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Article

A New Species of Free-Living Nematode (Enoplida: Enchelidiidae) from the Mangrove Wetlands of China

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Abstract: A new species, *Belbolla mangrove* sp. nov., isolated from the mangrove wetlands of Fujian and Zhejiang Provinces in China, is described and illustrated. *Belbolla mangrove* sp. nov. is characterized by a pharynx with four bulbs, a small gubernaculum with a short dorsocaudal apophysis, four weakly developed precloacal supplements, a conico-cylindrical tail with a terminal spinneret, and the absence of terminal setae. This new species differs from *B. vietnamica* by the absence of ocelli and the blunt and rounded proximal ends of the spicules. The 18S rDNA GenBank accession numbers of *B. mangrove* sp. nov. are provided.

Keywords: nematode taxonomy; *Belbolla mangrove* sp. nov.; mangrove wetland

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1. Introduction

Free-living marine nematodes are the most abundant meiofaunal animals in the marine environment. These organisms have short life cycles, and their distribution is closely related to the benthic environment. They are sensitive to environmental changes and pollution, particularly at small spatial and short time scales. Therefore, they have been considered one of the best indicator species of the marine environment [1]. However, there are still obstacles and challenges to their use as bioindicators on a global scale, mainly due to the limited available information, particularly regarding their identification. The taxonomic study of marine nematodes is an important basic work in current biodiversity research; however, a significant portion of marine nematode species remains unidentified.

Free-living marine nematodes are key players in coastal wetlands, especially in bioturbation [2]. The genus *Belbolla* was renamed by Andrássy to replace the previously used name *Bolbella* Cobb, 1920, because *Bolbella* was a homonym of *Bolbella* Giglio–Toss, 1915 (Orthoptera—Mantidae) [3]. The common characteristic features of the genus *Belbolla* include the presence or absence of ocelli, a large and narrow buccal cavity divided into two parts, the presence of three teeth, with the largest tooth being the ventro-sublateral one, and the presence of a series of bulb-like muscular pharynx swellings (ranging from 4 to 10) in the posterior section of the pharynx [4]. So far, 20 valid species of the genus *Belbolla* have been recorded from 11 countries, including Germany, the USA, Britain, Vietnam, Bangladesh, China, Korea, Russia, Pakistan, Indonesia, and France [5,6]. The main distribution habitat of the genus is marine, and five species have been previously described in China. One species, *B. zhangi* Guo & Warwick, 2001 is from the Bohia Sea [7], while the other four species,

namely *B. huanghaiensis* Huang and Zhang, 2005, *B. stenocephalum* Huang and Zhang, 2005, *B. warwicki* Huang and Zhang, 2005, and *B. sinica* Guo and Wang, 2022, are from the Yellow Sea [5]. On the other hand, only three species have been reported worldwide in estuarine and mangrove habitats. These include *B. longispiculata* Nasira, Shahina and Shamim, 2014 from the Indus River Estuary in Sindh, Pakistan; *B. gracilis* Gagarin and Thanh, 2016 from the Yen River Estuary mangrove in Vietnam; and *B. vietnamica* Gagarin and Nguyen Dinh Tu, 2016 from the Yen River Estuary mangrove in Vietnam. However, the genus *Belbolla* has not been studied and reported from mangrove wetlands in China. The classification and identification of *Belbolla* species are poorly known, and molecular identification based on DNA sequencing of the genus *Belbolla* has never been reported.

In China, the northernmost boundary of the natural distribution of mangrove wetlands is Fuding City in the Fujian Province, while the northern boundary of artificial introduction is Yueqing City in Zhejiang Province [8]. In recent years, we have investigated the biodiversity and community structure of free-living marine nematodes in mangrove wetlands in China, aiming to understand their spatial and temporal variation patterns and provide references for environmental monitoring. More than 50 new species of free-living nematodes have been discovered. Among them, a new species of the genus *Belbolla*, which has never been reported before, was discovered for the first time in the mangrove wetlands at the mouth of the Jiulong River and Ximen Island. The present study aims to provide a comprehensive description of this new species of *Belbolla*, enhancing our knowledge of the biodiversity of free-living nematodes in mangrove wetlands. Additionally, we report the 18S rDNA sequence of the new species *B. mangrove* sp. nov., which is essential to fill the DNA sequence gap of the genus *Belbolla*.

2. Materials and Methods

2.1. Sample Collection

Undisturbed sediment samples were taken from the Ximen Island mangrove wetland in Yueqin City, Zhejiang Province (28.34° N, 121.17° E) and the Jiulong Estuary mangrove wetland in Zhangzhou City (23.24° N, 117.55° E) of Fujian Province, China in December 2018 and May 2021, respectively. The samples were collected by inserting a syringe (2.9 cm inner diameter) into the sediment to a depth of 5 cm. The collected samples were fixed with 5% formalin and transported back to the laboratory. They were then stained with 0.1% rose Bengal for over 24 h [4]. A portion of the fresh samples was stored in a refrigerator at −20 °C for DNA extraction.

