Research Paper

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Integrative taxonomy of *Anaporrhutum mundae* sp. nov. (Trematoda: Gorgoderidae), a parasite of the Munda round ray *Urotrygon munda* (Urotrygonidae) in Costa Rica

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Abstract

A new species of trematode of anaporrhutine gorgoderid, from the gill chambers of the Munda round ray *Urotrygon munda* in Costa Rica is described, based on an integrative taxonomic approach that includes the use of light and scanning electron microscopy, ITS2 and 28S rDNA sequencing, and phylogenetic analysis. *Anaporrhutum mundae* sp. nov. can be distinguished from congeneric species by a combination of morphological traits and particularly by having the genital pore opening at the level of the intestinal bifurcation. The new species also can be distinguished from all other species of *Anaporrhutum*, except *A. euzeti* Curran, Blend & Overstreet, 2003, by having fewer testicular follicles per testis. *Anaporrhutum mundae* sp. nov. also differs from *A. euzeti* in its forebody shape and by having different morphology and location of the vitellaria. The study of the tegumental surface of *A. mundae* sp. nov., as revealed by scanning electron microscopy, allowed detection of new morphological characters for a member of Anaporrhutinae that may be of taxonomic value. These are: a stylet cavity dorsal to the oral sucker with a large penetration gland opening on each side of the cavity and small penetration gland openings located ventral to the stylet cavity, arranged in a circle around the mouth. This represents the first record of an *Anaporrhutum* species from Costa Rica. Further, *A. mundae* sp. nov. represents the first parasite described or reported in this host.

Introduction

Trematodes of the family Gorgoderidae Looss, 1899 comprise 3 sub-families: Anaporrhutinae Looss, 1901 (with seven genera infecting elasmobranchs and sea turtles), Degeneriinae Cutmore, Miller, Curran, Bennett & Cribb, 2013 (with a single genus infecting deep-sea fishes), and Gorgoderinae Looss, 1899 (with four genera infecting marine and freshwater ray-finned fish and amphibians) (Cutmore *et al.* 2013). Two- or three-host life cycles have been revealed only for some members of freshwater Gorgoderinae, with bivalves as first intermediate hosts, and insects, snails, crayfish, or amphibian tadpoles as second intermediate hosts (Campbell 2008). A single record of an unidentified gorgoderid cercaria infecting a marine bivalve *Lioconcha castrensis* (Linnaeus) has been also reported (Bott and Cribb 2005).

The Munda round ray *Urotrygon munda* Gill (Myliobatiformes: Urotrygonidae) is a relatively poorly known species distributed throughout the eastern Pacific from southern Baja California (Mexico) to northern Peru at depths of 4–51 m (Robertson and Allen 2015), which feeds primarily in shallow soft bottoms on caridean decapods (Flores-Ortega *et al.* 2011). According to Caira *et al.* (2022) and Pollerspöck and Straube (2023), no parasite species has been reported in this host. Additionally, of the 62 marine trematode species reported from both coasts of Costa Rica, no trematode species have been reported in elasmobranchs (Solano-Barquero *et al.* 2023). Furthermore, the only Anaporrhutinae reported from this area was *Plesiochorus cymbiformis* (Rudolphi, 1819) Looss, 1901 in the olive ridley sea turtle *Lepidochelys olivacea* (Eschscholtz) from the Pacific coast of Costa Rica (Santoro and Morales 2007; Solano-Barquero *et al.* 2023).

During a parasitological survey of fish from the Pacific coast of Costa Rica, some individuals of an anaporrhutine species were found in the gill chambers of the Munda round ray. These proved to represent a morphologically distinct, previously unknown species of *Anaporrhutum* Brandes

in von Ofenheim, 1900, which is described herein, based on morphological, ultrastructural, and molecular characters, and phylogenetic analysis.

Materials and methods

Sample collection

On 07 April 2023, three individuals of the Munda round ray were obtained from off Playa Cuajiniquil (Guanacaste province) on the Pacific coast of Costa Rica using nets at benthic depths ranging from 5 to 10 m. They included two females, with total length (TL) and total weight (TW) of 52 cm and 917 g, and 50.9 cm and 798 g, respectively, and one male, with 23.8 cm TL and 231 g TW. Fish were obtained under the framework of a project of the Centro de Investigación en Ciencias del Mar y Limnología of the University of Costa Rica "Proyecto BioMar -ACG" (see Cortés and Joyce 2020), aimed at studying the marine biodiversity of the Pacific coast of Costa Rica (permit no. ACG 019-2023), and a collaborative project between the Stazione Zoologica Anton Dohrn and the University of Costa Rica, aimed at studying the parasite biodiversity of elasmobranchs in the country.

Fish were refrigerated (4°C) and transferred to the laboratory, where they were studied within 6 h of fishing. During necropsy, eyes, skin, gills, mouth cavity, digestive tract (stomach and intestine), liver, heart, gonads, visceral cavity, mesenteries, and skeletal muscles of each fish were examined for metazoan parasites under a dissecting microscope (Axio Zoom V16, Zeiss, Switzerland) using the methods described in Santoro *et al.* (2022, 2023). When parasites were observed they were collected and washed in physiological saline solution and preserved in 70% ethanol for subsequent morphological and molecular analyses.

Morphological study

For light microscopy, trematodes were stained with Mayer's acid carmine, dehydrated through a graded ethanol series, cleared in methyl salicylate, and mounted in permanent slides in Canada balsam. Measurements (in micrometres) are reported as range values with mean ± standard deviation in parentheses followed by the total number (*n*) of observations. Measurements were obtained using a compound microscope (Axio Imager M1, Zeiss, Switzerland) and a dissecting microscope (Axio Zoom V16) equipped with the ZEN 3.1 imaging system (Zeiss, Switzerland) Drawings were made with the aid of a XP PEN Deco 02 drawing tablet (Deco, Italy) and Adobe Illustrator and Adobe Photoshop software (Adobe Systems Inc., United States).

For scanning electron microscopy analysis, a specimen was also fixed overnight in 2.5% glutaraldehyde, then transferred to 40% ethanol (10 min), rinsed in 0.1 M cacodylate buffer, postfixed in 1% OsO_4 for 2 h, and dehydrated in ethanol series, critical point dried, and sputter-coated with platinum. Observations were made using a JEOL JSM 6700F scanning electron microscope operating at 5.0 kV (JEOL, Italy).

