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Citation	Parasitology Research, 120(6), 2357-2362 https://doi.org/10.1007/s00436-021-07213-9
Issue Date	2021-06
Doc URL	https://hdl.handle.net/2115/85646
Rights	The final publication is available at link.springer.com
Type	journal article
File Information	Parasitol Res. 120(6) 2021.pdf

First report of marine horsehair worms (Nematomorpha: *Nectonema*) parasitic in isopod crustaceans

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Abstract

Nectonema, the only horsehair worm (Nematomorpha) genus found in marine environments, was previously known to be parasitic only in decapod crustaceans. We report *Nectonema* sp. as the first record of a marine nematomorph parasitic in isopod crustaceans. This is also the third record of marine nematomorphs from the North Pacific. Six infected isopods (*Natatolana japonensis*) collected from 1425 m depth in the Sea of Japan each contained one to seven (mean 2.33) nematomorphs in the body cavity in the pereon. There was no correlation between the host body length and number of parasites. For *Nectonema* sp., we describe and illustrate morphological features of the parasitic juvenile stage and present nucleotide sequences for the cytochrome *c* oxidase subunit I gene (COI or cox1; 451 nt), 18S rRNA gene (1777 nt), and region spanning the internal transcribed spacer 1 (ITS1) and the 28S rRNA gene including the 5.8S rRNA gene and ITS2 (1218 nt in total). In an 18S maximum-likelihood tree that included 24 nematomorph species, *Nectonema* sp. grouped with *N. agile* from the northwestern Atlantic; the 18S gene from these two taxa was divergent by 11.8% K2P distance, suggesting they are different species. *Nectonema* species may have a broader range of host groups than previously suspected, but may have been previously misidentified as nematode parasites.

Keywords Cymothoida · Deep sea · Endoparasite · Hairworm · Nectonematida · Peracarida

Introduction

Nectonema is the only nematomorph genus found in marine environments. There are five species described in this genus to date: *Nectonema agile* Verrill, 1879 (type species) from Atlantic Ocean and Mediterranean and Black Seas; *Nectonema melanocephalum* Nierstrasz, 1907 from Indonesia; *Nectonema munidae* Brinkmann, 1930 from Bergen and Norway; *Nectonema svensksundi* Bock, 1913 from Spitzbergen, Norway; and *Nectonema zealandica* Poinar and Brockerhoff, 2001 from New Zealand (Schmidt-Rhaesa et al. 2013). The occurrence records of this group in the North Pacific have been limited, with only two reports from Japan: Oku et al. (1983) and Yoshida (2016) reported unidentified *Nectonema* individuals from the brachyuran *Erimacrus isenbeckii* (Brandt, 1848) and the anomuran *Pagurus brachiomastus* (Thallwitz, 1891), respectively.

The life cycle of nematomorphs contains a larval stage, a parasitic juvenile stage, and a free-living adult stage (Hanelt and Janovy 2004). In *Nectonema*, juveniles have so far been reported only from crustaceans in the order Decapoda (shrimps, hermit crabs, crabs, etc.), among which more than 27 species have been reported as hosts (Schmidt-Rhaesa et al. 2013). In a single host species, *Nectonema* individuals at various developmental stages can be found (Huus 1932; Schmidt-Rhaesa 1996).

Here we report the first record of *Nectonema* (as *Nectonema* sp.) parasitic in a species in the order Isopoda, a morphologically diverse crustacean group with more than 10,000 described species. This is also the third record of marine nematomorphs from the North Pacific. We describe the morphology of this nematomorph and present nucleotide sequences for its cytochrome *c* oxidase subunit I (COI) gene, 18S rRNA (18S) gene, and ITS cluster, including the 3´ region of internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene, ITS2, and the 5´ region of the 28S rRNA gene.

Materials and methods

Isopods were collected from plastic jars that contained cut sardine bait and had been placed in baited traps (Saito et al. 2014: fig. 1J) during a cruise of R/V *Soyo-maru* (National Research Institute of Fisheries Science, Japan); the traps were recovered on 16 July 2014 at station Kago-4 (40°00.59′ N 135°57.63′ E), 1425 m depth, Sea of Japan. The isopods were picked from the jars by Ken Fujimoto and kept alive at 4°C until 30 July. Infected individuals were anesthetized with 35‰ $MgCl_2$ and dissected to extract nematomorphs. One infected isopod and one extracted nematomorph were photographed live. Isopods were fixed and preserved in ethanol; nematomorphs removed from them were fixed in DESS solution (Yoder et al. 2006) or 99% ethanol.