2.2. Meiofauna Extraction and Nematode Identification

The samples were rinsed with tap water to eliminate any remaining formalin and then filter through two sieves (with mesh size of 500 and 42 μm, respectively) until the water passing through the sieve appeared clean. Meiofauna was extracted using the flotation method (using Ludox™ (Sigma-Aldrich, Merck, Rahway, NJ, USA) with a specific gravity of 1.15) as described by Jonge et al. [9]. Afterwards, it was stained with 1% Rose Bengal for over 48 h to aid the detection of animals during sorting [10]. The meiofauna was sorted using a stereoscopic microscope (Nikon SMZ800 (Nikon, Tokyo, Japan). The nematodes were transferred to a cavity block filled with a solution consisting of a mixture of glycerol, pure ethanol, and distilled water at a ratio of 1:1:18. This allowed for the gradual evaporation of ethanol and some of the water before mounting specimens on slides [11]. Drawings were made with the aid of a camera lucida attached to the Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). Type specimens were deposited at Jimei University in Xiamen, China. Permanent slides were prepared as described by Shih et al. [12]. The holotype and paratypes were also deposited at Jimei University.

2.3. DNA Extraction and PCR Amplification

Total genomic DNA extraction was performed on three individuals of each newly discovered species of *Belbolla* mentioned in the previous section. The TIANamp Marine

Animals DNA Kit Dp324-03 (TIANGEN BIOTECH, CO., LTD., Beijing, China) was used for this purpose, following the manufacturer's instructions provided in the manual, with minor adjustments made to the volume of buffer solution used at each step. The volumes were modified as follows: 20 µL of buffer GA; 5 µL of proteinase K (20 mg/mL); 20 µL of buffer GB; 20 µL of 99% ethanol; 50 µL of buffer GD; 60 µL of buffer PW; and 30 µL of buffer TE. The genomic DNA was stored in a −20 °C refrigerator for further use. The primers utilized for the 18S rDNA were as follows: MN18F: 5′-CGCGAATRGCTCATTACAACAGC-3′ and Nem_18S_R: 5′-GGGCGGTATCTGATCGCC-3′ [13,14]. The volumes for each reaction were set at 25 µL, comprising 6 µL of DNA template, 12.5 µL of 2X Pro Taq Master Mix, 0.5 µL for each forward and reverse primer, and 5.5 µL of double distillation H_2O (ddH_2O). The thermal cycling conditions for PCR consisted of an initial denaturation step at 94 °C for 2 min, followed by 40 cycles of 94 °C for 35 s, 50 °C to 55 °C for 35 s, and 72 °C for 60 s. The final extension step was performed at 72 °C for 10 min. Following the PCR assay, 5 µL of each PCR product was subjected to electrophoresis on a 1% agarose gel. The gel was then strained with 0.5 µg/mL ethidium bromide and visualized using an ultraviolet trans-illumination system. All positive PCR amplicons were sequenced by Sangon Biotech Co., Ltd. (Shanghai, China).

2.4. Sequencing and Analyses

A total of three sequences were obtained from individual nematodes of *H. mangrove* sp. nov. in this study. All sequences were deposited in GenBank (Table 1). The 18S rDNA sequences of Enchelidiidae were retrieved from the GenBank and the sequences were selected for phylogenetic analysis. *Oncholaimus* sp. was used as an outgroup in the 18S rDNA sequence analysis. Sequences were aligned in the BioEdit version 7.0.9.0 sequence editor. A neighbor-joining (NJ) tree was constructed in MEGA 7 using the gamma-corrected Kimura 2-parameter distance method. The NJ tree was subsequently validated with bootstrap analysis using 1000 replicates [13]. Moreover, intergeneric thresholds based on gene sequences was calculated using Kimura 2-parameter distances (K2P) via MEGA 7.0 software. The parameter model was reconstructed with 1000 bootstrap replicates [12].

Table 1. GenBank accession numbers of the three male individuals of the new species, *Belbolla mangrove* sp. nov., discovered in this study.

No	Site	Habitat	Longitude	Latitude	Gender	GenBank Accession Number of 18S rDNA	Amplified Fragment Length (bp)
1	Jiulong River Estuary, Zhangzhou City, Fujian Province	mangrove	117.95° E	24.45° N	male	MZ229775	930 bp
2	Jiulong River Estuary, Zhangzhou City, Fujian Province	mangrove	117.95° E	24.45° N	male	MZ229776	935 bp
3	Ximen Island, Wenzhou City City, Zhejiang Province	mangrove	121.17° E	28.34° N	male	MZ229777	853 bp

2.5. Terminology and Abbreviations

The following measurements were conducted on nematodes, all in µm, with the following abbreviations: a, body length divided by maximum body diameter; b, body length divided by pharynx length; c, body length divided by tail length; c', tail length divided by anal body diameter; a.b.d., anal body diameter; c.b.d., corresponding body diameter; v.b.d., vulval body diameter; V, vulva distance from the anterior body end; V%, position of vulva as a percentage of body length from the anterior body end [4].