Molecular analysis

For the molecular study, genomic DNA was extracted from a fragment of two 70% ethanol-preserved specimens using the Quick-gDNA Miniprep Kit (Zymo Research, California). The 28S nuclear ribosomal DNA region was amplified using the primers LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGC-3') and 1500R (5'-GCTATCCTG AGGGAAACTTCG-3') (Littlewood 1994; Cutmore *et al.* 2010); the ITS2 region was amplified using the primers 3S (5'-GGTACC GGTGGATCACGTGGCTAGTG-3') and ITS2.2 (5'-CCTGGTTAGTTTCTTTTCCTCCGC-3') (Cribb *et al.* 1998; Cutmore *et al.* 2010). New specific primers also were designed for the 28S region to avoid any nonspecific amplifications; these were Anap28-F (5'-CAATGTGGTGTTYAGGTCGGTCTTC -3') and Anap28-R (5'-CTGYCGCTCAWTRCYTGGT -3'). Polymerase chain reactions (PCRs) for both 28S and ITS2 regions were performed in a 25 µL volume containing 1.5 µL of each primer 10 mM, 3 µL of MgCl2 25 mM (Promega, Wisconsin), 5 µL of 5× buffer (Promega), 0.6 µL of dNTPs 10 mM (Promega), 0.2 µL of Go-Taq Polymerase (5U/µL) (Promega), and 2 µL of total DNA.

PCR cycling parameters for the 28S region were as follows: an initial 94°C for 2 min, followed by 40 cycles of 94°C for 30 sec, 58°C for 45 sec, 72°C for 2 min, and a final 72°C extension for 10 min. PCR cycling parameters for the ITS2 region were as follows: an initial single cycle of 95°C for 3 min, 45°C for 2 min, 72°C for 90 sec, followed by 4 cycles of 95°C for 45 sec, 50°C for 45 sec, 72°C for 90 sec, followed by 30 cycles of 95°C for 20 sec, 52°C for 20 sec, 72°C extension for 90 sec, and a final 72°C extension for 5 min.

PCR products were purified using Agencourt AMPure XP (Beckman Coulter, United States), following the standard manufacturer-recommended protocol. Clean PCR products were Sanger sequenced from both strands using an Automated Capillary Electrophoresis Sequencer 3730 DNA Analyzer (Applied Biosystems, United States) and the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Life Technologies, United States). The obtained contiguous sequences were assembled and edited using MEGAX v. 11 (Kumar *et al.* 2018). Sequence identity was verified using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (Morgulis *et al.* 2008).

Phylogenetic analysis

Obtained sequences were cleaned and aligned with 28S and ITS2 sequences acquired from other members of the families Gorgoderidae and Allocreadiidae available in Genbank using MEGA10 (Kumar *et al.* 2018) and the MUSCLE algorithm (Edgar 2004) (Table 1). Also for the ITS2, sequences of members of family Prostogonimidae were acquired (Table 1). Then, a Bayesian inference (BI) phylogenetic tree was built for the 28S and ITS2 alignments using the BEAST package v2.6 (Drummond and Rambaut 2007) with General Time Reversible with gamma distribution and Hasegawa-Kishino Yano nucleotide substitution models, as based in Bayesian inference criteria. A total of 10^8 Montecarlo Markov Chains and a burn-in of 10% were estimated. Effective sample sizes larger than 300 were confirmed for each parameter with Tracer v1.7.2 (Rambaut *et al.* 2018). Finally, the converged tree was visualized with FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) In addition, a maximum likelihood (ML) phylogenetic tree was reconstructed with the nucleotide substitution models mentioned above and 1000 bootstraps using MEGA10. The phylogenetic tree for the 28S fragment was rooted using *Dicrocoelium dendriticum* (Rudolphi 1819) Looss 1899 (AF151939), *Paracreptotrematina limi* Amin & Myer, 1982 (HQ833706), and *Degeneria halosauri* (Bell, 1887) Campbell 1977 (AY222257) as outgroups, while *Provitellus chaometra* Wee, Cutmore & Cribb, 2019 (MK501980) was added as the outgroup for the ITS2 phylogenetic tree.

Table 1. Data from ITS2 and 28S sequences included in the phylogenetic analyses shown in Figures 4 and 5