Body length (BL) was measured from the anterior edge of the cephalothorax to the tip of the pleotelson for the isopods, and from the anterior to posterior tip of the body for the nematomorphs; the cephalothorax width (CW) of the isopods was measured at the widest portion of the cephalothorax.

Isopods were dissected with needles under a Nikon SMZ1500 stereomicroscope; detached appendages were mounted on glass slides in glycerin and observed with a Nikon E600 microscope. Two nematomorphs in DESS solution were transferred into a 1:3:6 mixture of glycerin, absolute ethanol, and deionized water and placed in a thermostatic chamber at 40°C for two days, after which they were mounted on glass slides in glycerin and observed with an Olympus BX51 microscope. Illustrations of nematomorphs were prepared with Inkscape 1.0 from digital micrograph images. Morphological terminology for *Nectonema* here follows Poinar and Brockerhoff (2001).

Total DNA was extracted from part of the body from each of four nematomorphs by using a NucleoSpin Tissue XS Kit (TaKaRa Bio, Japan). Primers used for PCR and sequencing are listed in Table 1. PCR amplification conditions for the ITS cluster and COI with TaKaRa Ex Taq DNA polymerase (TaKaRa Bio) were 94°C for 1 min; 35 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 1 min; and 72°C for 2 min. Conditions for 18S amplification with KOD FX Neo (Toyobo, Japan) were 94°C for 1 min; 45 cycles of 98°C for 10 s, 65°C for 30 s, and 68°C for 75 s; and 68°C for 3 min. PCR products for 18S were separated on a 2% agarose gel, excised with a micro spatula, and purified with a MagExtractor PCR & Gel Clean Up Kit (Toyobo). All nucleotide sequences were determined by direct sequencing with a BigDye Terminator Kit ver. 3.1 and a 3730 DNA Analyzer (Life Technologies, USA). Fragments were concatenated by using MEGA7 (Kumar et al. 2016).

The 18S dataset for a phylogenetic analysis included our 18S sequence from *Nectonema* sp. and 25 sequences from 23 nematomorph species and two outgroup taxa (a nematode and a tardigrade) taken from public databases (DDBJ 2021; Online Resource 1). Methods for alignment of all 18S sequences (1806 positions in the aligned dataset; Online Resource 2) and selection of the optimal substitution model (GTR + I + G) were as described by Homma et al. (2020). The Kimura (1980) 2-parameter (K2P) distance between the aligned *Nectonema* sequences was calculated with MEGA7. A maximum likelihood (ML) analysis was conducted in RAxML-NG (Kozlov et al. 2019), with nodal support values obtained by analysis of 1000 bootstrap pseudoreplicates. The ML tree was drawn by using FigTree v1.4.4 (Rambaut 2021).

Our nematomorph specimens were deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo (ICHUM-6178–6191); host isopods were deposited in the Seto Marine Biological Laboratory (SMBL-V0598–0603). The sequences we determined were deposited in the International Nucleotide Sequence Database through the DNA Data Bank of Japan, under the accession numbers LC605980–605988.

Results and discussion

The six infected isopods we observed (three males, BL 14.7–15.8 mm; three females, BL 13.5–16.9 mm) were identified as *Natatolana japonensis* (Richardson, 1904) in the suborder Cymothoida (Fig. 1). All infected individuals harbored nematomorphs internally in the cavity of the pereon. The nematomorph infections ranged from one to seven individuals per host (mean intensity = 2.33) (Table 2). The prevalence of infection is unknown because we lack data on the total number of isopods in the baited traps. No clear correlation was detected between host size (BL) and the number of parasites (N) ($N = 1.51 \times BL - 20.6$; $R^2 = 0.543$).