3. Results

3.1. Description of Belbolla mangrove sp. nov.

Class Enoplea Inglis, 1983
Order Enoplida Filipjev, 1929
Family Enchelidiidae Filipjev, 1918
Genus *Belbolla* Andrássy, 1973
Belbolla mangrove sp. nov.
(Figures 1 and 2; Tables 2 and 3)

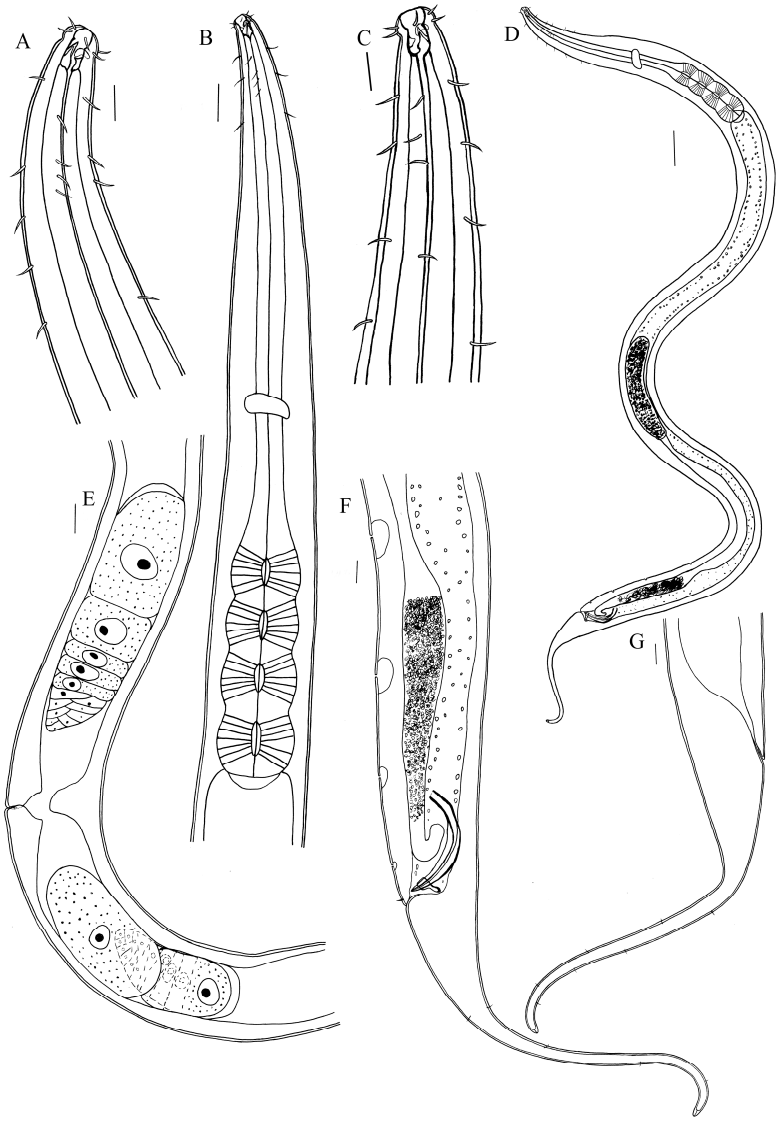

Figure 1. *Belbolla mangrove* sp. nov. (**A**) Lateral view of male head; (**B**) lateral view of female anterior part; (**C**) lateral view of female head; (**D**) lateral view of male body; (**E**) lateral view of ovaries; (**F**) lateral view of male posterior body part, showing the spicule and precloacal supplement; (**G**) lateral view of female tail end. Scale bars: 10 (**A**,**C**,**F**,**G**); 20 (**B**,**E**); 50 μm (**D**).