Species	Host	Geographical location	ITS2	28S	Reference
Allocreadium isoporum	*Barbatula barbatula*	Russia	MH143096		Petkevičiūtė *et al.* 2018
Allocreadium isoporum	*Pisidium milium*	Norway	OQ359135		Petkevičiūtė *et al.* 2023
Anaporrhutum mundae n. sp.	*Urotrygon munda*	Costa Rica	PP133644	PP133643 PP354907	This study
Anaporrhutum sp.	*Chiloscyllium punctatum*	Australia	KF013159	KF013184	Cutmore *et al.* 2013
Bunodera luciopercae	*Perca fluvialis*	Russia	FJ874917		Petkevičiūtė *et al.* 2010
Bunodera luciopercae	*Perca fluvialis*	Lithuania	MH143097		Petkevičiūtė *et al.* 2018
Cercaria duplicata (accepted as *Phyllodistomum elongatum*)	*Anodonta anatina*	Russia		KJ729514	Petkevičiūtė *et al.* 2015
Cercaria duplicata (accepted as *Phyllodistomum elongatum*)	*Anodonta anatina*	Lithuania		KJ729515	Petkevičiūtė *et al.* 2015
Crepidostomum auriculatum	*Acipenser schrenkii*	Russia		FR821371	Atopkin and Shedko 2014
Crepidostomum auriculatum	*Acipenser schrenckii*	Russia		MN524579	Atopkin *et al.* 2020
Crepidostomum auriculatum	*Acipenser ruthenus*	Russia		MN524581	Atopkin *et al.* 2020
Crepidostomum auriculatum	*Acipenser fulvescens*	USA		MN750364	Atopkin *et al.* 2020
Crepidostomum sp.	*Pisidium casertanum*	Ukraine		MH143119	Petkevičiūtė *et al.* 2018
Degeneria halosauri	*Halosauropsis macrochir*	NE Atlantic Ocean		AY222257	Olson *et al.* 2003
Dicrocoelium dendriticum	NI	NI		AF151939	Tkach *et al.* 2000
Gorgodera cygnoides	NI	NI		AF151938	Tkach *et al.* 2000
Gorgodera cygnoides	*Pelophylax ridibundus*	Bulgaria		AY222264	Olson *et al.* 2003
Nagmia floridensis	*Dasyatis sabina*	USA		EF032691	Curran *et al.* 2006
Nagmia sp.	*Stegostoma fasciatum*	Australia	KF013158	KF013192	Cutmore *et al.* 2013
Paracreptotrematina limi	*Paracreptotrematina limi*	USA		HQ833706	Curran *et al.* 2011
Phyllodistomum brevicecum	*Umbra limi*	Canada		KC760204	Razo-Mendivil *et al.* 2013
Phyllodistomum inecoli	*Profundulus punctatus*	Mexico		KM659387	Pérez-Ponce de León *et al.* 2015b
Phyllodistomum inecoli	*Profundulus* sp.	Mexico		KM659389	Pérez-Ponce de León *et al.* 2015b
Phyllodistomum lacustri	*Ameiurus melas*	USA		EF032692	Curran *et al.* 2006
Phyllodistomum spinopapillatum	*Profundulus balsanus*	Mexico		KM659388	Pérez-Ponce de León *et al.* 2015b
Plesiochorus cymbiformis	*Caretta caretta*	Australia	ON062958	ON062962	Corner *et al.* 2022
Plesiochorus cymbiformis	*Eretmochelys imbricata*	Australia	ON062959	ON062961	Corner *et al.* 2022
Plesiochorus elongatus	*Caretta caretta*	Brazil	MK577499 MK577500 MK577501		Werneck *et al.* 2019
Plesiochorus sp.	*Caretta caretta*	USA	KF013154	KF013180	Cutmore *et al.* 2013
Plesiochorus sp.	*Eretmochelys imbricata*	Australia	ON062960	ON062963	Corner *et al.* 2022
Prosthogonimus ovatus	*Bithynia tentaculata*	Germany	MN726999 MN727000		Schwelm *et al.* 2020
Provitellus chaometra	*Gnathanodon speciosus*	Australia	MK501980		Wee *et al.* 2019
Staphylorchis cymatodes	*Carcharhinus sorrah*	Australia		HM486320	Cutmore *et al.* 2010
Staphylorchis cymatodes	*Chiloscyllium punctatum*	Australia	HM486321	HM486318	Cutmore *et al.* 2010
Staphylorchis cymatodes	*Sphyrna lewini*	Australia		HM486319	Cutmore *et al.* 2010

NI: Not indicated
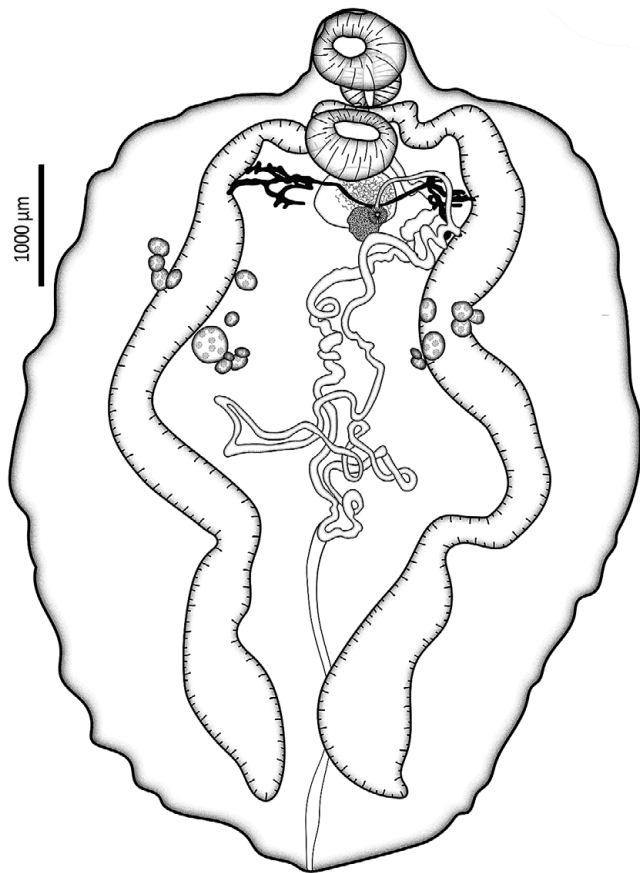
Figure 1. Drawing of *Anaporrhutum mundae* sp. nov., holotype ventrally mounted.
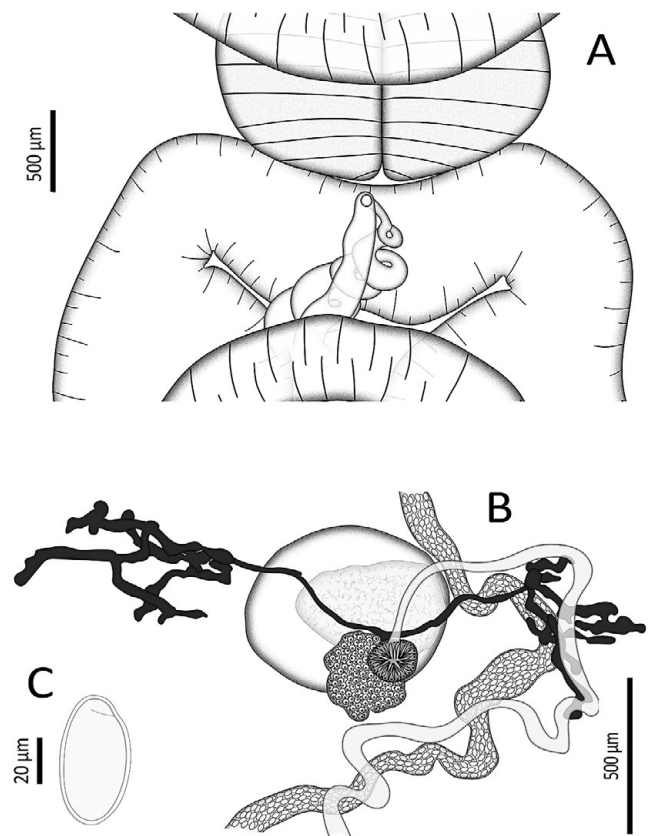

Figure 2. Drawing of *Anaporrhutum mundae* sp. nov., (A) paratype, ventral view of terminal genitalia; (B) holotype, ventral view of ovarian complex; (C) egg.

