvus* in cephalic setae length (7–10 µm vs. 1 µm), amphideal fovea size and position (21–33% c.b.d., 27–30 µm from anterior end vs. 45–50% c.b.d., 8–10 µm from anterior end), spicules length (1.2–1.3 cloacal body diameters vs. 1.5–1.6 cloacal body diameters), and tail length (86–100 µm vs. 61–78 µm) [42]; it differs from *B. teutonicus* in amphideal fovea size and position (21–33% c.b.d., 27–30 µm from anterior end vs. 60% c.b.d., 8 µm from anterior end) [43].

Table 5. Morphological differentiating data of *Bolbolaimus* with gubernaculum apophysis.

Species	Body Length	Spicule Length	a	b	c
<i>B. crassiceps</i>	1095–1120 µm	23–27 µm	35–47	7–7.5	10.6–12.2
<i>B. parvus</i>	401–471 µm	28–31 µm	14–19	4.5–5.5	5.1–7.2
<i>B. teutonicus</i>	1100–1220 µm	27–28 µm	40–46	7.1–7.8	11.6–12.1
<i>B. distalamphidus</i>	546–556 µm	20–22 µm	32.1–32.7	5.3–5.6	5.8–6.3

Bolbolaimus distalamphidus sp. nov. forms a well-supported clade with *B. major* in the phylogenetic topology tree, but it differs by 7.45% (206 in 2325 bp, including 27 gaps), with p-distance of 0.0907 ± 0.0058 . Based on the morphological difference of the gubernaculum shape and amphideal fovea size and position, *Bolbolaimus distalamphidus* sp. nov. should be considered as a new species within the superfamily Microlaimoidea.

3.3. Molecular Phylogenetic Relationships and Discussion

The ML trees topology based on combined SSU and LSU sequences within the family Chromadoridae and the superfamily Microlaimoidea are mainly identical to the BI trees. Only the BI trees are shown in Figures 7 and 8.

3.3.1. Phylogenetic Analysis Within Family Chromadoridae

In the phylogenetic analysis of the family Chromadoridae, LSU and SSU sequences from the same specimens representing three subfamilies (Chromadorinae, Euchromadorinae, and Hypodontolaiminae) were analyzed, with 25 sequences from 9 genera retrieved from Genbank. Sequences of Harpagonchinae and Spilipherinae were excluded from the analysis due to the absence of both SSU and LSU from the same specimen in the Genbank.