The nematomorphs ranged in BL from 23.7 mm to 94.0 mm (mean, 58.3 mm; N = 12 intact individuals; Online Resource 3). An abbreviated description of individual ICHUM-6181 (Fig. 2) is as follows. Anterior and posterior ends rounded. Cuticle colorless. Epidermis with single cell layer. Cuticular natatory bristles not observed. Cephalic papillae absent. Septum distinct. Anterior chamber translucent, with ca. 10 giant cells. Anterior region not pigmented. Mouth opening at anterior apex. Oral cavity with eight (?) bifurcated hooks protruding from cuticular wall. Sclerotized proboscis with two anterior scissor-shaped spines. Pharynx sclerotized, connecting with intestine posterior to septum. Body filled with mesenchyme between septum and subposterior region. Gonads not observed. Subposterior region with posterior opening (gonopore?) surrounded by elongate epidermal cells.

COI and ITS-cluster sequences were determined from four nematomorphs (ICHUM-6182–6184, 6188). The four COI sequences (accession numbers LC605980–605983; 451 nt long, translating to 150 amino acids) were identical, as were the four ITS-cluster sequences (accession numbers LC605984–605987; 1218 nt long). No *Nectonema* COI and ITS-cluster sequences have previously been deposited in public databases (DDBJ 2021). The 18S sequences (accession numbers LC605988, 605989) determined from two nematomorphs (ICHUM-6184, 6188; 1777 nt long) were identical, and were 11.8% divergent in K2P distance from the only *Nectonema* 18S sequence available in public databases (*N. agile*; Bleidorn et al. 2002). In the ML tree for 18S (Fig. 3), *Nectonema* sp. and *N. agile* formed a clade with 100% bootstrap support, which was the sister group to a Gordiida clade having 87% bootstrap support; the relationships within Gordiida differed from those in previous studies (e.g., Bleidorn et al. 2002; Tobias et al. 2017), which may have resulted from the difference in the dataset used.

Morphology-based species identification is difficult in *Nectonema*, and Schmidt-Rhaesa (2005) noted that the five known species may have been described based on specimens at different developmental stages, making the descriptions not directly comparable. It should be noted that our *Nectonema* samples differ from juveniles of three species (*N. agile*, *N. munidae*, and *N. zealandica*; no juveniles have been reported in the other two species) in having ca. 10 giant cells and lacking cephalic papillae. Molecular identification is currently unavailable for this group because no molecular markers have been determined for all named *Nectonema* species. Identification of our material to species was not possible, as no adult specimens were obtained.

On the basis of the 11.8% K2P divergence, *Nectonema* sp. is likely not conspecific with *N. agile*. Furthermore, its occurrence in an isopod indicates that *Nectonema* may use other, undetected groups besides Decopoda for hosts. In the parasitic stage, nematomorphs resemble nematodes, and infections of unusual hosts in the past might have been misidentified as nematode infections. To understand true diversity of marine nematomorphs, host surveys targeting non-decapods as well as decapods and integrative taxonomic approaches will be necessary.

Acknowledgments

We thank Ken Fujimoto, Shiro Sawadaishi, and the crew of R/V *Soyo-maru* for collecting specimens and providing facilities; Matthew H. Dick for reviewing the manuscript and editing our English; and two anonymous reviewers for improving this manuscript with their comments.

Funding

This research did not receive any specific grant support from public, commercial, or non-profit funding agencies.

Competing interests

We declare no competing interests.

Author contributions

KK conceived and designed the study, collected samples, and conducted the molecular analysis. JF identified the host isopods. KK and DS made morphological observations of the nematomorphs. DS made drawings. KK wrote the first draft of the manuscript, and all authors commented on the first draft and read and approved the final draft.

Supplementary information

This work includes the following online supplementary information.

Online Resource 1. Species included in the phylogenetic analysis based on 18S rRNA sequences.

Online Resource 2. Aligned sequences used for phylogeny reconstruction.

Online Resource 3. Details of the animals studied.

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Fig. 1 *Nectonema* sp. parasitic on *Natatolana japonensis*. **a**, **b** *N. japonensis* containing *Nectonema* sp., dorsal and ventral views, fresh specimen (SMBL-V0598). **c** *Nectonema* sp., fresh specimen (ICHUM-6178)

