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Heterocheilus floridensis sp. n. (Nematoda: Heterocheilidae) from the West Indian manatee *Trichechus manatus* (Trichechidae, Sirenia) in Florida, USA

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Abstract: Morphological data are used to describe a new nematode species, *Heterocheilus floridensis* sp. n. (Heterocheilidae) from the digestive tract of the Florida manatee *Trichechus manatus latirostris* (Harlan) (Trichechidae, Sirenia) from Florida, USA. Examination by light and scanning electron microscopy revealed that the new species differs from the related *Heterocheilus tunicatus* Diesing, 1839 mainly by having dentigerous ridges on the inner surface of the lips, a median unpaired papilla located anterior to the cloaca, and a considerably larger body size. Sequence data for subunits I and II of mitochondrial cytochrome oxidase gene, 18S small subunit and 28S ribosomal RNA genes were provided for molecular characterisation of the new species. However, the current unavailability of homologous sequence data for congeneric specimens precluded a molecular assessment of the morphological species hypothesis, and ascaridoid phylogenetic hypotheses could not be advanced. Specimens of *Heterocheilus* sp. collected from the Antillean manatee *Trichechus manatus manatus* Linnaeus in Puerto Rico, on loan from the US National Museum of Natural History, were morphologically consistent with the new species, so apparently all congeneric nematodes reported from both subspecies of the West Indian manatee *Trichechus manatus* Linnaeus and previously identified as *H. tunicatus* belong rather to *H. floridensis* sp. n. *Heterocheilus hagenbecki* (Khalil et Vogelsang, 1932) Sprent 1980 is here considered to be a *species inquirenda*. A key to valid species of *Heterocheilus* Diesing, 1839 is provided.

Keywords: Parasitic nematode, Ascaridoidea, taxonomy, aquatic mammal, marine mammal, North America

The nematode genus *Heterocheilus* Diesing, 1839 (Heterocheilidae, Ascaridoidea) includes gut parasites that are specific to manatees (*Trichechus* spp.) (Trichechidae, Sirenia) (Sprent 1980, 1983). These hosts are large-bodied (maximum length 3.0–3.5 m, maximum weight 450–1620 kg, depending on species), aquatic, herbivorous mammals with essentially an allopatric distribution of species in tropical and subtropical waters of the Eastern or Western Atlantic or the Amazon River basin (Marsh et al. 2011). The only other extant sirenian, the Indo-Pacific dugong *Dugong dugon* (Müller) (Dugongidae), also hosts a gut-infecting ascaridoid, *Paradujardinia halicoris* (Owen, 1833) which has some morphological affinity to *Heterocheilus* spp. (Sprent 1980).

The three valid species of manatee belong to the genus *Trichechus* Linnaeus and are reported to host two different species of *Heterocheilus*. The African manatee *Trichechus senegalensis* Link, distributed along the western coast of Africa, is host to *H. domningi* Sprent, 1983. Conversely, type species, *Heterocheilus tunicatus* Diesing, 1839, is reported to infect both its type host, the Amazonian manatee *Trichechus inunguis* (Natterer in von Pelzeln, 1883), and

the West Indian manatee *Trichechus manatus* Linnaeus. *Trichechus inunguis* is distributed solely in the Amazon River basin of South America, including a very limited area of sympatry with the euryhaline *T. manatus*.

The latter species is distributed in shallow marine waters of the Caribbean and Atlantic coasts of the Americas from Brazil to the southeastern United States where it ranges up rivers well inland (Marsh et al. 2011). *Trichechus manatus* is further represented by two subspecies, the Antillean manatee *T. manatus manatus* Linnaeus, which is distributed sparsely throughout the species' range except for the southeastern United States, where it is replaced by the Florida manatee *Trichechus manatus latirostris* (Harlan). Infrequently, specimens of *T. m. latirostris* have been documented well north of the subtropics during the summer. However, migration to Florida's thermal refugia is necessary to survive cold winter temperatures (Marsh et al. 2011). Both subspecies of *T. manatus* have been reported to host *H. tunicatus* (Sprent 1980, 1983).

All sirenians are listed as vulnerable to extinction by the IUCN and among their symbionts are endemic, host-specific parasites, each representing a unit of biological di-

versity that is existentially imperiled to at least the same degree as its host. In Florida specifically, *T. m. latirostris* is threatened by watercraft collisions, loss of warm water habitat and Florida Red Tide (blooms of toxin producing dinoflagellate *Karenia brevis* [Davis]) (Runge et al. 2017), and it is protected by both state and federal law (Florida Manatee Sanctuary Act, Endangered Species Act, and US Marine Mammal Protection Act). Considerable public and private resources are applied to its study and conservation, including robust law enforcement, continuous population monitoring, habitat restoration, maintenance of manatee refuges and sanctuaries, routine recovery of carcasses for study, and rescue (i.e., physical and veterinary intervention to protect individual animals in distress, sometimes including capture, rehabilitation, and release).

Data on manatee symbiont diversity, life history, and host interactions are directly relevant to host conservation and potentially useful toward that end. One manatee parasite species is implicated as a pathogen (Weisbrod et al. 2021), while others may prove to be commensal or even beneficial to host health. Some might have value as biological markers or provide unrecognised ecosystem services (see Marcogliese 2005, Johnson et al. 2013, Wood and Johnson 2015). Ultimately, all wildlife parasites are irreplaceable components of the global natural heritage and are inherently worthy of scientific attention (see Gómez and Nichols 2013, Carlson et al. 2020), and knowing which existentially threatened species exist is important.

Diesing (1839) erected *Heterocheilus* to accommodate *H. tunicatus*, a species he described from specimens collected by Johann Natterer in the Amazonian manatee *T. inunguis* in Brazil. In this description, Diesing (1839) omitted or misinterpreted some morphological features. Most consequentially, he stated erroneously that the nematode's vulva was in the tail region. Drasche (1884) perpetuated the error by not correcting it when he re-examined and redescribed *H. tunicatus* from Diesing's (1839) type material.

Later, Khalil and Vogelsang (1932) described *Typhlophorus hagenbecki* Khalil et Vogelsang, 1932 based on nematodes collected from a Caribbean specimen of *Trichechus manatus* that had died in the Hamburg Zoo. Evidently, unaware of *H. tunicatus*, they discerned similarities between their specimens and the type species of *Typhlophorus* [correctly *Typhlophoros*] Linstow, 1906, *T. lamellaris* Linstow, 1906 from the gharial *Gavialis gangeticus* (Gmelin). Their description placed the vulva of *T. hagenbecki* anterior to the middle of the body and indicated that a gubernaculum was present. These characteristics were described incorrectly, or not at all, in diagnoses of *Heterocheilus* and *Typhlophoros*, which later prompted the erection of a third genus, *Plicatolabia* Mozgovoy in Skryabin, Shikhobalova et Mozgovoy, 1951, into which *T. hagenbecki* was transferred as the type species, *Plicatolabia hagenbecki* (Khalil et Vogelsang, 1932) Mozgovoy in Skryabin, Shikhobalova et Mozgovoy, 1951 (see Mozgovoy 1953).