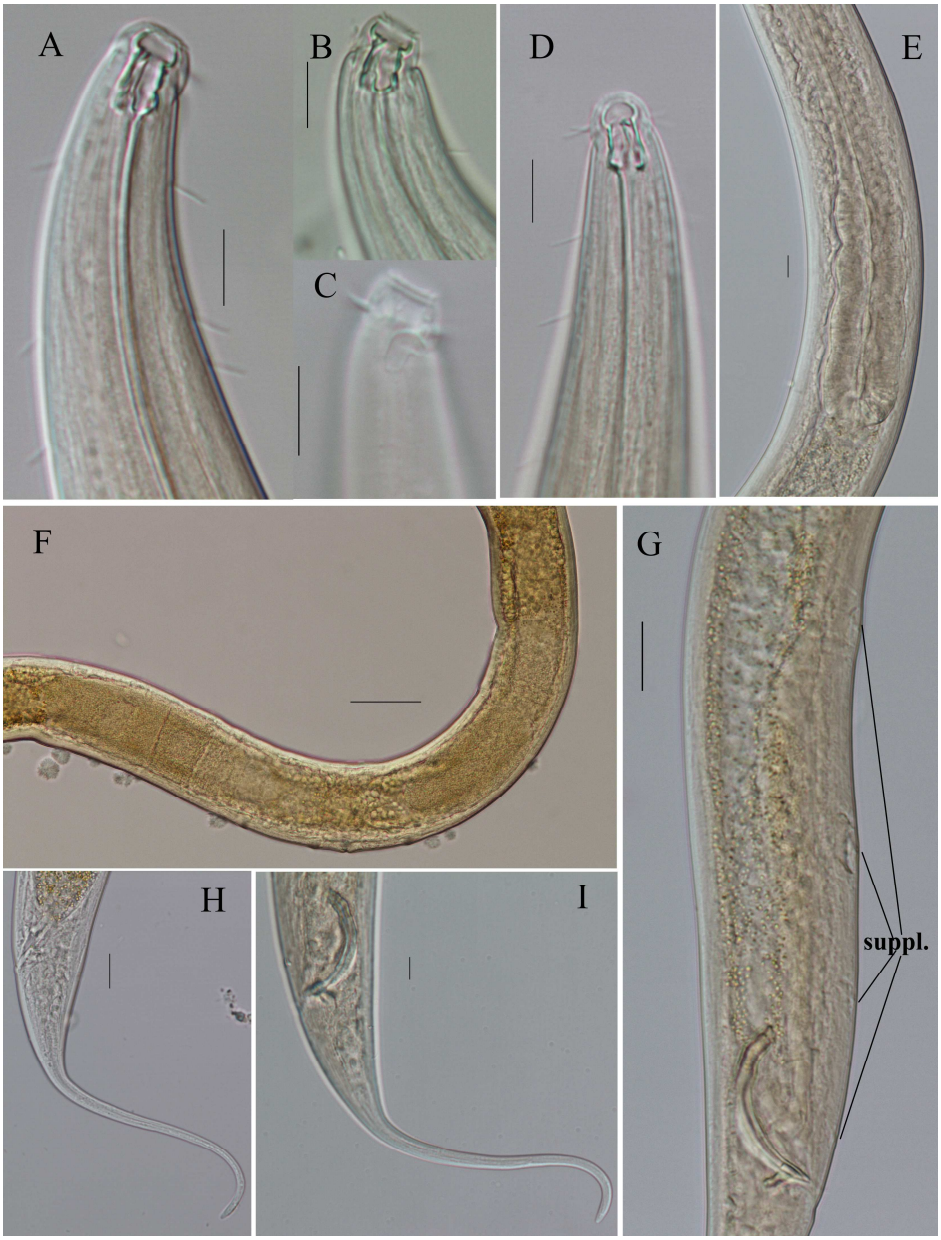

Figure 2. *Belbolla mangrove* sp. nov. (**A**) Lateral view of male head end; (**B**) lateral view of labial papillae; (**C**) lateral view of amphideal fovea; (**D**) lateral view of male head end; (**E**) lateral view of male pharynx; (**F**) lateral view of ovaries; (**G**) lateral view of male posterior body part, showing the spicule and precloacal supplement; (**H**) lateral view of female tail end; (**I**) lateral view of male tail end. Scale bars: 10 (**A**–**D**); 20 (**E**,**G**–**I**); 50 μm (**F**).

A new species of the genus *Belbolla* was discovered in the mangrove sediments of China, adding to the existing 20 valid species documented in the literature (summarized in Table 2).

Table 2. The valid species of the genus *Belbolla* reported in literature.

Valid Species	Habitat
B. asupplementata Juario, 1974	Marine
B. californica (Allgén, 1951) Andrássy, 1973	Marine
B. gallanachmorae (Inglis, 1961) Andrássy, 1973	Marine
B. gracilis Gagarin & Thanh, 2016	Estuary mangrove
B. heptabulba (Timm, 1961) Andrássy, 1973	Marine
B. huanghaiensis Huang & Zhang, 2005	Marine
B. hoonsooi Rho, Lee, Lee & Choi, 2021	Marine
B. insula Belogurov, Fadeeva & Belogurova, 1983	Marine
B. intarma Belogurov & Belogurova, 1980	Marine
B. koreensis Rho, Lee, Lee & Choi, 2021	Marine
B. longispiculata Nasira, Shahina & Shamim, 2014	Estuary
B. sinica Wang, Guo & Wang, 2022	Marine
B. stenocephalum Huang & Zhang, 2005	Marine
B. sundaensis (Micoletzky, 1930) Andrássy, 1973	Marine
B. teissieri (Luc & De Coninck, 1959) Andrássy, 1973	Marine
B. tenuidens (Cobb, 1920) Andrassy, 1973	Marine
B. vietnamica Gagarin & Nguyen Dinh Tu, 2016	Estuary mangrove
B. warwicki Huang & Zhang, 2005	Marine
B. wonkimi Rho, Lee, Lee & Min, 2020	Marine
B. zhangi Guo & Warwick, 2001	Marine

Table 3. Measurements of individuals of the new species, *Belbolla mangrove* sp. nov. discovered in this study (-: not applicable).