Fig. 2 *Nectonema* sp. (ICHUM-6181). Anterior (**a**, **b**, **e**, **f**) and posterior (**c**, **d**) portions and sclerotized proboscis with two anterior scissor-shaped spines (**b'**) of *Nectonema* sp., in glycerin (ICHUM-6181), as microphotographs (**a**, **c**, **e**, **f**) and line drawings (**b**, **b'**, **d**). Arrowheads in **e** indicate giant cells. Abbreviations: ac, anterior chamber; bh, bifurcated hook; cu, cuticle; du, duct (gonoduct?); ep, epidermis; gc, giant cell; in, intestine; me, mesenchyme; mo, mouth opening; mu, muscle; ph, pharynx; po, posterior opening (gonopore ?); pr, proboscis; se, septum; sp, scissor-shaped spine

Fig. 3 ML tree for 18S sequences (1806 positions) from nematomorphs, including *Nectonema* sp. from isopods. Values near nodes are bootstrap values ≥ 80%; black circles indicate 100% bootstrap support. Outgroup taxa (the tardigrade *Milnesium tardigradum* and the nematode *Deontostoma magnificum*) are not shown

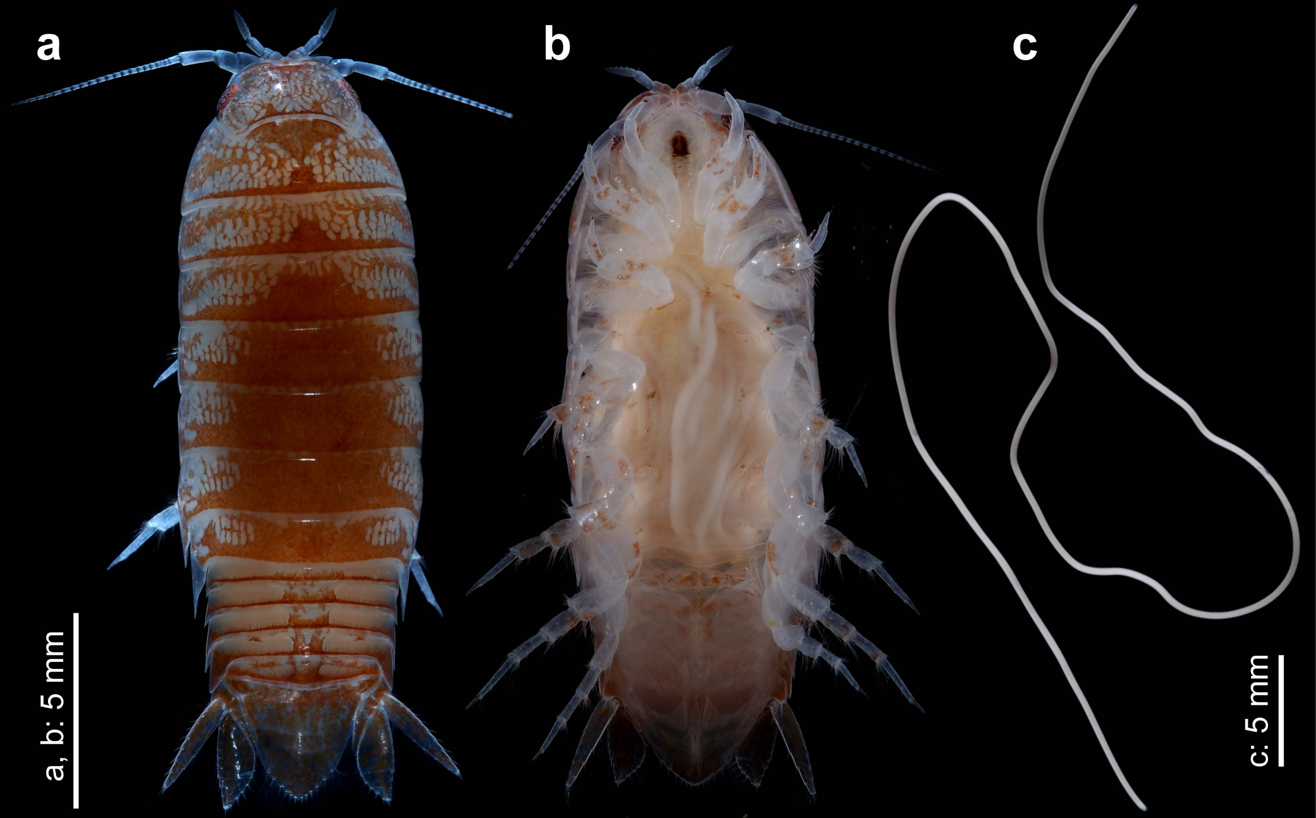
Table 1. List of PCR and cycle sequencing (CS) primers used in this study.

