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Linking phenotypic to genotypic metacestodes from *Octopus maya* of the Yucatan Peninsula

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A B S T R A C T

Octopus maya is an endemic species of the Yucatan Peninsula. This species sustains the octopus' fishery in the region and is the only cephalopod cultured in Mexico. It is known that *O. maya* harbor a large richness and abundance of metacestodes that have been tentatively identified by light microscopy alone. Since the larval stages of some orders of marine cestodes lack the taxonomic characteristics shown by the adult stages and on which cestode taxonomy is based, identification down to the species level is often unattainable. Hence, the goal of this study was to characterize the parasites, for the first time, at morphological and molecular levels. A total of 60 octopuses were collected from September to December 2017 from four fishery landing ports in Yucatán: Sisal, Progreso, Dzilam de Bravo, and Rio Lagartos (15 hosts per locality). Morphology of metacestodes was characterized by light and Scanning Electron Microscopy (SEM), while the genes 18S and 28S rDNA were sequenced for molecular characterization. Based on phenotypic characters and molecular data, seven taxa of metacestodes were identified, four of them belonging of order Trypanorhyncha: *Eutetrarhynchus* sp., *Kotorella pronosoma*, *Nybelinia* sp., *Prochristianella* sp. 1; and the three remaining taxa belonging to the order Oncho-proteocephalidea: *Acanthobothrium* sp., *Phoreiobothrium* sp., and *Prosobothrium* sp. This work provides, for the first time, molecular support to the morphological characterization of metacestodes recorded in *Octopus maya.*

1. Introduction

Octopus maya is an endemic species of the Yucatan Peninsula that sustains the most important cephalopod fisheries in Mexico and is the main target species to develop the octopus' commercial culture in this country (Avendaño et al., 2019, 2020). To date, the biology, physiology, and impact of climate change on *O. maya* health and reproduction have been studied and applied to aquaculture and fishery of this species (Moguel et al., 2010; Rosas et al., 2014; Gallardo et al., 2017; Guillén-Hernández et al., 2018a; Roumbedakis et al., 2020; Ángeles-González et al., 2021).

Parasitological studies however are still insufficient. The available data reveal that *O. maya* found around the Yucatan Peninsula are infected by 20 parasite taxa that include the marosporidians *Aggregata* sp., digeneans (i.e., *Podocotyle* sp., *Lecitochirium* sp., *Parvatrema* sp., *Dollfustrema* sp., Cryptogonimidae gen. sp., Opecoeliidae gen. sp., *Stephanostomum* sp., Bucephalidae gen. sp.), cestodes (i.e., *Echeneibothrium* sp., *Prosobothrium* sp., *Otobothrium* sp., *Nybelinia* sp., *Eutetrarhynchus* sp., *Prochristianella* sp., 'Tetraphyllidea gen. sp.'), nematodes (i.e., Spiruridae gen. sp., Philometridae gen. sp., Anisakidae gen. sp.), and crustacean (*Octopicola* sp.). Most of the parasites were found in larval stages except the protozoan *Aggregata* sp. and the crustacea *Octopicola* sp., suggesting the role of *O. maya* as an intermediate and paratenic host in the life cycle of most of these parasites (Hochberg, 1990; Guillén-Hernández et al., 2018a).

Cestode larvae is one of the most diverse parasitic groups infecting *O. maya.* The most abundant (1034 parasites on average) and prevalent (up to 98%) is the unidentified species of genus *Prochristianella*. (order Trypanorhyncha) (Guillén-Hernández et al., 2018a). *Prochristianella* sp. primarily infects the anterior salivary glands of the octopus and destroy the epithelial secretory gland cells causing haemocytic infiltration, replacement of glandular tissue by connective tissue and, presumably, dysfunction of salivary gland cells (Guillén-Hernández et al., 2018b; Cruz-Quintana et al., 2019).

Knowledge about cephalopod parasitology has increased in the last decade, however, some gaps remain to be unresolved such as the

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identification of helminth larval stages (Roumbedakis et al., 2018). Metacestodes from the order Trypanorhyncha, 'Tetraphyllidea', and Onchoproteocephalidea have been recorded in different cephalopod species, mainly infecting the digestive tract (Hochberg, 1990; Caira and Jensen, 2017; Roumbedakis et al., 2018; Tedesco et al., 2020). The precise identification of some species in larval stages are complicated since it requires observing the morphological features exhibited by adult stages, lacking in metacestodes. Hence, molecular markers (18S and 28S rDNA) and phylogenetics, coupled with morphological data that are being used to identify metacestodes as precisely as possible (Tedesco et al., 2020; Adán-Torres et al., 2022).

In the present study, we aim to identify the cestodes parasitizing *O. maya* in areas of its distribution in Yucatan using both morphological and molecular data. This study is important to complement the data yet available and increase our knowledge about the parasite life cycles and the role of cephalopods in cestodes biology.