Characters	Holotype	Paratypes					
	♂**1**	♂**2**	♂**3**	♂**4**	♀**1**	♀**2**	♀**3**	♀**4**
Body length (μm)	1454	1608	1482	1465	1825	1649	1790	1632
Head diameter (μm)	10.6	9.4	10.0	9.2	10.2	9.8	10.0	9.5
Length of cephalic setae (μm)	4.0–5.4	4.8–5.7	4.0–5.1	4.0–5.2	5.0–6.0	4.0–4.7	4.0–5.1	4.0–5.1
Buccal cavity length (μm)	11.2	11.3	11.2	9.6	12.0	12.0	12.5	11.6
Buccal cavity diamtere (μm)	5.2	5.5	3.7	4.2	6.1	5.0	4.5	4.6
Amphids from the anterior end (μm)	4.7	5.7	5.6	5.9	5.1	4.0	4.3	5.9
Amphid c.b.d. (μm)	11.2	10.2	9.7	12.0	11.3	9.6	10.0	9.9
Nerve ring from the anterior end (μm)	180	195	177	159	210	190	197	179
Nerve ring c.b.d. (μm)	47	52	52	47	45	50	54	47
Pharynx length (μm)	327	350	352	343	390	386.5	405	379.5
Pharynx c.b.d. (μm)	52	60	54	52	59	60	70	55
Maximum body diameter (μm)	52	61	54	55	66	62	77	58
a.b.d. (μm)	34.6	37.3	36.0	33.4	34.2	34.5	39.0	33.0
Tail length (μm)	182	207	197	195	190	217	212	206
Spicule length along chord (μm)	45.8	42.0	42.6	42.7	-	-	-	-
Spicule length long arc (μm)	57.9	54.0	53.0	53.8	-	-	-	-
Gubernaculum length (μm)	6.5	4.7	7.2	8.0	-	-	-	-
Apophysis length (μm)	6.8	6.3	6	5.7	-	-	-	-
Supplements 1 distance from cloacal opening (μm)	21.5	22.4	22.0	21.0	-	-	-	-
Supplements 2 distance from cloacal opening (μm)	60.5	58.9	53.0	50.0	-	-	-	-
Supplements 3 distance from cloacal opening (μm)	106.2	104.3	103	95.6	-	-	-	-
Supplements 4 distance from cloacal opening (μm)	167.4	180.3	173	168	-	-	-	-
V (μm)	-	-	-	-	1048	899	909	814
Precloacal seta length (μm)	2.3	2.0	2.0	2.1	-	-	-	-
v.b.d. (μm)	-	-	-	-	62.0	54.5	67.6	54.5
V%	-	-	-	-	57.4	54.5	50.8	49.9
a	28.0	26.4	27.4	26.6	27.7	26.6	23.2	28.1
b	4.4	4.6	4.2	4.3	4.7	4.3	4.4	4.3
c	8.0	7.8	7.5	7.5	9.6	7.6	8.4	7.9
c′	5.3	5.5	5.5	5.8	5.6	6.3	5.4	6.2

3.1.1. Type Material

Holotype: one male (♂1: WZ2018120212107); paratypes: three males (♂2: WZ2018120211113, ♂3: ZZ20210509114, ♂4: ZZ20210509107) and four females

(♀1: WZ2018120212115, ♀2: ZZ20210509112, ♀3: ZZ20210509112, ♀4: ZZ20210509111). Among them, WZ2018120212107 and WZ2018120211113 were obtained from Ximen Island mangrove wetland in Wenzhou City of Zhejiang Province; while ZZ20210509114, ZZ20210509107, ZZ20210509112, ZZ20210509112, and ZZ20210509111 were from Jiulong Estuary mangrove wetland in Zhangzhou City of Fujian Province.

3.1.2. Type Locality and Habitat

Intertidal muddy sediment was collected from the mangrove wetlands of Ximen Island, (28.34° N, 121.17° E). Specimens were found in the surface layer of the sediment at a depth of 0–5 cm.

3.1.3. Etymology

The species was named after the mangrove habitat.

3.1.4. Measurements

The measurements of the new species are summarized in Table 3.