Gene	Primer	Sequence	Reaction	Source
COI	F1	CCTACTATGATTGGTGGTTTTGGTAATTG	PCR	Kanzaki and Futai (2002)
	R2	GTAGCAGCAGTAAAATAAGCACG	PCR, CS	Kanzaki and Futai (2002)
18S	SR1	TACCTGGTTGATCCTGCCAG	PCR	Nakayama et al. (1996)
	SR12	CCTTCCGCAGGTTCACCTAC	PCR	Nakayama et al. (1996)
	18S-b3F	CCTGAGAAACGGCTACCACAT	CS	Kakui and Shimada (2017)
	18S-b4F	TGCGGTTAAAAAGCTCGTAGTTG	CS	Kakui et al. (2011)
	18S-b4R	TCCAACTACGAGCTTTTTAACC	CS	Kakui et al. (2011)
	18S-b5F	GATCGAAGGCGATYAGATACC	CS	This study
	18S-b6F	CCTGCGGCTTAATTTGACTC	CS	Kakui et al. (2011)
	18S-a6R	AACGGCCATGCACCAC	CS	Kakui et al. (2011)
	18S-b8F	GGTCTGTGATGCCCTTAGATG	CS	Kakui et al. (2011)
ITS cluster	NC5	GTAGGTGAACCTGCGGAAGGATCATT	PCR, CS	Zhu et al. (1998)
	NC2	TTAGTTTCTTTTCCTCCGCT	PCR, CS	Zhu et al. (1998)
	NC13	ATCGATGAAGAACGCAGC	CS	Zhu et al. (1998)

Table 2. Size of isopod host (*Natatolana japonensis*) and nematomorph (*Nectonema* sp.) individuals. All measurements are in millimeters. BL, body length; CW, cephalothorax width. SMBL, Seto Marine Biological Laboratory.

Isopods				Nematomorphs	
SMBL-	Sex	BL	CW	No. of parasites	BL
V0598	Female	16.9	3.8	7	26.9–94.0 (mean 58.1; n = 6[*])
V0599	Male	14.7	3.4	2	40.2–84.9 (mean 62.6; n = 2)
V0600	Male	15.8	3.8	1	87.8
V0601	Female	14.9	3.3	2	23.7–68.6 (mean 45.2; n = 2)
V0602	Female	13.5	3.2	1	46.0
V0603	Male	15.1	3.6	1	42.1[**]