However, Sprent's (1980) re-examination of the type specimens of *H. tunicatus* from the collection of the Vienna Natural Museum showed that the vulva of this spe-

cies is in fact located anterior to the middle of the body. Consequently, Sprent (1980) designated *Plicatolabia* as a junior synonym of *Heterocheilus*, to which he transferred its type species as *H. hagenbecki*. Based on light and scanning electron microscopical studies of the type specimens, Sprent (1980) provided a detailed redescription of *H. tunicatus* and considered *H. hagenbecki* its junior synonym.

Sprent (1980) also made brief mention of specimens of *H. tunicatus* collected from *T. m. latirostris*, and later (Sprent 1983) addressed these and some additional specimens from *T. m. manatus* in more detail, while also describing another, still valid, species of *Heterocheilus*, *H. domningi* from the African manatee *T. senegalensis* in Senegal (Sprent 1983). Thereby, Sprent (1983) authoritatively established that, while the euryhaline sister species *T. manatus* and *T. senegalensis* harboured distinct species of *Heterocheilus*, a single species, *H. tunicatus* infected both *T. manatus* and its type host the exclusively freshwater species *T. inunguis*. His description of *H. domningi* (see Sprent 1983) was as thorough and detailed as his redescription of *H. tunicatus* (Sprent 1980), and both studies employed SEM.

However, new observations suggest that Sprent (1983) may have overlooked some important morphological differences in specimens of *Heterocheilus* sp. collected from *T. manatus*. This apparent lapse has theretofore gone unchecked. Subsequent authors (e.g., Beck and Forrester 1988, Mignucci-Giannoni et al. 1999a, b, Bando et al. 2014, Wyrosdick et al. 2018, Lucot et al. 2020, Delgado-Estrella et al. 2022) have also identified the nematodes from *T. manatus* as *H. tunicatus* without reference to SEM observations. The present, unpremeditated study was initiated in response to serendipitous SEM observation of specimens of *Heterocheilus* sp. collected from *T. m. latirostris*. The specimens substantially differ morphologically from *H. tunicatus*, and they are here considered representatives of a new, third species of *Heterocheilus* from manatees. This species is morphologically described and molecularly characterised below. Each extant sirenian species is now reported to harbour a unique, host-specific, gut-infecting ascaridoid.

MATERIAL AND METHODS

Opportunely available nematodes were collected from two female specimens of the Florida manatee *Trichechus manatus latirostris* that had died in the wild. These hosts were recovered, transported to the Marine Mammal Pathobiology Laboratory (MMPL) of the Florida Fish and Wildlife Conservation Commission (FWC) in St. Petersburg, refrigerated overnight (4° C), and then necropsied (methods of Bonde et al. 1983) the following respective day as part of the FWC's routine manatee mortality monitoring and research activities. The first (196 cm in length, Field ID: MEC10239) was recovered, in badly decomposed condition (as defined by Bonde et al. 1983), from Merritt Island, Brevard County, along the Atlantic coast of Florida, USA on 28 December 2010. It was determined to have died naturally from cold stress.

During necropsy, unmoving (apparently dead) nematodes were observed along with digeneans in the lumen of the stomach and duodenum. These were removed from gut contents, fixed in 10%

Fig. 1. Portion of the excised gut of a Florida manatee with a longitudinal incision exposing numerous specimens of *Heterocheilus floridensis* sp. n. in the lumen. Note, the image foreground has been digitally altered to visually declutter the figure and emphasise the subject. The unmodified image is available upon request.

neutral buffered formalin, and shipped to the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic for study. These specimens were stored without examination until 2022 when unexpected morphological characteristics were observed using SEM. Accordingly, additional nematode specimens were collected as source material for molecular sequencing and vouchers. These were isolated by scientists at MMPL from the gut of a second host specimen (277 cm in length, Field ID: MNW23030), which was recovered from the Crystal River, Citrus County, along the Gulf of Mexico coast of Florida on 2 February 2023. It had died from chronic effects of watercraft impact trauma. These nematodes were frozen, thawed at 4° C, then preserved in 95% ethanol or glycerine, or fixed in 10% neutral buffered formalin.

Five ethanol-preserved specimens were processed for gene sequencing at the FWC's Fish and Wildlife Research Institute (FWRI) headquarters in St. Petersburg, Florida, USA. Sequences were obtained from posterior portions of specimens while anterior portions were saved as morphological vouchers. A macroscopic photograph (Fig. 1) of nematodes *in situ* during necropsy in the gut of a third specimen of *T. m. latirostris* (a 301 cm long male collected on 25 October 2022, Field ID: MSE22106) was provided by MMPL staff for inclusion here. Finally, 13 ethanol-fixed, voucher specimens of *Heterocheilus* sp. from the stomach of *Trichechus manatus* collected by A. Mignucci-Gianonnoni in Humacao, Puerto Rico, were borrowed from the Smithsonian National Museum of Natural History, (USNPC 087819.03) and examined under a light microscope at the FWRI.

For light microscopical examination (LM) of the type specimens, the nematodes were cleared with glycerine and drawings were made with the aid of a Zeiss microscope drawing attachment. Voucher and Puerto Rican specimens were examined with

a microscope equipped with DIC optics and a DP71 camera. Photomicrographs were taken of two specimens, one glycerine-preserved from Florida and one uncleared, formalin-fixed, ethanol-preserved specimen from Puerto Rico. Four type specimens (two males and two females) used for scanning electron microscopical (SEM) examination were postfixed in 1% osmium tetroxide (in phosphate buffer), dehydrated through a graded acetone series, critical-point-dried and sputter-coated with gold; they were examined under a JEOL JSM-7401F scanning electron microscope at an accelerating voltage of 4 kV, GB low mode. All measurements in the species description are in micrometres unless otherwise indicated.

DNA extraction, PCR amplification and sequencing

Tissues from each of five ethanol-preserved specimens of nematodes collected from the digestive tract of *T. m. latirostris* were placed in biomasher tubes (RPI Research Products Mount Prospect, Chicago, Illinois). The ethanol was evaporated in an incubator at 37°C for one hour and the genomic DNA was extracted by using the PureGene DNA isolation kit (Gentra Systems Inc., Minneapolis, Minnesota), and pellets were rehydrated in 100 µl of deionised water. The mitochondrial DNA (mt) partial cytochrome oxidase subunit 1 (COI) was PCR amplified with the primers Nem-COI5P (5′-CATTTRTTTTGRTTTTTTGG-3′) and NemCOI3P (5′-ACYACATRATAAGTATCRTG-3′) (de Buron et al. 2011), and the small subunit 18S rDNA (18S) with primers PhilonemaF (5′-GCCTATAATGGTGAAACCGCGAAC-3′) and PhilPCRr (5′-CCGGTTCAAGCCACTGCGATTA-3′) for PCR and ameb620f (5′-GCCAGCACCCGCGGTAATTCC-3′) and ameb620r (5′-GGAATTACCGCGGGTGCTGGC-3′) as internal sequencing primers (Černotíková et al. 2011). PCR reactions and protocols for COI and 18S followed those of Moravec et al. (2021).