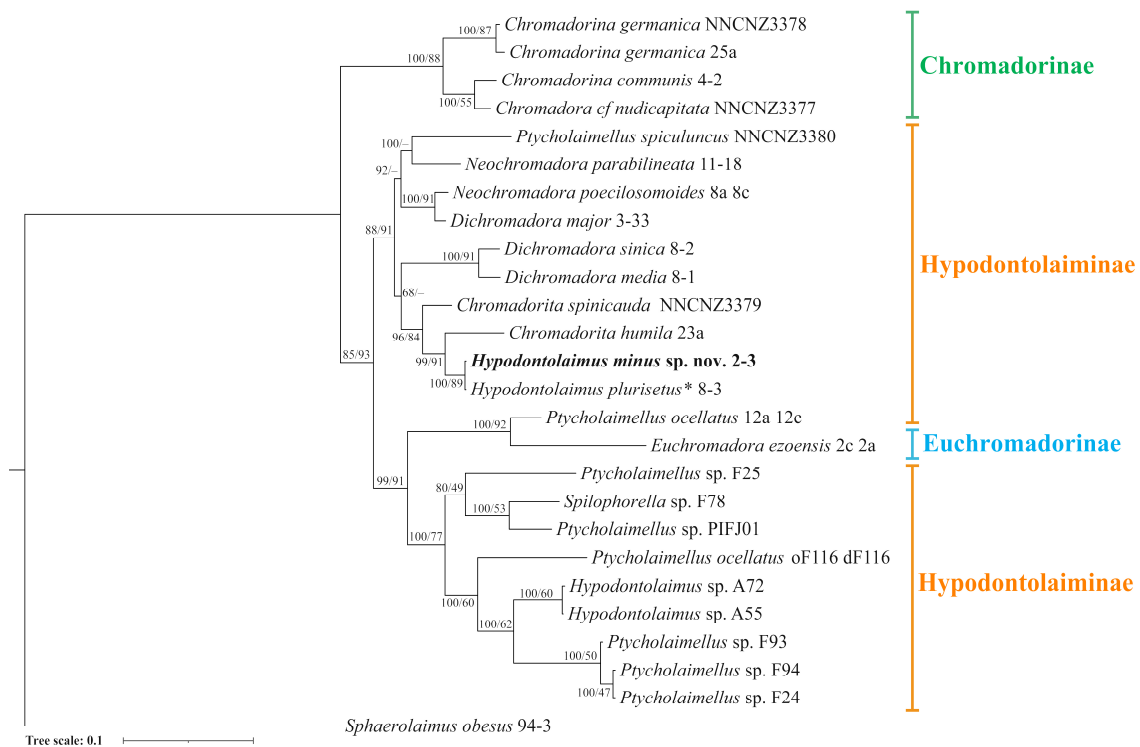


Figure 7. Bayesian inference tree based on combined SSU and LSU sequences within the family Chromadoridae. Posterior probability on the left and bootstrap values on the right are listed on the corresponding clade. Sequences obtained in this study are shown in bold. Subfamilies are given on the right. The scale represents substitutions per site; * means it was formerly labeled as *Dichromadora multisetosa*.

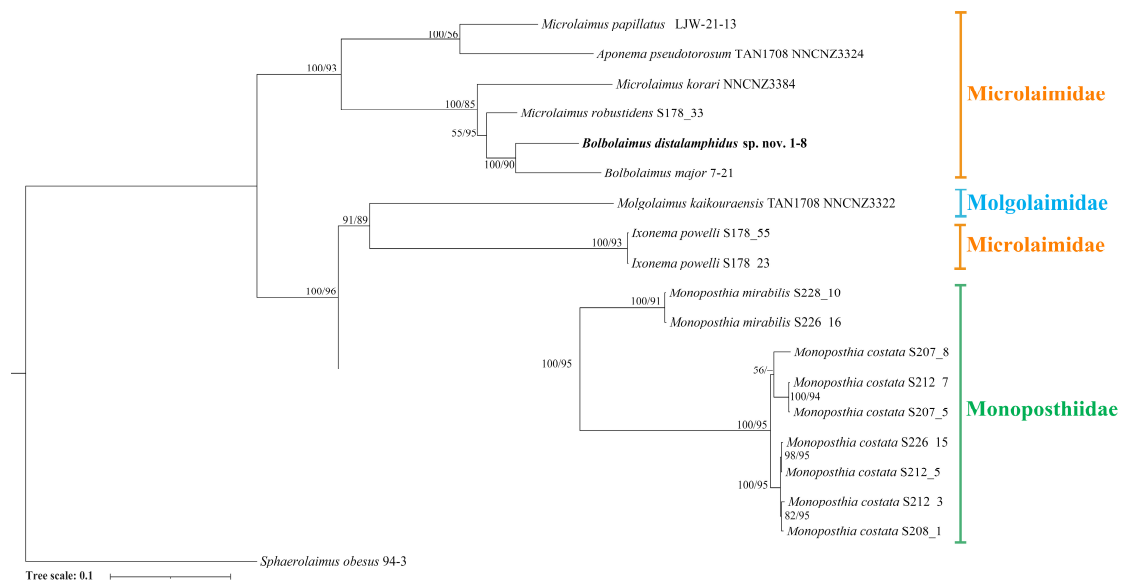


Figure 8. Bayesian inference tree based on combined SSU and LSU sequences within the superfamily Microlaimoidea. Posterior probability on the left and bootstrap values on the right are listed for the corresponding clade. Sequences obtained in this study are shown in bold. Families are given on the right. The scale represents substitutions per site.

Within the subfamily Chromadorinae, four sequences belonging to two genera, *Chromadora* and *Chromadorina*, formed a well-supported clade (100% posterior probability and 88% bootstrap value) that appeared as a sister taxon to other genera of Chromadoridae. This finding is consistent with the results of Guo et al. based on SSU analysis [9]. The mean

p-distance between genera within Chromadorinae was the smallest among the studied subfamilies (0.0537 ± 0.0048). These two genera share high morphological similarity with respect to cuticle with homogeneous punctations, buccal cavity with three solid teeth, amphideal fovea transverse slit shaped, and precloacal supplements cup shaped (when present) [8].