3.1.5. Morphological Description

The body is slender and cylindrical, tapering towards both ends, with a length ranging from 1454.0–1825.0 µm and a maximum body diameter ranging from 52.0–77.0 µm, approximately 4.9–7.7 times the width of the cephalic region, which is located around the level of posterior esophagus end. The cuticle is smooth without lateral differentiation. The head diameter is 9.2–10.6 µm and distinctly narrowed. The anterior part of the head is slightly separated through a constriction at the level of cephalic setae. There are six inner labial setae, each approximately 1.0–1.3 µm long. There are six outer labial setae and four cephalic setae that form a circle, with each seta measuring about 4.0–5.7 µm in length. Numerous cervical setae were scattered over the anterior region of the pharynx, reaching lengths of up to 5.0–6.0 µm. The buccal cavity has a thick wall, is large and wide, and is divided into two chambers by a cuticular ring: (i) a spacious anterior chamber, and (ii) a narrower and longer posterior chamber. The total length of the buccal cavity is 9.6–12.5 µm, with a width of 4.2–6.1 µm. There was a large right subventral tooth and two less prominent teeth (dorsal and left subventral). The amphideal fovea was pocket-like and located at the level of the posterior buccal chambers. Ocelli are absent. The pharynx measures 327.0–405.0 µm in length, gradually expanding posteriorly and modified into four pharyngeal bulbs with a corresponding diameter of 52.0–70.0 µm at the end of the pharynx. The nerve ring was located at 46.4–55.7% of the pharynx length, with a corresponding body diameter of 47.0–54.0 µm. The cardia was short and conoid. The tail was conico-cylindrical, tapering with the distal third being cylindrical. In males, the tail length was 5.3–5.8 times the anal body diameter, and in females, it was 5.4–6.3 times the anal body diameter. The tip of the tail was slightly swollen and had a terminal spinneret, without any terminal setae. The caudal glands were poorly visible.

Male: Diorchic, with the anterior testis on the right side of the intestine, while the posterior testis was located at the same position as the vas deferens. The spicules were equal in length, curved with rounded and blunt proximal ends, and tapered distally. They measured 53.0–57.9 µm as an arc (1.4–1.7 times the anal body diameter), and 42.0–45.8 µm as a chord (Table 3). The gubernaculum was small, measuring 4.7–8.0 µm in length, 1.4–1.7 times the anal body diameter. There was a precloacal seta located close to the cloaca, measuring 2.0–2.3 µm in length. Four precloacal supplements were weakly developed, appearing pore-like on the cuticle and resembling vacuoles internally. The size of the vacuoles increased as they were positioned further away from the anus. The arrangement of the supplements is as follows: The first supplement was located 21.0–22.4 µm from the cloacal opening, the second supplement 50.0–60.5 µm, the third supplement 95.6–106.2 µm, and the fourth, which was the most anterior, was positioned 167.4–180.3 µm from the cloacal opening.

Females: The general appearance was similar to that of the male. The ovaries were paired and reflexed, both located to the right of the intestine. The vulva was transversely positioned at 49.9–57.4% of the body length (Figures 1 and 2, Table 3).

3.2. Differential Diagnosis

Belbolla mangrove sp. nov. is characterized by a total body length of 1454.0–1825.0 µm. The cuticle is smooth, and the buccal cavity is divided into two chambers by a cuticular ring. The amphideal fovea is pocket-like, and there are four pharyngeal bulbs. The species possesses a small gubernaculum with a dorsocaudal apophysis and weakly developed precloacal supplements. The tip of the species is slightly swollen and has a terminal spinneret, without any terminal setae. Currently, *B. vietnamica* is the only known species in the *Belbolla* genus with four pharyngeal bulbs.

Belbolla mangrove sp. nov. differs from 19 valid species of the genus *Belbolla* in having four pharyngeal bulbs, the last one (the 20th) also having four bulbs, and it also differs from all other species in the shape of the precloacal supplements. The new species, *Belbolla mangrove* sp. nov., closely resembles *B. vietnamica* as they both possess four pharyngeal bulbs. However, the newly discovered species in this study differs from *B. vietnamica* in five aspects: (1) the presence or absence of ocelli (absence in the new species versus presence in *B. vietnamica*); (2) the shape of proximal spicule ends (blunt and round in the new species versus strongly bent in *B. vietnamica*), (3) the shorter length of the gubernacular dorsocaudal apophysis (6.0–6.8 µm in the new species versus 16.0–19.0 µm in *B. vietnamica*); (4) the development of the precloacal supplements (weakly developed in the new species versus well-developed in *B. vietnamica*); and (5) the presence or absence of terminal setae (without terminal setae in the new species versus with three terminal setae in *B. vietnamica*). The new species shared weakly developed precloacal supplements with *B. longispiculata*; however, *B. longispiculata* differs from the new species in its eight pharyngeal bulbs, long spicules, and gubernaculum.

3.3. DNA Sequences Results

A total of three sequences of the 18S rDNA gene were obtained from three male individuals of *B. mangrove* sp. nov. All sequences were deposited in GenBank. The detailed information of individual specimens includes collection date, site, habitat, longitude, latitude, GenBank number, and the length of the amplified fragment (Table 1). Intergeneric thresholds of K2P distance divergence are as follows: 1.6–8.3% for 18S rDNA.

Table 4. Comparison of the major morphological features of *Belbolla mangrove* sp. nov. discovered in this study and other species in the genus *Belbolla* reported in the literature ("-" not documented in the literature).