For the mt cytochrome oxidase subunit 2 (COII), we used the primers Rhigo_COXII_For (5′-TCHACYACAATAGG-YATAAAMCT-3′) and Rhigo_COII_Rev (5′-GWTATATR-GRTTGGTTYCATAA-3′) (Zhang et al. 2022) following the amplification protocol for that of COI. For 28S rDNA (28S) sequence, we used two primer pairs: F1: 5'-AGCGGAG-GAAAAGAAACTAA and R1 5'-ATCCGTGTTTCAAGACGGG (Nadler and Hudspeth 1998), and F2: 5'-AGCATATCATTTAG-CGGAGG and R2: 5'-TCGGAAGGAACCAGCTACTA (Nadler and Hudspeth 2000). For both 28S primers, PCR reactions followed a step-down profile: initial denaturation at 94°C for 2 min; 3 cycles each for 45 s at (94, 52 and 72°C), 4 cycles each for 40 s at (94, 50 and 72°C), 8 cycles each for 35 s at (94, 49 and 72°C); 22 cycles each for 30 s at (94, 48 and 72°C), and a final extension at 72 °C for 15 min.

Amplified products were gel-purified (Agilent Technologies, Santa Clara, California) and sequenced from both directions by using Big Dye Terminator v 1.1 (Applied Biosystems, Inc., Waltham, Massachusetts). Cycle-sequenced products were precipitated and resuspended in Hi-Di Formamide and visualised on a 3130 Genetic Analyser (Applied Biosystems, Inc.). Raw sequences were aligned and edited by using Sequencher (version 4.0; Gene Codes Corporation, Ann Arbor, Michigan) and deposited in GenBank (www.ncbi.nlm.nih.gov/genbank/). The two regions of 28S were concatenated during alignment.

RESULTS

Family Heterocheilidae Railliet et Henry, 1915

Heterocheilus floridensis sp. n.   Figs. 1–5

ZooBank number for species:
urn:lsid:zoobank.org:act:C90D866B-EC8C-4066-A9AA-7FB8726BF89B

Description: Fresh nematodes (Fig. 1) beige to pinkish, with dark brown intestine sometimes visible through cuticle in live specimens. Worms of even width and with thick, transversely striated cuticle (Figs. 3E,F, 4A,C,F–H). Anterior end cone-shaped (Fig. 2A). Lips with elongation of posterior region, forming long, tongue-like process; anterior parts of each lip with 2 lateral processes at level of labial papillae (Figs. 3A–D, 4A,B). Dorsal lip with conical, rounded rostral process projecting forward anterior to subventral lips (Fig. 3B,C); subventral lips slightly asymmetrical, with concave anterior process articulating with rostral process of dorsal lip (Figs. 3A,B,D, 4A,B). Inner surface of each lip with transverse dentigerous ridge consisting of about 30–40 distinct denticles (Figs. 3C, 4D,E, 5A–D). Dorsal lip bears 2 subdorsal double papillae (Fig. 3B); each ventrolateral lip with 1 double subventral papilla, 1 small single papilla and 1 amphid situated laterally (Fig. 3D). Inner surface of anterior region of dorsal lip with 2 distinct submedian pores (Fig. 3C). Dorsal lip slightly larger than subventral lips. Between each posterior labial prolongation are 4 conspicuous longitudinal ridges, 2 long, much wider ridges and 2 short narrow ridges; long ridges are parallel to each other, initiating anteriorly near labial lateral processes; in contrast, anterior and posterior ends of short ridges curved to respective long ridge, appearing to be continuous with its posterior end (Figs. 3B,D, 4A). Interlabia and lateral alae absent. Cuticle of anterior half of body with prominent, wide striations, subdivided into rod-like longitudinal folds (Fig. 3E). Deirids inconspicuous, difficult to observe, located short distance posterior to level of nerve ring (Fig. 2A). Digestive tract starting with short vestibule at level of posterior labial prolongations (Fig. 2A). Oesophagus long, narrow, without distinct extension at posterior portion, terminating in small, transversely oval ventriculus; ventricular appendix or lobes absent; long intestinal caecum present (Fig. 2A). Nerve ring encircles oesophagus approximately at one sixth of its length; excretory pore approximately at level of nerve ring (Fig. 2A). Tail of both sexes conical.

Male (9 specimens; measurements of holotype in parentheses): Length of body 38.6–59.2 (49.6) mm, maximum width 1.36–2.04 (1.90) mm; posterior third of body somewhat narrowed. Lips including posterior prolongations 612–1,006 (843) long. Length of oesophagus 4.22–6.25 (6.25) mm, representing 11–13% (13%) of body length; maximum width 299–367 (340). Nerve ring, excretory pore and deirids 1.16–1.54 (1.36) mm, 1.09–1.54 (1.36) mm and 1.36–2.08 (1.82) mm, respectively, from anterior extremity. Ventriculus 177–313 (249) long and 286–340 (340) wide. Intestinal caecum 0.60–4.57 (3.06) mm long and 95–258 (163) wide. Posterior end of body curved ventrally (Figs. 1F, 3C). Spicules equal or slightly unequal, alate, with somewhat extended proximal and rounded distal ends; length of spicules 1.16–1.32 (1.22) mm, representing 2.0–3.4% (2.5%) of body length (Figs. 2F,G, 4C,G). Small gubernaculum present, 136–231 (190) long, Y-shaped in ventral view. Genital papillae flat, difficult to observe. Total of 8 pairs of subventral papillae present, 4 being preanal and 4 postanal; postanal pairs of papillae arranged in 2 groups, anterior group of 2 pairs located just posterior to cloaca, whereas posterior group consisting of 2 pairs located at short distance from tail tip; in addition, 1 median preanal papilla present just anterior to anterior cloacal lip (Figs. 2C,F, 4C,F–H). Pair of small lateral phasmids present, located between 2 posteriormost pairs of postanal papillae (Figs. 2C,F, 4H). Tail conical, pointed, 558–816 (748) long (Figs. 2C,F, 4C,F).