With regard to subfamily Hypodontolaiminae, six genera are recognized: *Chromadorita*, *Dichromadora*, *Ptycholaimellus*, *Hypodontolaimus*, *Neochromadora*, and *Spiliphorella*. Molecular phylogenetic analyses based on concatenated SSU and LSU sequences revealed that members of Hypodontolaiminae form two paraphyletic clades with robust statistical support (85% posterior probability and 93% bootstrap value). Notably, none of the genera *Dichromadora*, *Ptycholaimellus*, *Neochromadora*, and *Hypodontolaimus* constituted monophyletic assemblages. The analysis incorporated four species of *Dichromadora*, with only *D. media* and *D. sinica* forming a strongly supported monophyletic group (100% posterior probability and 100% bootstrap value). *D. major* clusters with *Neochromadora poecilosomoides* (100% posterior probability and 91% bootstrap value), while *D. multisetosa* (8–3) (taxonomically revised as *H. plurisetus*) groups with *Hypodontolaimus minus* sp. nov. (100% posterior probability and 89% bootstrap value). The phylogenetic topology obtained for *Dichromadora* is largely congruent with observations by Liang et al., wherein *D. media* and *D. sinica* are characterized by shared morphological attributes, including a buccal cavity with a diminutive dorsal tooth and the absence of precloacal supplements [10].

The genera *Hypodontolaimus* and *Dichromadora* exhibit considerable morphological similarity, including a homogeneous cuticle with two longitudinal differentiations, cup-shaped precloacal supplements (when present), asymmetrically swollen peribuccal cavity, and a pharynx featuring a single distinct posterior bulb. The primary diagnostic character differentiating these genera is the dorsal tooth morphology: triangular and acute in *Dichromadora*, versus S-shaped with a dorsal apophysis in *Hypodontolaimus*. Molecular phylogenetic analysis reveals that *Hypodontolaimus minus* sp. nov. and a specimen identified as *D. multisetosa* (8–3) form a well-supported clade. Upon re-examination of the voucher specimen of *D. multisetosa* (8–3), significant morphological similarities to *Hypodontolaimus plurisetus* were observed, particularly in the structure of the spicules and gubernaculum; cuticle both have four files of longitudinal somatic setae and buccal cavity with large dorsal tooth and dorsal apophysis. Based on these combined morphological characteristics, we conclude that the specimen previously identified as *D. multisetosa* and whose sequences were deposited in GenBank (SSU accession number OR479915; LSU accession number OR479917) should be taxonomically reassigned as *Hypodontolaimus plurisetus*.

In the phylogenetic tree topology, species of genus *Ptycholaimellus* are distributed across different clades, with interspecific p-distance reaching 0.1542 ± 0.0056 . This value substantially exceeds the mean p-distance among genera within the subfamily Hypodontolaiminae (0.1256 ± 0.006). Notably, a similar pattern is observed in the comparison between two sequences identified as *Ptycholaimellus ocellatus* (specimen vouchers: 12a 12c and oF116/dF116), where the intraspecific p-distance value is remarkably high (0.16 ± 0.01). In the molecular phylogenetic reconstruction, these two *P. ocellatus* sequences segregate into distinct clades, with *P. ocellatus* (voucher: 12a 12c) clustering with species of the genus *Euchromadora*. This pronounced intraspecific divergence suggests the possible existence of cryptic species within the nominal taxon *P. ocellatus*.

The genus *Euchromadora*, the sole representative of Euchromadorinae included in this molecular analysis, does not form a sister clade to other subfamilies, as might be expected based on traditional taxonomy. Instead, it clusters with *Ptycholaimellus ocellatus* (specimen voucher: 12a 12c) with robust statistical support (100% posterior probability and 92% bootstrap value). The phylogenetic position of Euchromadorinae within Chromadoridae

may require reconsideration as additional molecular data become available in public repositories such as GenBank.

3.3.2. Phylogenetic Relationships Within the Superfamily Microlaimoidea

In the phylogenetic reconstruction of the superfamily Microlaimoidea, 18 sequences representing 10 species were analyzed. Microlaimoidea has been recently reviewed by Leduc et al., who recognized four constituent families: Microlaimidae, Monoposthiidae, Molgolaaimidae, and Aponchiidae [2]. The family Aponchiidae was not included in the present molecular phylogenetic analysis due to the absence of conspecific LSU and SSU sequence data. The taxonomic placement of genera within Microlaimoidea remains challenging due to the lack of clear synapomorphies distinguishing it from Chromadorida and Desmodorida, with current classification schemes largely informed by molecular phylogenetic evidence [2].