Taxa	Number of Pharynx Bulb	Supplements	Spicule Length(µm)	AmphidsFovea	Ocelli	*a* (µm)	*b* (µm)	*c* (µm)	References
B. asupplementata Juario, 1974	7	absent	38.9	pocket-like	not described	42	3.7	12.3	[15]
B. californica (Allgén, 1951) Andrássy, 1973	7–9	2 horseshoe-like	-	not described	not described	20.78	3.73	14.9	[16]
B. gallanachmorae (Inglis, 1961) Andrássy, 1973	8	2 winged	69	pocket-like	not described	34.9	4	12.9	[17]
B. gracilis Gagarin & Thanh, 2016	7	2 complex structure	50	pocket-like	not described	31	9	6.7	[18]
B. heptabulba (Timm, 1961) Andrássy, 1973	7	2 winged	30–34	not described	present	25–29	3.4–3.7	11.8–14.7	[19]
B. hoonsooi Rho, Lee, Lee, & Choi, 2021	8	2 winged	127	not described	absent	30	5	13	[20]
B. huanghaiensis Huang & Zhang, 2005	9	2 winged	137	not described	not described	38.5	4.5	14.8	[21]
B. insula Belogurov, Fadeeva, & Belogurova, 1983	10	2 winged	182	pocket-like	-	19.8	3.5	13.4	[22]

Table 4. *Cont.*

Taxa	Number of Pharynx Bulb	Supplements	Spicule Length(μm)	AmphidsFovea	Ocelli	*a* (μm)	*b* (μm)	*c* (μm)	References
B. intarma Belogurov & Belogurova, 1980	9	2	144	-	-	-	-	-	[23]
B. koreensis Rho, Lee, Lee, & Choi, 2021	8	2 winged	119	not described	absent	34.0	5.0	12.0	[20]
B. longispiculata Nasira, Shahina, & Shamim, 2014	8	5 papillae	216	not described	not described	32.97	4.7	12.9	[24]
B. *Sinica* wang, Guo & Wang, 2022	7	2 wined	36	not observed	absent	36.0	3.9	13.9	[5]
B. stenocephalum Huang & Zhang, 2005	8	2 winged	98	not described	not described	36.3	3.7	12.3	[21]
B. sundaensis (Micoletzky, 1930) Andrássy, 1973	8	2	-	crescent	present	28	4.16	13.6	[25]
B. teissieri (Luc & De Coninck, 1959) Andrássy, 1973	8	3 papillae	39	oval	not described	36.5	3.7	11.7	[26]
B. tenuidens(Cobb, 1920) Andrassy, 1973	8	2 winged	-	-	not described	-	-	-	[27]
B. vietnamica Gagarin & Nguyen Dinh Tu, 2016	4	4 papillae	55	not described	present	22	3.8	7.5	[28]
B. warwicki Huang & Zhang, 2005	7	2 long pocket-shaped	37	not described	not described	40.8	3.4	12.7	[21]
B. wonkimi Rho, Lee, Lee, & Min, 2020	9	2 winged	-	oval	absence	-	4	11	[6]
B. zhangi Guo & Warwick, 2001	8–9	2 winged	56	not obvious	not described	34.7	4.6	13.5	[7]
B. mangrove sp. nov.	4	4	57.9	pocket-like	absent	28	4.4	8	This study

4. Discussion

So far, the genus *Belbolla* has been found in various localities of intertidal and shallow subtidal habitats worldwide, including the Atlantic Ocean, Indian Ocean, and Pacific Ocean [6]. The genus *Belbolla* is characterized by multiple pharyngeal bulbs, which serve as an important feature for classification [23]. *Belbolla* has been subdivided into three groups based on the number of pharyngeal bulbs. The first group is characterized by having seven or eight pharyngeal bulbs in males, and it comprises fifteen species. The second group is characterized by having nine or ten pharyngeal bulbs, and it comprises five species. The third group is characterized by having four pharyngeal bulbs in males, and only includes the species *B. vietnamica*. Huang et al. [26] provided a binary key for identifying the 12 species of the genus *Belbolla* based on the number of pharyngeal bulbs, number, shape, and size of precloacal supplements, the length and shape of the spicules, and the presence or absence of the gubernaculum and apophysis. Interestingly, the number of pharyngeal bulbs can be a variable feature within the same species. For *B. zhangi*, the most anterior bulb sometimes appears unclear, while for *B. californica*, the number of pharynx bulbs ranges from seven to nine [7,21]. Additionally, the presence or absence of ocelli was also an important feature within this genus. Among the total of 21 species (including the species newly described here), *B. heptabulba*, *B. vietnamica*, and *B. sundaensis* were reported to have ocelli [24,27,28]. One species was explicitly described as lacking ocelli, while no information regarding this feature was available for the remaining species. The major morphological features of the 21 species of the genus *Belbolla* were summarized in Table 4.