[*] One damaged specimen (length 50.1 mm; part of body missing) excluded. [**] Data from damaged specimen lacking part of body.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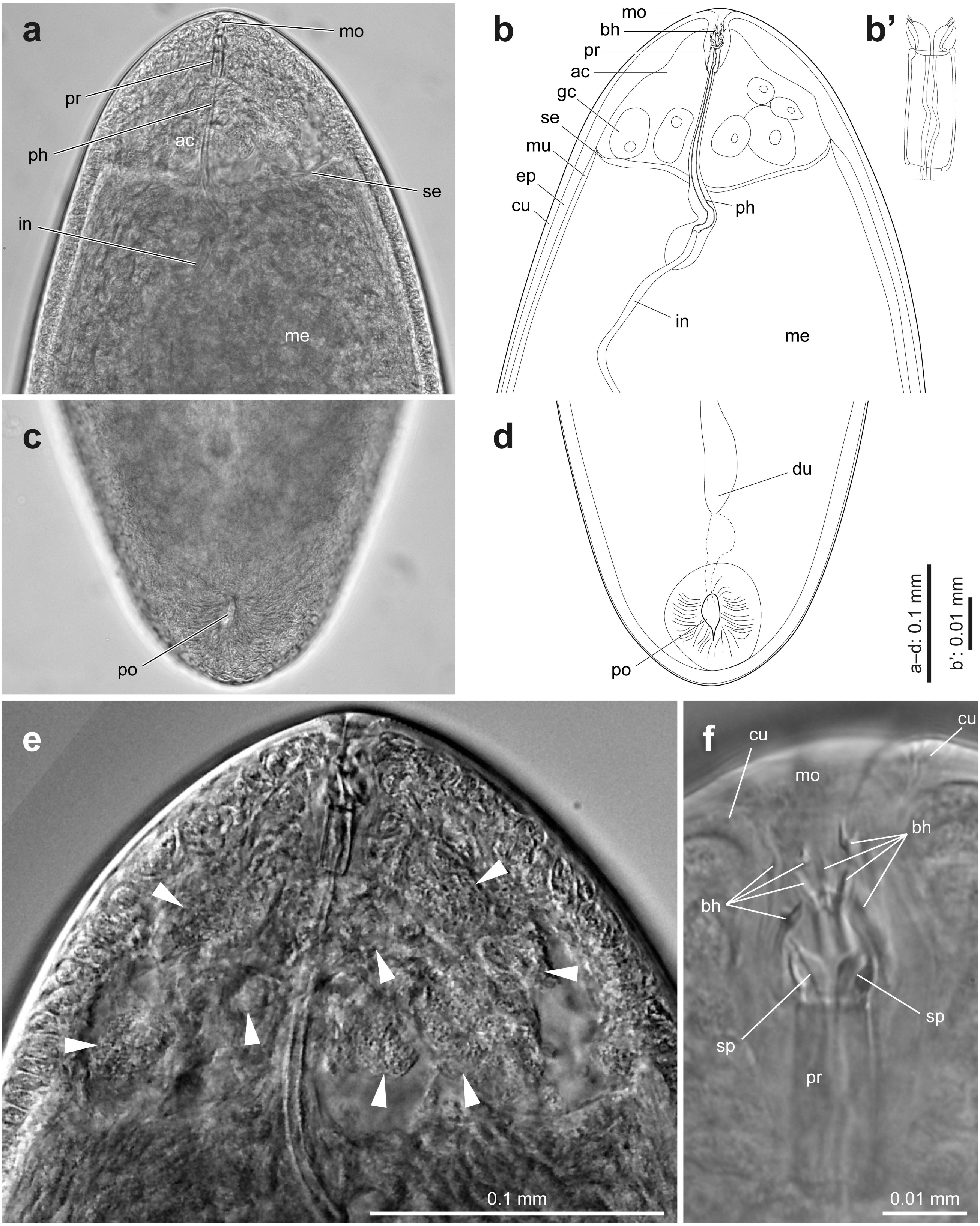
c
a, b: 5 mm
c: 5 mm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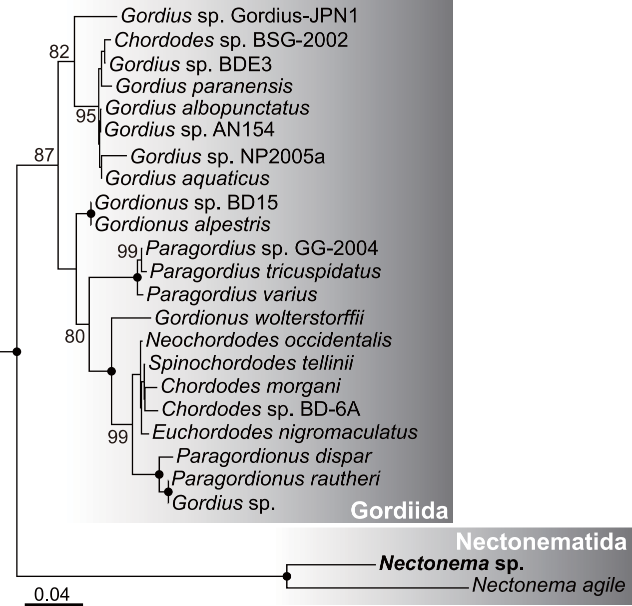
Gordius sp. Gordius-JPN1
Chordodes sp. BSG-2002
Gordius sp. BDE3
Gordius paranensis
Gordius albopunctatus
Gordius sp. AN154
Gordius sp. NP2005a
Gordius aquaticus
Gordionus sp. BD15
Gordionus alpestris
Paragordius sp. GG-2004
Paragordius tricuspidatus
Paragordius varius
Gordionus wolterstorffii
Neochordodes occidentalis
Spinochordodes tellinii
Chordodes morgani
Chordodes sp. BD-6A
Euchordodes nigromaculatus
Paragordionus dispar
Paragordionus rautheri
Gordius sp.
82
95
87
99
80
99
Gordiida
Nectonematida
Nectonema sp.
Nectonema agile
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