Results

Description *(see Figures 1–3)*

ZooBank: urn:Isid:zoobank.org:act:991D0E3E-4C1E-4928-9510-69833DB834E1.

  Family. Gorgoderidae Looss, 1899

  Subfamily. Anaporrhutinae Looss, 1901

  Anaporrhutum mundae Santoro, López-Verdejo, Angulo, Rojas, & Solano-Barquero, 2024 sp. nov. in Santoro, López-Verdejo, Angulo, Rojas, Cortés, Pacheco-Chaves & Solano-Barquero, 2024

  Based on six mounted mature specimens studied by optical microscopy and one mature specimen studied by SEM. Body large, flat, oval, 5588–7152 (6624 ± 653.5; $n = 7$) long, 2782–5022 (3712 ± 904.1; $n = 7$) wide; length/width ratio 1:0.4–0.7 (0.5 ± 177.1; $n = 7$). Body divided into small forebody and large hindbody; forebody 1270–1782 (1425 ± 190.9; $n = 7$) long, representing 18–24% (21 ± 2.5; $n = 7$) of total body length (Figure 1). Tegument wrinkled, lacking spines. Stylet cavity large, dorsal to the oral sucker on the anterior end of the body; a large penetration gland opening is present on each side of this cavity (Figures 3B, 3D, 3E). Penetration gland openings small, symmetrically arranged ventral to the stylet cavity (Figure 3D). Oral sucker round, 547–705 (636 ± 62.1; $n = 7$) long, 612–743 (668 ± 48; $n = 7$) wide (Figures 3A, 3B). Penetration gland openings small, arranged in a ring around the mouth (Figures 3B, 3C). Prepharynx absent. Pharynx 315–410 (357 ± 33.1; $n = 7$) long, 408–479 (442 ± 28.9; $n = 7$) wide. Oesophagus short, 76–99 (87 ± 7.8; $n = 7$) long (straight line from posterior end of pharynx to intestinal bifurcation). Caeca bifurcation in forebody, slightly sinuous, lacking diverticula, terminating near to posterior margin of body (Figure 1). Genital pore median, opening in forebody, posterior to margin of pharynx and at the level of intestinal bifurcation (Figures 2A, 3A, 3F). Ventral sucker round, 547–658 (586 ± 41.9; $n = 7$) long, 523–742 (606 ± 87.6; $n = 7$) wide (Figure 3F). Oral sucker width to ventral sucker width ratio 1:0.8–1 ($1:0.9 \pm 288$; $n = 7$). Testes pre-equatorial, consisting of two opposite groups of round to oval unlobed follicles prevalently intercaecal (Figure 1), right group 7–9 (8 ± 0.8; $n = 7$) in number, left group 7–11 (8 ± 1.8; $n = 7$) in number. Follicles highly variable in size, right group 109–186 (157 ± 29.6; $n = 20$) long, 92–309 (147 ± 54.6; $n = 20$) wide; left group 114–312 (175 ± 56.2; $n = 20$) long, 92–251 (138 ± 41.9; $n = 20$) wide. Seminal vesicle elongate, sinuous. Pars prostatica short. Ejaculatory duct short, opening into genital atrium. Cirrus-sac absent. Ovary pretesticular, median, irregularly shaped, 167–250 (212 ± 33.9; $n = 6$) long, 115–253 (162 ± 51.9; $n = 6$) wide (Figure 2B). Large seminal receptacle pre-ovarian, round to oval, 358–488 (436 ± 48.9; $n = 6$) long, 229–333 (278 ± 41.4; $n = 6$) wide. Mehlis's gland globular, surrounding ootype, 86–106 (96 ± 8.7; $n = 5$) long, 78–112 (90 ± 14.7; $n = 5$) wide (Figure 2B). Vitellarium tubular, in two opposite, intercaecal, dendritic fields at level of ovary; fields consist of 4–5 main branching, winding tubes with finger-like projections branching from main tubes (Figures 1, 2B); dextral fields 264–703 (517 ± 158.6; $n = 5$) long, 316–528 (419 ± 87.2; $n = 5$) wide; sinistral fields 339–524 (438 ± 65.6; $n = 6$) long, 324–663 (532 ± 123.8; $n = 6$) wide. Uterus intercaecal, extending posteriorly to level of posterior limit of caeca, before turning extending
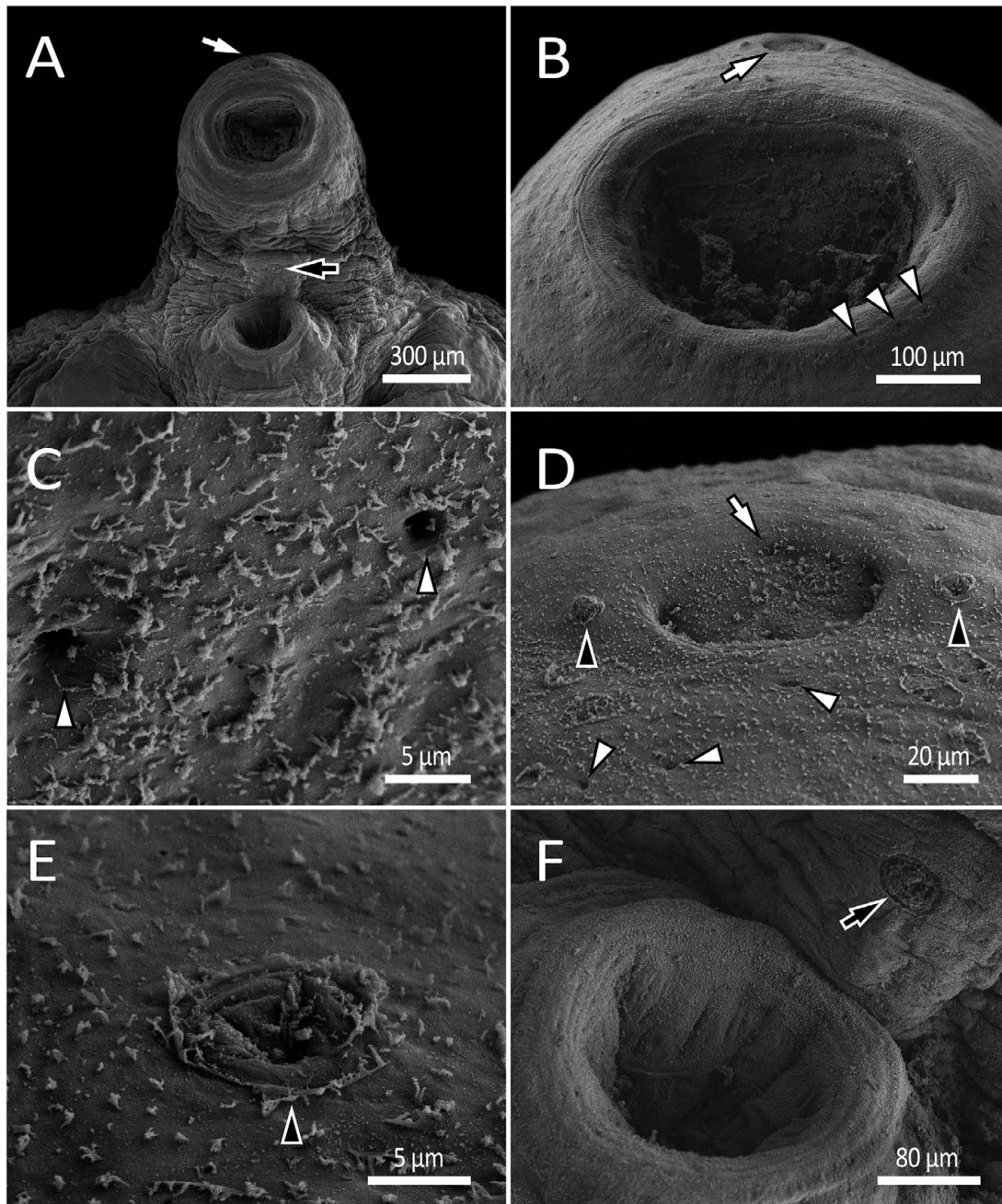
Figure 3. Scanning electron microscopy of *Anaporrhutum mundae* sp. nov. ventrally mounted: (A) anterior extremity showing the oral sucker, the genital pore, and the ventral sucker; (B) oral sucker showing the stylet cavity and the small penetration gland openings arranged in a ring around the mouth; (C) magnification of two penetration gland openings around the mouth; (D) magnification of the stylet cavity showing a large penetration gland opening on each side and smaller penetration gland openings ventral to the cavity; (E) magnification of a large penetration gland opening on the side of the stylet cavity; (F) magnification of ventral sucker and genital pore. White arrows indicate the stylet cavity; black arrows indicate the genital pore; white arrowheads indicate the small penetration gland openings; black arrowheads indicate the larger penetration gland openings.