Female (6 gravid specimens; measurements of allotype in parentheses): Length of body 45.4–63.6 (63.6) mm; maximum width 1.64–1.97 (1.66) mm. Lips including posterior prolongations 748–979 (843) long. Length of oesophagus 5.65–7.00 (7.00) mm, representing 11–13% (11%) of body length; maximum width 177–326 (218). Nerve ring, excretory pore and deirids 1.29–1.47 (1.29) mm, 1.18–1.70 (1.43) mm and 1.69–2.07 (1.97) mm, respectively, from anterior extremity. Ventriculus 190–272 (204) long and 286–367 (286) wide. Intestinal caecum 0.50–5.17 (5.17) mm long and 136–326 (163) wide. Vulva situated in anterior region of body, 15.0–21.9 (21.9) mm from anterior extremity, at 30–35% (34%) of body length; vagina directed posteriorly from vulva (Figs. 2B, 3F). Eggs numerous, oval, thin-walled, with uncleaved contents (Fig. 2D), 163–244 (163–177) long and 122–177 (122–136) wide. Tail
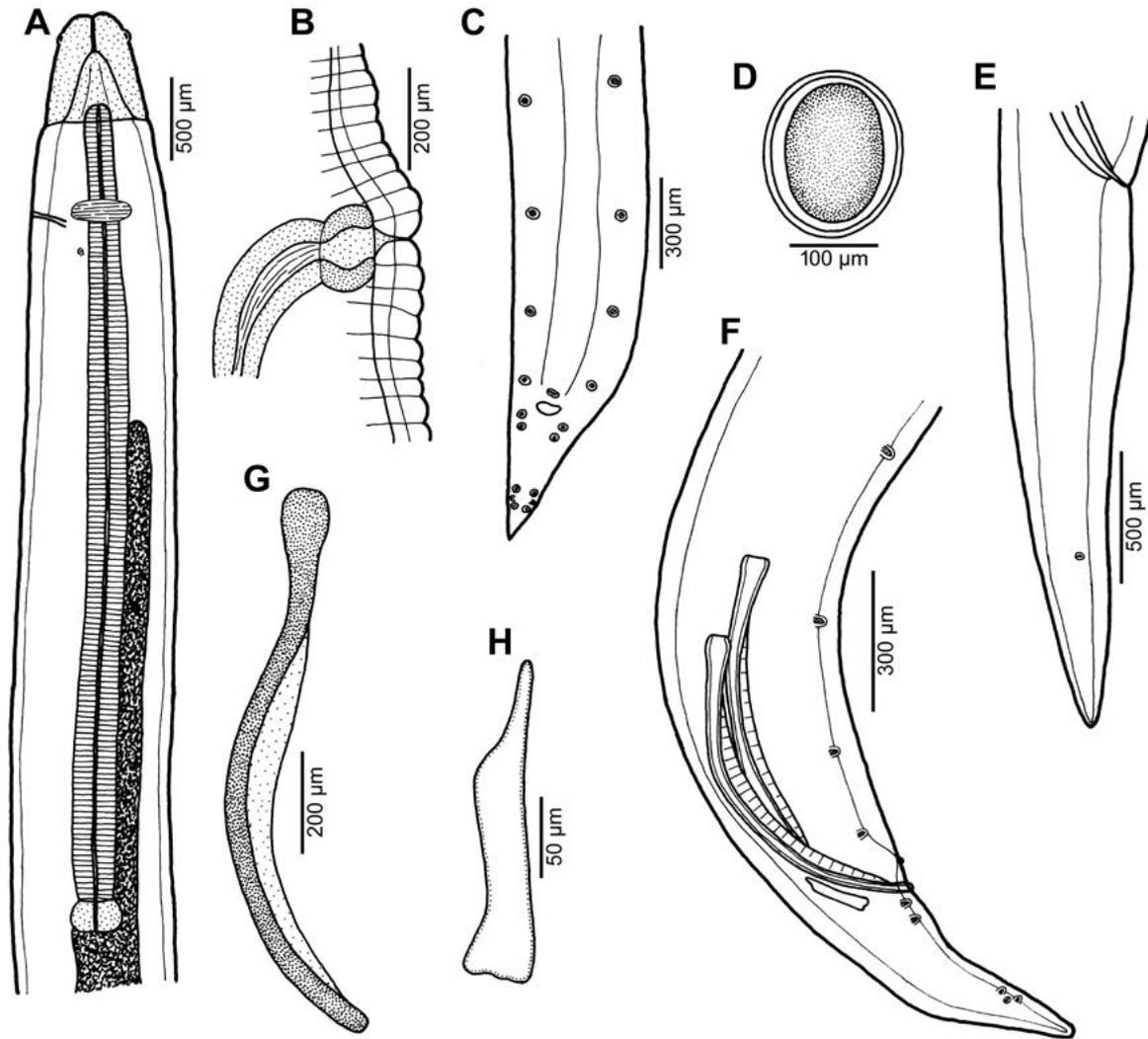
Fig. 2. *Heterocheilus floridensis* sp. n. from the Florida manatee *Trichechus manatus latirostris* (Harlan). **A** – anterior end of male, lateral view; **B** – vulva, lateral view; **C** – posterior end of male, ventral view; **D** – egg; **E** – tail of female, lateral view; **F** – posterior end of male, lateral view; **G** – spicule, lateral view; **H** – gubernaculum, lateral view.

1.77–2.60 (2.60) mm long, with pointed tip; small lateral phasmids present short distance from tail tip (Fig. 2E).

Newly collected voucher specimens, Florida Gulf Coast: male (n = 1) body length 46.2 mm; females (n = 11) body length 45.5–81.0 (mean 58.9) mm.

Borrowed voucher specimens (USNPC 87819.03), Puerto Rico: males (n = 7) body length 32.0–59.3 (mean 49.9) mm; females (n = 6) 34.8–72.8 (mean 58) mm.

Type host: *Trichechus manatus latirostris* (Harlan), Florida manatee; total body length 196 cm.

Site of infection: Digestive tract (stomach and duodenum).

Additional hosts: *Trichechus m. latirostris* (total body length 277 cm) and *Trichechus manatus manatus* Linnaeus, Antillean manatee.

Type locality: Banana River, a saltwater sub-basin of the Indian River Lagoon landward of Cape Canaveral along the Atlantic coast in the city of Merrit Island, Brevard County, Florida, USA, 28.37072° N, 80.66303° W (collected December 28, 2010).

Additional localities: Kings Bay, a freshwater basin at the headwaters of the Crystal River on Florida's Gulf of Mexi-co coast in the city of Crystal River, Citrus County, Florida, 28.889761°N, 82.594049°W (collected February 3, 2023); Manatee Pocket, a saltwater embayment just inside the Saint Lucie Inlet on Florida's Atlantic coast in the city of Stuart, Martin County, Florida, 27.153°N, 80.195°W (collected October 25, 2022); Humacao, Puerto Rico, 18.1496833°N, 65.8273861°W (Centrid) (collected February 10, 1997).

Deposition of type specimens: Holotype, allotype and 7 paratypes (5 males and 2 females) in the Helminthological Collection of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic (Cat. No. N-1276). Two paratypes (male and female) in the Smithsonian, Washington D.C., USA (Cat. No. USNM 1548395).

Additional material: Eight voucher specimens in the Fish and Wildlife Research Institute Invertebrate Specimen Collection, St. Petersburg, Florida (Cat. No. FSBC I 169018).

Etymology: The species name *floridensis* relates to the region (Florida, USA) from where this parasite is described.