Whether the number of pharyngeal bulbs, the shape of the supplements, or the shape of the spicules and gubernaculum constitutes an important feature for distinguishing different species within the genus *Belbolla* requires further evidence. Marine nematodes are morphologically diverse, and traditional identification methods are morphologically based and therefore limited, while the development of DNA barcoding technology has provided new technical support for species identification. Currently, molecular markers such as the nuclear small subunit ribosomal RNA (18S rDNA), large subunit ribosomal RNA (28S rRNA), and the mitochondrial cytochrome oxidase subunit I (COI) are commonly used for taxonomic identification of marine nematodes [29]. Taxonomic identification of nematodes using various DNA fragments has been a common approach in the past

decades [30]. Previous studies have indicated that 18S rDNA serves as a suitable molecular marker for nematode identification [31–34]. However, no species of the genus *Belbolla* has ever been sequenced. The GenBank database was searched, and a total of 27 sequences belonging to 18S rDNA were found in the family Enchelidiidae, which is the taxonomic family level of the genus *Belbolla*. However, most of the sequences have not been assigned exact species names, indicating that the molecular sequence data in the database have significant limitations in providing taxonomic evidence.

Therefore, in this study to better complement and enhance the database, we first provided 18S rDNA sequences of *B. mangrove* sp. nov. and compared them with the genera *Bathyeurystomina*, *Calyptronema*, *Eurystomina*, *Pareurystomina*, and *Polygastrophora*. Subsequently, other genera were analyzed, and a taxonomic phylogenetic tree was constructed using the sequence data (Figure 3). The results show that *B. mangrove* sp. nov. is a separate species and exhibits good differentiation from species of other genera. In addition, due to no other species of the genus *Belbolla* being sequenced, the reference sequences were limited, and therefore we established intergeneric thresholds of K2P distance divergence as follows: 1.6–8.3% for 18S rDNA.

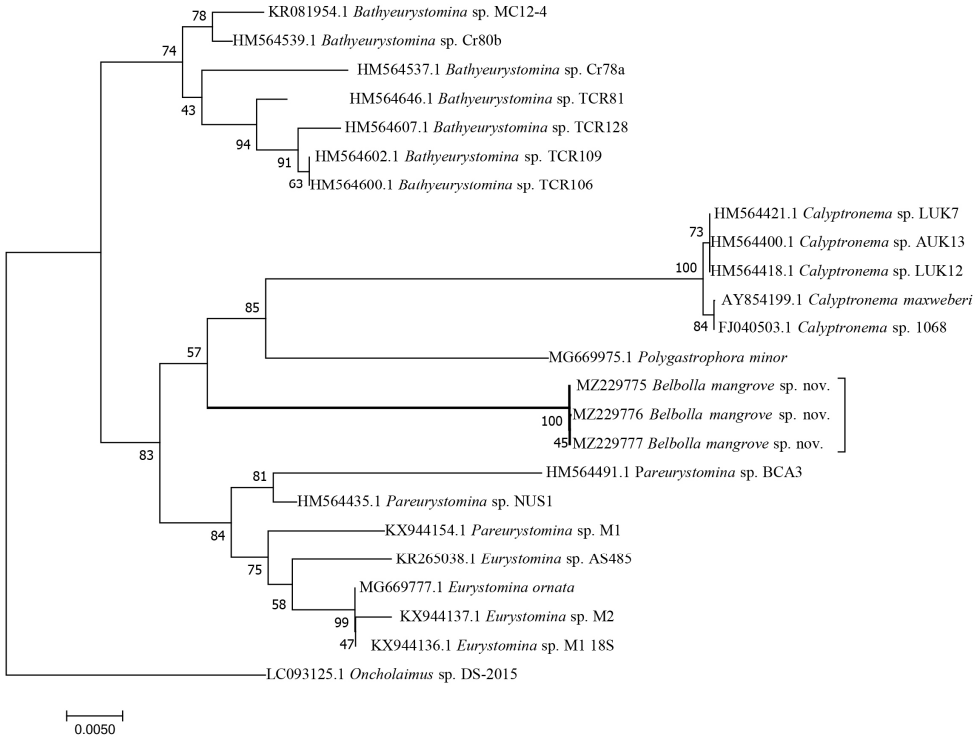

Figure 3. Neighbor-joining tree based on a 643 bp region of the 18S rDNA gene.

5. Conclusions

A new species of the genus *Belbolla* has been discovered from surface muddy intertidal sediments in two mangrove wetlands of China. This newly discovered species was named *B. mangrove* sp. nov. in recognition of its association with the mangrove habitat, marking the first report of the genus *Belbolla* in Chinese mangroves. The taxonomic and distinct morphological features of this species were described and its DNA sequence was obtained to provide fundamental data for molecular identification. The main morphological characteristics of all species within the genus were also summarized to facilitate species identification. These findings contribute to our understanding of the biodiversity of free-living nematodes in mangrove sediments, providing a foundation for further biodiversity research, which is crucial for the conservation of coastal wetlands.

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