anteriorly to reach the dorsal level of ventral sucker (Figure 1). Metraterm short, opening into genital atrium. Operculated eggs (from distal region of uterus), 47–66 (56 ± 5.7; $n = 20$) long, 22–33 (29 ± 2.7; $n = 20$) wide, with tick wall, 1.6–2.8 (2.2 ± 0.2; $n = 20$) wide (Figure 2C). Excretory vesicle H-shaped.

Taxonomic summary

Type-host: Munda round ray *Urotrygon munda* Gill, 1863 (Myliobatiformes: Urotrygonidae).

Type-locality: Off Playa Cuajiniquil (10°56'04.38″N, 85° 42'14.09″W), Guanacaste province, north Pacific coast of Costa Rica (collected on 07 April 2023).

Location in the host: Gill chambers.

Type-material: Holotype (MHNG-PLAT-0157452), two paratypes and an SEM preparation (MHNG-PLAT-0157451) in the Parasite Collection of the Natural History Museum of Geneva in Geneva (Switzerland); two paratypes in the Helminthological Collection of Costa Rica (CHCR-207-1; CHCR-207-2) at the Universidad de Costa Rica, San José (Costa Rica).

Prevalence and intensity: 2 (both females) of 3 individuals infected with 1 and 9 flukes.

Etymology: The new species is named after its host species.

Remarks

Currently, there are seven recognized species of *Anaporrhutum*. These are: *A. albidum* Brandes in Ofenheim, 1900, *A. mantae* Nagaty & Abdel-Aal, 1961, *A. stunkardi* Tandon, 1969, *A. narayani* Simha, Rao & Rao, 1971, *A. sinicum* Cao, 1990, *A. torpedoense* Khan & Begum, 1991, and *A. euzeti* Curran, Blend & Overstreet, 2003 (von Ofenheim 1900; Nagaty and Abdel 1961; Tandon 1969; Simha *et al.* 1971; Cao 1990; Khan and Begum 1991; Curran *et al.* 2003). *Anaporrhutum mundae* sp. nov. can be distinguished from all these species by having the genital pore opening at level of the intestinal bifurcation (whereas it opens just posterior to caecal bifurcation in all congeneric species). Moreover, it has a different location in the host (gill chambers *vs* pericardium and body cavity). Additionally, the new species can be distinguished from all other congeners except *A. euzeti* by having fewer testicular follicles per testis (7–11 *vs* 6–13 in *A. euzeti*, 12–25 in *A. albidum*, 13 in *A. sinicum*, 19–24 in *A. mantae*, 25–26 in *A. stunkardi*, 26 in *A. narayani* and 23–35 in *A. torpedoensis*) (von Ofenheim 1900; Nagaty and Abdel 1961; Tandon 1969; Simha *et al.* 1971; Cao 1990; Khan and Begum 1991).