**Remarks.** As mentioned above, Sprent (1980) employed both LM and SEM to redescribe in detail the type
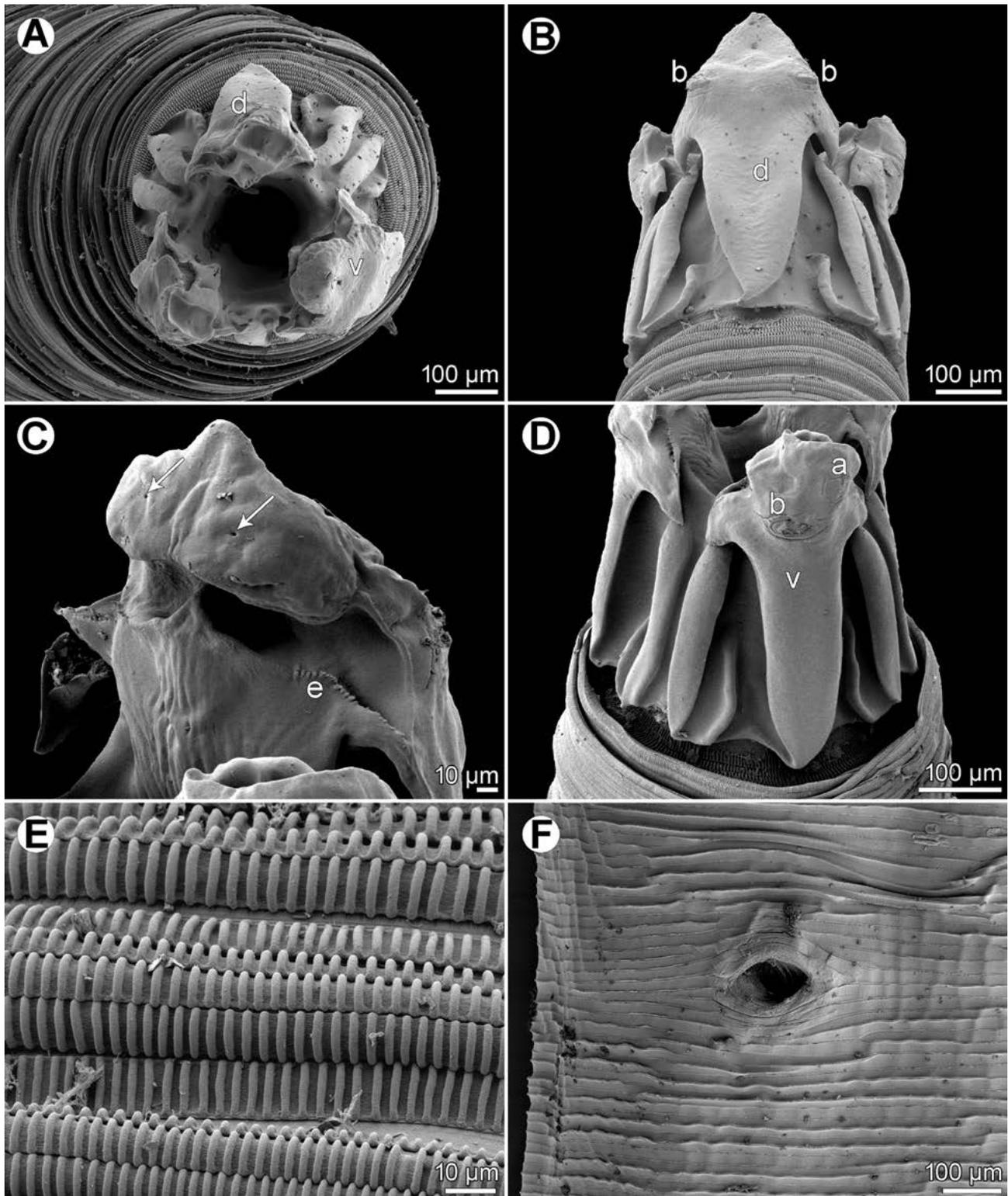
Fig. 3. *Heterocheilus floridensis* sp. n. from the Florida manatee *Trichechus manatus latirostris* (Harlan), scanning electron micrographs. **A** – cephalic end, apical view; **B** – dorsal lip, dorsal view; **C** – inner structure of anterior part of dosal lip (arrows indicate pair of distinct pores); **D** – subventral lip, subventral view; **E** – structure of cuticle at short distance posterior to lips; **F** – vulva, ventral view. *Abbreviations*: a – single cephalic papilla and amphid; b – double cephalic papilla; d – dorsal lip; e – denticles; v – subventral lip.

species of *Heterocheilus*, *H. tunicatus*, from the type specimens collected from the Amazonian manatee *T. inunguis* in Brazil. He stated that the lips of *H. tunicatus* are without dentigerous ridges, and this is observable in his electron micrographs (see figs. 3 and 4 of Sprent 1980). The absence of dentigerous ridges is also stated in Sprent's generic diagnosis of *Heterocheilus*.