The phylogenetic position of genus *Molgolaimus* Ditlevsen, 1921 has been debated since Jensen's work in 1978 [44]. *Molgolaimus* was initially classified within the family Microlaimidae based on similarities in head morphology, amphideal fovea shape, cephalic sensilla arrangement, and buccal cavity structure [35]. Subsequently, Jensen established the family Molgolaaimidae to accommodate *Molgolaimus*, distinguishing it from Microlaimidae based on reproductive system characteristics (one or two testes in males, ovaries outstretched in females in Microlaimidae versus one anterior testis in males, reflexed ovaries in females in Molgolaaimidae) [44]. Leduc et al. proposed that *Molgolaimus* should be assigned to the superfamily Microlaimoidea based on integrated morphological and molecular analyses, reinstated the family Molgolaaimidae, and recommended that Microlaimoidea should encompass Microlaimidae, Monoposthiidae, Molgolaiminae, and Aponchiidae [2]. The present rDNA-based phylogenetic tree topology corresponds with Leduc et al. [2], showing *Molgolaimus* clustering with *Ixonema* to form a strongly supported sister clade to the family Monoposthiidae (100% posterior probability and 96% bootstrap value).

The genus *Aponema* was previously assigned to Molgolaaimidae by Jensen based on its dorso-caudally directed gubernaculum apophysis and distinctly sclerotized amphideal foveae [44]. Subsequently, Tchesunov and Leduc et al. suggested its transfer to Microlaimidae based on outstretched ovaries (contrasting with the reflexed ovaries in Molgolaaimidae), despite lacking molecular evidence [2,45]. In the present phylogenetic reconstruction, the genera *Aponema*, *Microlaimus*, and *Bolbolaimus* form a well-supported clade (100% posterior probability and 93% bootstrap value), supporting the placement of genus *Aponema* within Microlaimidae. The genera *Bolbolaimus* and *Microlaimus* exhibit high morphological similarity and are currently distinguished primarily based on anterior end morphology [9,40]. The combined rDNA phylogeny further indicates that neither *Bolbolaimus* nor *Aponema* forms a sister clade to *Microlaimus*; rather, they are nested within the *Microlaimus* clade.

The genus *Ixonema* does not cluster with other Microlaimid genera, but forms a moderately supported clade (100% posterior probability and 90% bootstrap value) with the genus *Molgolaimus*. However, these two genera exhibit limited morphological similarity, with principal differences in male and female reproductive systems. Additionally, *Ixonema* differs from other Microlaimid genera in possessing caudal glands with three separate outlets, whereas other genera have a common outlet. Nine sequences representing two species of *Monoposthia* form a well-supported monophyletic clade (100% posterior probability and 95% bootstrap value), with the two distinct species forming separate subclades.

Additional sequence data deposited in Genbank may further elucidate phylogenetic relationships within the superfamily Microlaimoidea and potentially clarify deeper evolutionary connections among the orders Microlaimida, Chromadorida, and Desmodorida.

4. Conclusions

Hypodontolaimus minus sp. nov. can be differentiated by relatively small body length, four files of sublateral somatic setae, spicules L-shaped, proximal end slightly swollen, distal end tapered with posterior pointed hook, and gubernaculum with dorsal caudal apophysis. Phylogenetic analysis shows similarity to *H. plurisetus*, but based on the difference in spicules shape, it should be considered as a new species.

Bolbolaimus distalamphidus sp. nov. can be differentiated by small body size, amphideal fovea unispiral oval and far from the anterior end, spicules slightly curved, and gubernaculum with anterior pointed apophysis. Phylogenetic analysis supports that it should be considered as a new species.

According to the phylogenetic tree within Chromadoridae, the subfamily Chromadorinae is shown as a monophyletic clade, while the genera of subfamily Hypodontolaiminae are shown as paraphyletic group, and the genus *Ptycholaimellus* shows high intraspecific diversity. Phylogenetic analysis within the superfamily Microlaimidea supports the conclusion of Leduc et al. [2], who suggested that *Molgolaimus* should be ascribed to Microlaimidea, and Microlaimidea should include Microlaimidae, Monoposthiidae, Molgolaiminae, and Aponchiidae.

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Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article.

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

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