Anaporrhutum mundae sp. nov. most closely resembles *A. euzeti*, which was described from the Gulf of California (Mexico) in *Myliobatis longirostris* Applegate & Fitch, *Narcine entemedor* Jordan & Starks, *Hypanus dipterurus* (Jordan & Gilbert) and *Urobatis maculatus* Garman (Curran *et al.* 2003). However, *A. mundae* sp. nov. is smaller than *A. euzeti* (5.5–7.1 *vs* 7.2–17.5 mm long), and it differs from *A. euzeti* in forebody shape, having a different location of the genital pore and having the vitellaria divided in two transversal tubular dendritic fields in the intercecal field *vs* follicular vitellaria divided in two opposing bunches medial or ventral to the caeca.

The ultrastructural characters observed in *A. mundae* sp. nov. using scanning electron microscopy analysis, i.e., a large stylet cavity dorsal to the oral sucker with a large penetration gland opening on each side of the cavity, small penetration gland openings ventral to the stylet cavity, and small penetration gland openings arranged in a ring around the mouth are suspected to be of taxonomic relevance. However, to date, there are no ultrastructural data for other species of *Anaporrhutum* for comparison.

Molecular and phylogenetic analyses

Two 28S rDNA sequences (899 bp and 901 bp) and two identical ITS2 (458 bp) were generated from two individuals of *A. mundae* sp. nov. Sequences were deposited in GenBank under the accession numbers PP133643 and PP354907 for the 28S and PP133644 for ITS2. The BLASTn search of the sequences obtained retrieved a percentage of identity of 93.8% and 93.6% for the 28S and 94.2% for ITS2 with the only sequences of the genus available in GenBank (KF013184: 1144 bp, 28S; KF013159: 437 bp, ITS2), corresponding to an unidentified *Anaporrhutum* sp. found in the brownbanded bamboo shark *Chiloscyllium punctatum* Müller & Henle in Australia.

Because BI and ML trees had similar topologies, only the BI trees of both 28S (Figure 4) and ITS2 (Figure 5) are shown. BI and ML phylogenetic tree separated 28S sequences according to their families and subfamilies. For instance, both sequences obtained from

A. mundae sp. nov. clustered together and with another *Anaporrhutum* sp. (KF013184). In addition, the subfamily Anaporrhutinae formed three clusters: one with *Anaporrhutum* spp. and *Staphylorchis cymatodes* (Johnston, 1913) Travassos, 1922, a second group with *Plesiochorus* spp. Looss, 1901, and a third one with *Nagmia* spp. Nagaty, 1930. The subfamily Gorgoderinae included sequences available for *Phyllodistomum* spp. Braun, 1899, *Gorgodera cygnoides* (Zeder, 1800) Looss, 1899 and *Cercaria duplicata* (accepted as *Phyllodistomum folium* (Olfers, 1816) Braun, 1899). Sequences available for the family Allocreadiidae included *Crepidostomum* spp. Braun, 1900. All tree branches had good confidence with posterior probabilities (PP) larger than 0.726, except in the division of subfamilies Anaporrhutinae and Gorgoderinae, which had a PP = 0.449. However, the ML tree showed a value of 87 bootstraps in this same subdivision.

The phylogenetic BI and ML tree for the ITS2 sequences grouped sequences as in the 28S gene with high PP and bootstrap values. However, sequences of the subfamily Gorgoderinae were not available for the analysis. In addition, the low number of sequences available led to the formation of two clusters in the subfamily Anaporrhutinae in the BI tree: one with *Plesiochorus* spp. and a second group with *S. cymatodes*, *Anaporrhutum* spp. and *Nagmia* sp. In the ML tree, the two groups were also observed, but *Nagmia* sp. clustered with *Plesiochorus* spp. instead of *Anaporrhutum* spp. and *S. cymatodes* sequences.

Discussion

Members of the family Gorgoderidae infecting elasmobranchs belong to five genera within the subfamily Anaporrhutinae Loss, 1901. These are *Anaporrhutum*, *Nagmia*, *Petalodistomum* Johnston, 1913, *Probolitrema* Looss, 1902, and *Staphylorchis* Travassos, 1922 (Curran *et al.* 2003; Cutmore *et al.* 2010). In the most recent review of Gorgoderidae, most of genera of Anaporrhutinae were divided into a number of subgenera (see Campbell 2008); however, because none of the subgenera proposed have received general acceptance by the scientific community (Cutmore *et al.* 2010, 2013), we follow in recognising them as full genera.

The specimens described here agree well with the diagnostic morphological characters of the genus *Anaporrhutum* as described by Curran *et al.* (2003) and Campbell (2008). In particular, the main characters used for their identification as belonging to *Anaporrhutum* were the caeca without diverticula, the testicular follicles traversing the caeca, the vitellaria medial or ventral to the caeca, and the H-shaped excretory vesicle.

Species of the genus *Anaporrhutum* had historically been erected based solely on their traditional morphological characters. Unfortunately, many of them are still poorly known and inadequately described. For instance, in the original descriptions of the species of *Anaporrhutum*, there is no mention of any tegumental structures. Only Curran *et al.* (2003), in the original description of *A. euzeti*, reported the occurrence of an ellipse-shaped opening pit at the anterior end of the oral sucker; however, no other information or figures were provided.