In the same paper, Sprent (1980) mentions that "since going to press, specimens from *T. m. latirostris* have been observed, corresponding to above description but measur-
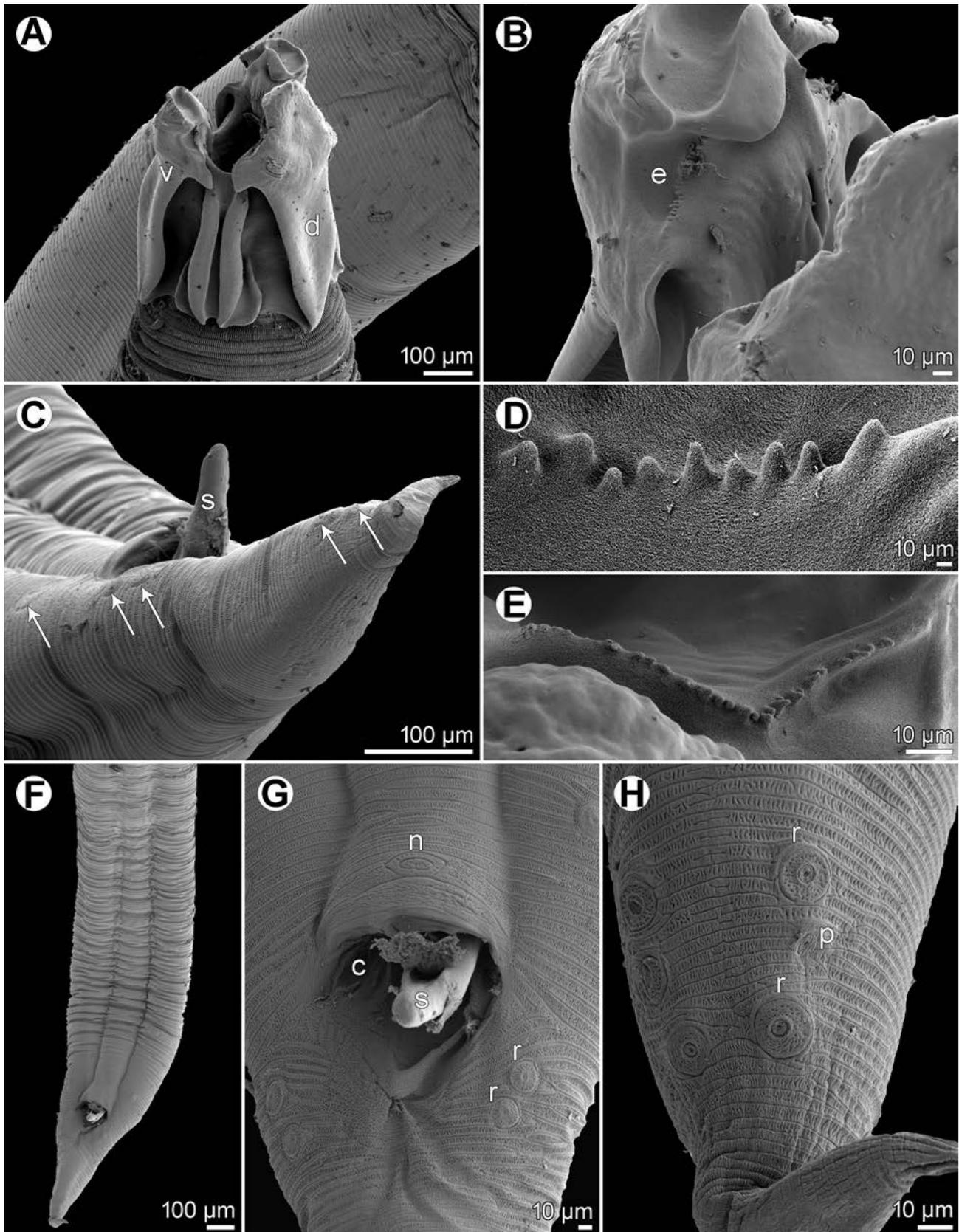
Fig. 4. *Heterocheilus floridensis* sp. n. from the Florida manatee *Trichechus manatus latirostris* (Harlan), scanning electron micrographs. **A** – cephalic end, lateral view; **B** – inner structure of anterior part of subventral lip; **C** – tail of male, sublateral view (arrows indicate caudal papillae); **D** – detail of denticles on lip; **E** – lip denticles (lower magnification); **F** – posterior end of male, ventral view; **G** – region of cloaca, ventral view; **H** – tail region of two last pairs of postanal papillae, ventral view. *Abbreviations*: c – cloaca; d – dorsal lip; e – denticles; n – median preanal papilla; p – phasmid; r – postanal papilla; s – spicule; v – subventral lip.
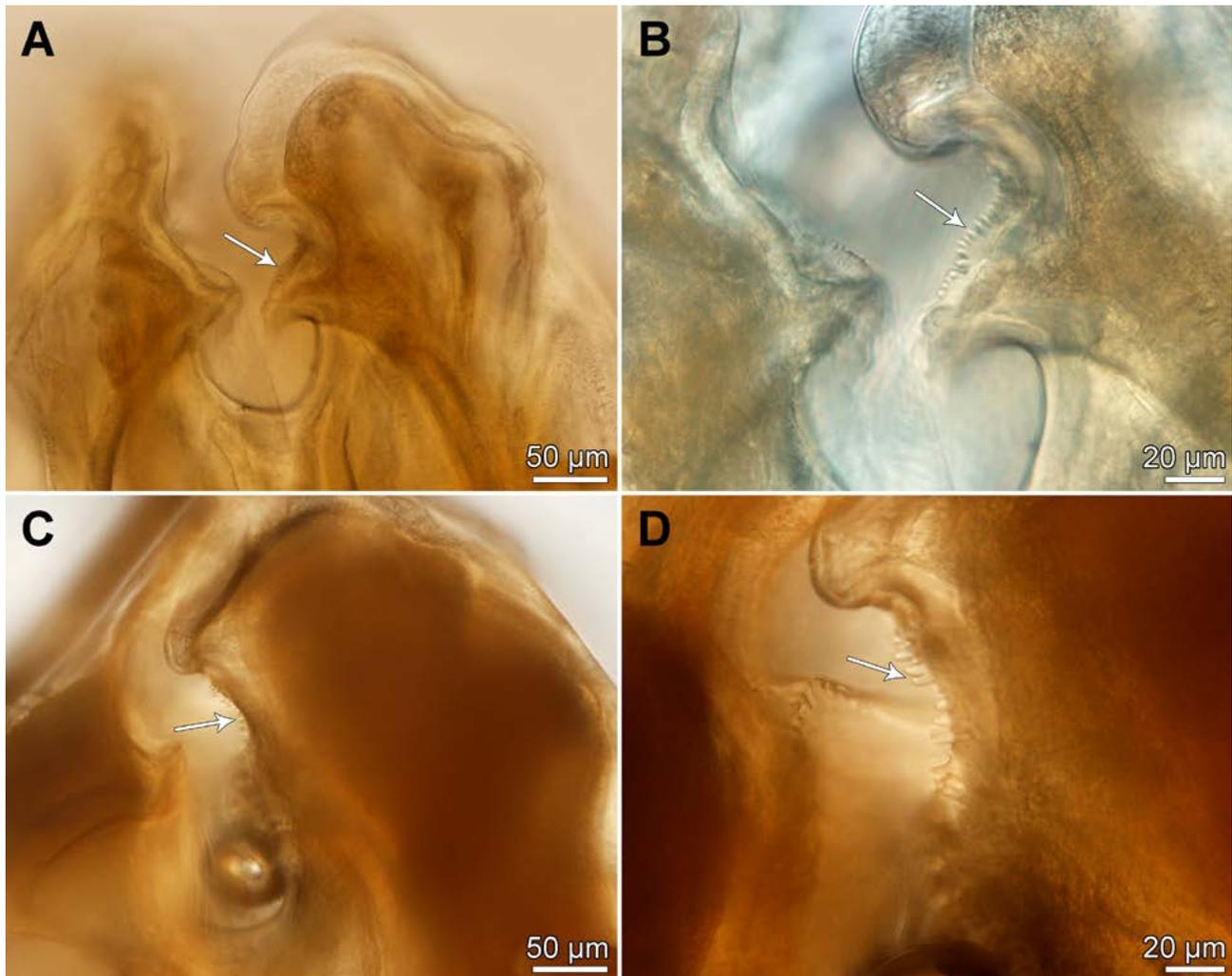
Fig. 5. Photomicrographs with DIC illumination showing the cephalic end of each of two specimens of *Heterocheilus floridensis* sp. n. from the Florida manatee *Trichechus manatus latirostris* (Harlan). **A, B** – a newly collected voucher specimen from the gut of the Florida manatee *Trichechus manatus latirostris* (Harlan) on the Florida coast of the Gulf of Mexico (FSBC I 169018). **C, D** – a borrowed voucher specimen from the stomach of the Antillean manatee off eastern Puerto Rico in the Caribbean Sea (USNPC #087819.03). Arrows indicate diagnostically important dentigerous ridges on the inner surface of the lips.

ing up to 68 mm (females) and 58 mm (males)". Later, he (Sprent 1983) provided measurements of specimens (five males and five females) from *T. m. latirostris* collected in Florida and from others collected from *T. manatus* (as *Manatus americanus*) in the Washington National Zoological Park, USA, deposited in the US National Parasite Collection. He again identified all these specimens as *H. tunicatus*, mentioning that they "corresponded in all respects with Diesing's specimens, except that the females attained a length of 80 mm and the males 62.5 mm". However, it should be noted that none of these specimens from *T. manatus* was unambiguously examined by SEM and Sprent did not explicitly and specifically confirm the absence/presence of dentigerous ridges on the lips.

Presence/absence of dentigerous ridges on lips is an important taxonomic feature in ascaridoid nematodes, sometimes considered to be of generic significance (e.g., Mozgovoy 1953, Anderson et al. 2009). However, the presence of dentigerous ridges on the inner side of lips may be easily overlooked in specimens studied only by

LM, as, for example, Mašová et al. (2010) demonstrated in the case of the ascaridoid *Multicaecum heterotis* Petter, Vassiliadès et Marchand, 1979, a parasite of the osteoglossiform fish *Heterotis niloticus* (Cuvier) in Africa. Because no dentigerous ridges on lips were reported in the original description of *M. heterotis*, Khalil (1984) transferred this species to *Brevimulticaecum* Mozgovoy in Skryabin, Shikhobalova et Mozgovoy, 1951. However, when Mašová et al. (2010) used SEM to examine *M. heterotis*, dentigerous ridges were confirmed to be present.

Heterocheilus floridensis sp. n. is morphologically similar to *H. tunicatus*, but differs distinctly from it in the presence (*vs* absence) of dentigerous ridges on the inner surface of the lips. Moreover, the unpaired, median papilla anterior to the cloaca of the new species has not been reported for *H. tunicatus*. The two species also differ in body size (males and females of *H. floridensis* 34–59 mm and 45–81 mm, respectively, *vs* 26.5–27.3 mm and 31.5–31.8 mm, in *H. tunicatus,* respectively) and in the lengths of the spicules: 0.85–1.32 mm (*vs* 0.75–0.77 mm) (our data

Table 1. Number of observed sequence differences for COI (below diagonal), and COII (above diagonal) between specimens pairs of voucher specimens of *Heterocheilus floridensis* sp. n. (labels correspond to individual vial numbers within the lot for FWRI Cat. No. FSBC I 169018) and the specimen of '*H. tunicatus*' from Nadler and Hudspeth (1998).

Specimen ID	MNW2030-1	MNW2030-2	MNW2030-3	MNW2030-4	MNW2030-5	'*H. tunicatis*'
MNW2030-1	---	na	na	na	na	na
MNW2030-2	0	---	0	0	1	2
MNW2030-3	0	0	---	0	1	2
MNW2030-4	3	3	3	---	1	2
MNW2030-5	2	2	2	3	---	1
'*H. tunicatus*'	*na	na	na	na	na	---
* not available						

on *H. floridensis* combined with those reported by Sprent 1983 for specimens from *T. m. latirostris*). Whereas *H. floridensis* sp. n. is parasitic in both subspecies of the West Indian manatee, *T. manatus*, in the USA and Caribbean, *H. tunicatus* occurs in geographic isolation of *H. floridensis* sp. n. as the parasite of the Amazonian manatee *Trichechus inunguis* in South America.

Regarding *Heterocheilus hagenbecki* from *T. m. manatus* in the Caribbean, Sprent (1980) synonymised it with *H. tunicatus*. However, the host species, collection locality and body size (length of male 43 mm, of female 50 mm) indicate that it may be identical with *H. floridensis*, although the spicules are reported to be unusually short (0.57 mm). Since the type specimens are not available (see Sprent 1980) and the species description is problematic in some respects (Sprent 1980), it is considered here as a *species inquirenda*.

Based on the present data, the generic diagnosis of *Heterocheilus* provided by Sprent (1980) should be modified to indicate "dentigerous ridges present or absent".

Key to the valid species of *Heterocheilus*

1 Three interlabiar cuticular prominences present between each posterior labial expansion, one median shield-shaped, anteriorly bifid process and two triangular processes partly covered by lateral flange. Dentigerous ridges on lips absent. Anterior end of oesophagus approximately in mid-way between posterior ends of lips and excretory pore. Oesophageal part of body with conspicuous, elongate, cuticular plaques on body surface. Parasitic in *T. senegalensis*; Senegal .... ***H. domningi***

- Two long and two short ridges present between each posterior labial expansion. Dentigerous ridges on lips present or absent. Anterior end of oesophagus near level of posterior ends of lips. Oesophageal part of body without conspicuous, elongate, cuticular plaques on body surface. Parasites of *Trichechus* spp. in the Americas 2

2 Dentigerous ridges on lips absent. Body length of male 27 mm, of female 32 mm. Spicules 0.75–0.77 mm long. Parasitic in *T. inunguis*; South America (Amazon River basin) .... ***H. tunicatus***

- Dentigerous ridges on lips present. Body length of male 34–59 mm, of female 45–81 mm. Spicules 0.85–1.32 mm long. Parasitic in *T. m. latirostris* in southern USA and in *T. manatus manatus* in Caribbean .... ***H. floridensis*** sp. n.

Molecular characterisation of *Heterocheilus floridensis* sp. n.

DNA sequences AF179913 (COII), U94373 (18S), and U94759 (28S), which were accessioned in GenBank under the name '*Heterocheilus tunicatus*' by Nadler and Hudspeth (2000), were based on a specimen collected from a *T. m. latirostris* in the Crystal River, Florida, USA. We consider that specimen to share a taxonomic identity with the five *de novo* sequenced specimens of *H. floridensis* sp. n. characterised molecularly herein. From these five specimens, we obtained three mt gene haplotypes of 404 base pairs (bp) for COI (GenBank accession nos. PP 262122, PP262123, PP262124), two mt gene haplotypes of 606 bp for COII (PP265928, PP265929). No stop codons or indels were observed within COI or COII. Based on our haplotypes *H. floridensis* and those previously accessioned under the name '*H. tunicatus*' by Nadler and Hudspeth (2000), intraspecific variation ranged from 2–3 bp (COI) to 1 bp (COII) (Table 1). For rDNA genes, we obtained one 18S sequence of 1,635 bp (PP259039), and one 28S sequence of 1,016 bp (PP259040). No intraspecific sequence variation was observed among the specimens for 18S or 28S.