The present study of the tegumental surface of *A. mundae*, as revealed by scanning electron microscopy, allowed us to detect new morphological characters for a member of Anaporrhutinae that may be of systematic interest. Within the Gorgoderidae, these structures (i.e., the frontal pit, the stylet cavity, and the penetration gland openings) have been reported in some members of Gorgoderinae in which four types of presumed sensory papillae
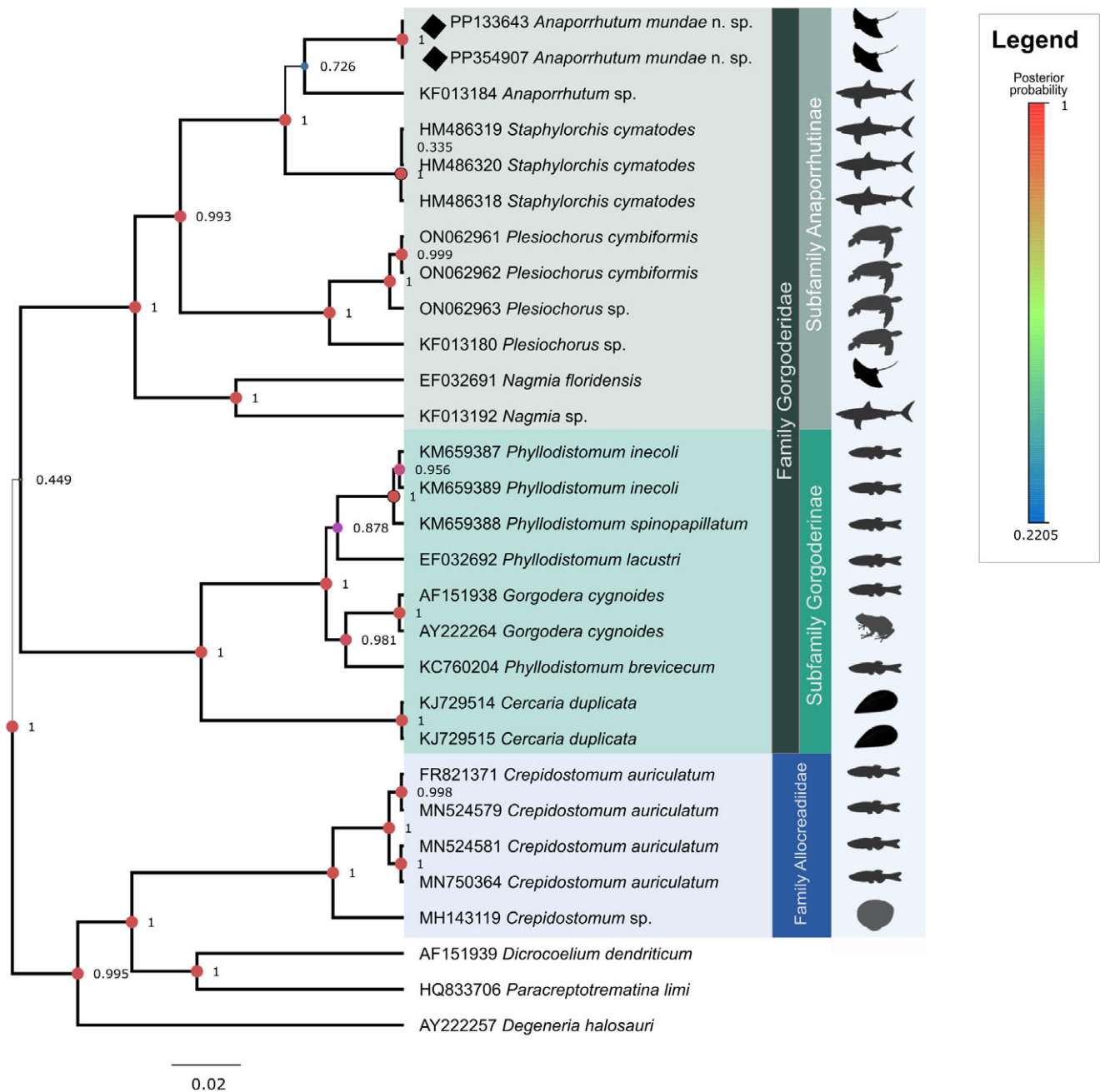
Figure 4. Bayesian inference phylogenetic tree for the 28S gene fragment of sequences derived from organisms of the families Gorgoderidae and Allocreadiidae. The tree was reconstructed using a General Time Reversible with gamma substitutions nucleotide substitution model. Line width and node circles are proportional to the posterior probabilities. The host group from which the organism was collected is indicated as a cartoon.

(here not found) were also described (Bakke and Bailey 1987; Bakke and Hoole 1988; Hoole *et al.* 1983; Mata-López and León-Règagnon 2006). According to Hoole *et al.* (1983), the frontal pit and stylet cavity in juvenile and adult gorgoderids might be a vestigial organ with no active function, although the function of the penetration gland openings remains enigmatic. The presence of penetration gland pores occurring anterior and lateral to the frontal pit have been described in metacercarial cysts and adults of *Gorgoderina vitelliloba* (Olsson, 1876) infecting *Rana temporaria* Linnaeus (Hoole *et al.* 1983). In *Gorgoderina festoni* Mata-López, 2005, penetration gland openings were arranged around the frontal pit (Mata-López and León-Règagnon 2005). Moreover,

Mata-López and León-Règagnon (2006), who studied six species of *Gorgoderina* Looss, 1902 infecting amphibians in Mexico, observed the occurrence of a frontal pit and/or stylet cavity in all species, but it varied in shape and size among species. In contrast, the penetration glands showing different arrangement patterns were observed only in *G. diaster* Lutz, 1926, *G. parvicava* Travassos, 1922, and *G. megacetabularis* Mata-López, León-Règagnon & Brooks, 2005. Comparative analyses on the detailed tegumental topography of gorgoderids of the genus *Phyllodistomum* clearly indicates that scanning electron microscopy is a pivotal tool to discriminate among closely related species (Pérez-Ponce de León *et al.* 2015a; Petkevičiūtė *et al.* 2020),
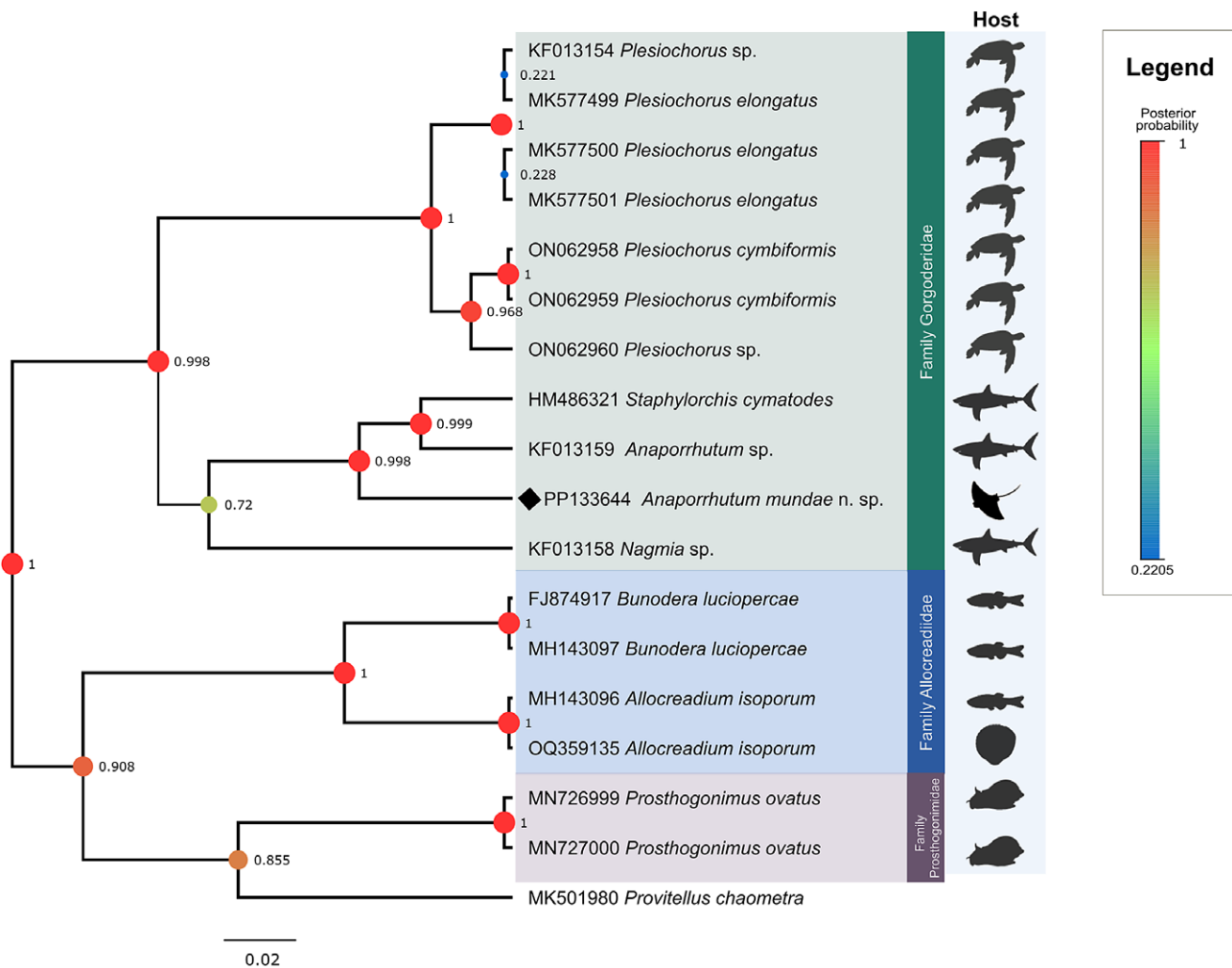
Figure 5. Bayesian inference phylogenetic tree for the ITS2 fragment of sequences derived from organisms of the families Gorgoderidae, Allocreadiidae, and Prostogonimidae. The tree was reconstructed using a Hasegawa-Kishino-Yano nucleotide substitution model. Line width and node circles are proportional to the posterior probabilities. The host group from which the organism was collected is indicated as a cartoon.

because it is the only reliable method that can reveal the presence of small tegumental structures not easily visible under the light microscope. The comparative re-examination of species of *Anaporrhutum* using scanning electron microscopy can provide useful information in the taxonomic study of the group. This could also afford understanding of whether in this genus, the ultrastructural characters here described can be used as diagnostic characters.

Concerning the molecular characterisation, we presented the first 28S rDNA and ITS2 sequences for an identified species of *Anaporrhutum*. In particular, we also amplified the ITS2 rDNA because it has been used extensively in trematode taxonomy (including also Gorgoderidae), possessing sufficient variability to distinguish even closely related species (Nolan *et al.* 2006; Cutmore *et al.* 2010, 2013). Unfortunately, at present only ITS2 and 28S rDNA sequences of four taxa of Anaporrhutinae from elasmobranchs are available in GenBank for comparison. These are those of unidentified species of *Anaporrhutum* and *Nagmia* from *Chiloscyllium punctatum* and *Stegostoma tigrinum* (Forster) respectively, and *Staphylorchis cymatodes* from *Carcharhinus sorrah* (Müller & Henle), *Sphyrna lewini* (Griffith & Smith), and *C. punctatum*, all from Australia (Cutmore *et al.* 2010, 2013), and

Nagmia floridensis Markell, 1953 from *Hypanus sabinus* (Lesueur) and *Rhinoptera bonasus* (Mitchill) from the coasts of the United States of America (Olson *et al.* 2003; Curran *et al.* 2006). The present phylogenetic analyses included only a relatively small subset of the 32 putative species of Anaporrhutinae listed in WoRMS (2023) thus far. Nevertheless, our results are congruent with those of Cutmore *et al.* (2013) who suggested that taxa infecting elasmobranchs host-switched into sea turtles. Unfortunately, due to the scarcity of molecular data from Anaporrhutinae, it is not currently possible to suggest other conclusions about the relationships among members of this subfamily.

Finally, the site of infection of *A. munda* was also of remarkable interest. Indeed, we found the new species in the gill chambers of *U. munda*, but most records of members of *Anaporrhutum* are from the body-cavity of elasmobranchs. Some exceptions are those reported by von Ofenheirn (1900) and Curran *et al.* (2003), who found *A. albidum* and *A. euzeti* in the pericardium of the ray *Aetobatis narinari* (Euphrasen) and the pericardium and body cavity of several other ray species, respectively, and Simha *et al.* (1971), who reported *A. narayani* from the buccopharyngeal cavity of *Mobula mobular* (Bonnaterre). Based on the present findings, the

Munda round ray represents a new host record and Costa Rica a new geographical record for *Anaporrhutum* species (see Solano-Barquero *et al.* 2023). Further, according to Caira *et al.* (2022) and Pollerspöck and Straube (2023), *A. mundae* represents the first parasite described or reported for this host.

In conclusion, previous studies (Cutmore *et al.* 2010, 2013) and the present results show that the species of the genus *Anaporrhutum* need revision based on both molecular analysis and detailed morphological redescriptions (including light and scanning electron microscopy), emphasising the need to use the integrative taxonomy for accurate species identification and to provide insights into their poorly known life history.

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