DISCUSSION

Representatives of all known species of gut-infecting ascaridoids in sirenians have now been studied using SEM (Sprent 1980, 1983; this study). Taxonomic conclusions reported herein are based solely on morphological evidence. Our combined morphological and molecular analyses suggest parsimoniously that all specimens of *Heterocheilus* sp. collected in Florida to date and sourced for gene sequencing, including those of Nadler (1992), Nadler and Hudspeth (2000) and Nadler et al. (2000), are conspecific with respect to *H. floridensis* sp. n. Thus, among all species within *Heterocheilus*, only *H. floridensis* sp. n. has been molecularly characterised, and only using specimens collected in Florida.

The resulting phylogenetic deficit in taxonomic sampling can be remedied with relative ease once new material becomes available for gene sequencing (morphological voucher specimens will be necessary for full resolution). Additional sequencing of specimens from related species will be necessary to reconstruct the phylogenetic positions of *H. floridensis* sp. n. and other members within the genus. Of most immediate taxonomic importance is comparison of *H. floridensis* sp. n. with its congeners *H. tunicatus* and *H. domningi* collected from the Amazonian manatee

Trichechus inunguis and the African manatee *Trichechus senegalensis*, respectively.

It may also be worthwhile to challenge, with sequence data, the morphological identification of specimens collected from *Trichechus manatus manatus* in the Caribbean. Finally, this study did not address the possibility that a cryptic sister species of *Heterocheilus* (*H.* cf. *tunicatus*) might exist within the same host, perhaps in different localities or infection sites within the gut. Such is the case for species of *Anisakis* Dujardin, 1845, infecting the pilot whale *Globicephala melas* (Traill), a single specimen of which Mattiucci et al. (2014) found hosted both *Anisakis pegreffii* Campana-Rouget et Biocca, 1955 and *A. berlandi* Mattiucci, Cipriani, Webb, Paoletti, Marcer, Bellisario, Gibson et Nascetti, 2014.

Considering morphology alone, Sprent (1980) suggested that sirenian ascaridoids collectively formed a suprageneric group of sister taxa sharing a recent common ancestor with others parasitising aquatic reptiles, and to some extent this bears out with the scantly available molecular evidence. In the phylogenetic reconstruction of Tkach et al. (2010), a close relationship was reported between members of *Heterocheilus* and their newly described species *Krefftascaris sharpiloi* Tkach, Kuzmin et Snyder, 2010, a parasite of Australian side-necked turtle *Chelodina rugosa* Ogilby. This premise was in keeping with Sprent's (1980) proposed subfamily Heterocheilinae. A more recent molecular phylogenetic analysis (Li et al. 2018), based on mt12S, COI, COII, internal transcribed spacer, 18S and 28S sequences also supports this close relationship. This broad phylogeny, which includes Nadler and Hudspeth's (2000) specimen of *H. floridensis* n. sp. (as *H. tunicatus*), positions *Heterocheilus* in a family-level clade (the Heterocheilidae) among aquatic reptile-infecting ascaridoids, basal to the Anisakidae, Acanthocheilidae, Toxocaridae, Ascarididae and Raphidascarididae.

With our morphological-based species description of *H. floridensis* sp. n., each of the four extant sirenian species is reported to be infected by a host-specific ascaridoid, and the existence of these host-parasite species pairs may be useful for comparative phylogeography, as parasites can provide information about host evolutionary history (Whiteman and Parker 2005). Unfortunately, no vouchers useful for such studies were retained by Steller (1753) during his necropsy of a specimen of Steller's sea cow *Hydrodamalis gigas* (Zimmermann), a massive sirenian (around twice the length of the largest specimens of extant manatees) driven to extinction by human exploitation about 250 years ago (Marsh et al. 2011). Steller (1753) reported that it hosted in its stomach, pylorus and duodenum, an intense infection by "white worms half a foot long" (about twice the length of the largest specimens of *Heterocheilus* spp.).

Stiles and Hassall (1899) summarised other authors' taxonomic speculations about these worms in the intervening years and observed that "As Steller's sea cow is now extinct it can never be demonstrated what species Steller found…and all time used in speculating as to the affinities of this worm with corresponding parasites in other hosts may be considered lost". Of course, technological advancements have since exponentially reduced the amount of physical material necessary to move from speculation to hypothesis testing. Should naturally or anthropogenically preserved remains of *H. gigas* or its gut contents be unexpectedly discovered, it may be hoped that the integrated taxonomy of its symbionts will be a component of the ensuing study.

Fortunately, morphological and molecular data useful for study of phylogenetic relationships, and the geographical distribution of extant sirenian-infecting ascaridoids, remains obtainable, if not easily so by most parasitologists. These data may be useful in the further elucidation of the recent evolutionary histories of their hosts or provide insights complementary to current host population-genetic and phylogeographical studies, particularly once more is known of the nematodes' life histories.

No such life cycle data are available for any species of *Heterocheilus*, which limits the scientific utility they might have in the study of their definitive hosts. The mode of infection of these unique ascaridoids may be similar to that observed in species of *Paradujardinia* Travassos, 1933, which Jueco (1997) suggested are acquired when dugongs ingest eggs located on or among the sea plants on which they feed, without the involvement of an intermediate host. Sprent (1980) put forward the hypothesis that epifaunal organisms on food plants, particularly crustaceans, might serve as intermediate hosts. Also, whereas *T. manatus* primarily feeds on plants, it is conditionally omnivorous, sometimes consuming items ranging in diversity from scavenged fish flesh to its own faeces (Marsh et al. 2011). As suggested by Marsh et al. (2011), coprophagia could be relatively common in colder months when *T. m. latirostris* congregates in thermal refugia, rapidly depleting any available forage.

Given the intense research and conservation effort devoted to the Florida manatee over the past half century, it seems ostensibly remarkable that the conspicuous *H. floridensis* sp. n. has gone unrecognised, particularly given that specimens were examined by Sprent (1980, 1983) while his attention was directly focused on the genus. Certainly, the present study was not initiated out of scepticism, despite the retrospective implausibility of Sprent's (1980, 1983) conclusions that two host species so substantially different in ecology and spatial distribution would harbour the same parasitic nematode. However, whereas large, endangered mammals draw more research attention than smaller species of low conservation concern (Trimble and Van Aarde 2010), the reverse is true for helminth parasites of these groups (Poulin et al. 2023). Poulin et al. (2023) noted that this may be partly because of some issues that Shaw et al. (2021) identified as impediments to experimental replication studies on the behaviour and cognition of endangered species. Nevertheless, for parasites of the Florida manatee, these obstacles are here demonstrated as surmountable. Although their charisma may be less easily appreciated than their hosts', manatee parasites have intrinsic value, and there is much to discover about their roles in the broader ecosystem and their relevance to their hosts' behaviour, health and conservation.

Author contribution. František Moravec initiated the study, carried out the identification and morphological analysis of all type material, and drafted the manuscript. Micah D. Bakenhaster obtained nematode specimens and examined those collected from the Gulf Coast of Florida and Puerto Rico. Seifu Seyoum performed molecular assays, sequence alignments and GenBank submissions. Michael D. Tringali interpreted molecular findings. All authors contributed substantially to the manuscript text, participated in its revision and approved its final draft.

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