2. Material and methods

2.1. Octopus collection and parasitological examination

Octopus samples were collected (15 host/locality) from September to December 2017, from the local fishery developed in Yucatan, Mexico, by traditional fishing methods ("garete" fishing) located near Sisal (21°10′ N, 90°1′ W), Progreso (21° 19′ N, 89° 42′W), Dzilam de Bravo (21° 19′ N, 88° 35′W), and Rio Lagartos (21° 24′ N, 88° 02′W) (Fig. 1). The specimens were immediately transported in coolers to the Laboratorio de Patologia Acuatica at the Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV, Merida City, Yucatan, Mexico). Once in the laboratory, the dorsal mantle length (DML) and total length (TL) of each octopus were measured to the nearest millimeter (mm) with a measuring tape (±0.1 mm); while the total weight (g) was recorded to the nearest 0.1 g with an Ohaus E0B120 weighing scale (±0.01 g). A longitudinal cut in the ventral mantle was performed to remove the digestive tract (buccal mass, digestive gland, ink sac, intestine, esophagus, and caecum), gills, eyes, and muscle to examine for metacestodes under a stereomicroscope (Motic SMZ–168) according to Guillén-Hernández et al. (2018a). No cestodes were found alive. Cestodes were removed from the tissues and fixed in formalin for phenotypic analysis and in 96% ethanol for molecular analysis.

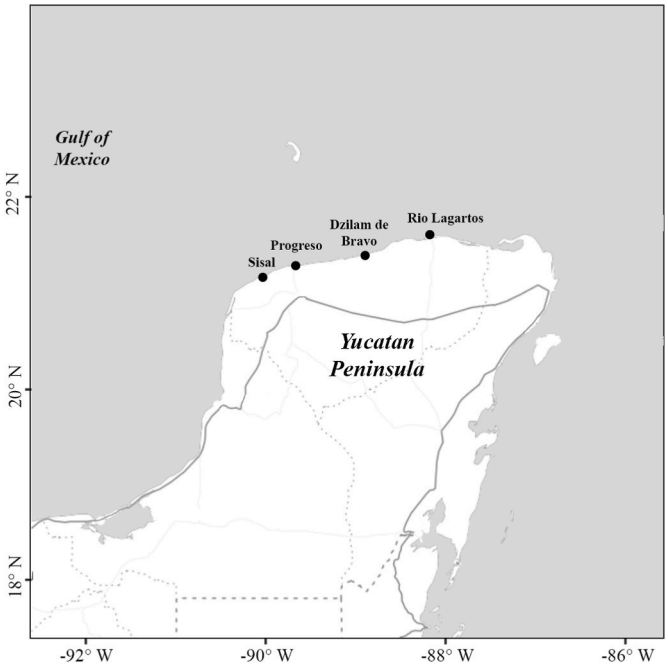

Fig. 1. Sampling localities where specimens of *Octopus maya* were collected in Yucatan, Mexico.

2.2. Light and Scanning Electron Microscopy (SEM)

For light microscopy, the metacestodes were stained with Mayer–-Schubergs Carmine technique, mounted in Canada balsam (Palm, 2004), and identified according to Khalil et al. (1994), Palm (2004) and Jensen and Bullard (2010). Drawings were made from photomicrographs with the help of Wacom Intous Tablet and Corel Painter 6 software. For ultrastructural analysis by SEM, the larvae were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C for 12 h, washed three times with phosphate-buffered saline (PBS) 1X at room temperature, and post-fixed in 1% OsO_4 for 1 h. The parasite samples were dehydrated in an ascending ethanol series (30%, 50%, 70%, 90, 100%), critical point dried in CO_2 in a K850 Critical Point Dryer (Quorum Technologies, East Sussex, United Kingdom), sputter-coated in a Quorum Q150R ES (Quorum Technologies, East Sussex, United Kingdom) 60% gold-palladium, and examined in a Jeol-7600F0 (JEOL Ltd., Tokyo, Japan) operated at 5–25 kV.

2.3. DNA extraction, PCR amplification, and sequencing

Genomic DNA (gDNA) was individually extracted from whole specimens that were previously fixed in 96% ethanol using the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The 18S rDNA was amplified with the primers WormA (5′–GCGAATGGCTCATTAAATCAG–3′) and WormB (5′–CTTGTTACGACTTT TACTTCC–3′) (Littlewood and Olson, 2001); while the 28S rDNA was amplified using the primers 391 (5′–AGCGGAGGAAAAGAAACTAA–3′) (Nadler and Hudspeth, 1998) and 536 (5′–CAGCTATCCTGAGGGAAAC–3′) (García-Varela and Nadler, 2005). The PCR mix in each tube contained: 12.5 µl of Green GoTaq Master Mix (Promega, Madison, WI, USA), 1 µl of each primer (10 µM), 8.5 µl of double-distilled sterile water, and 2 µl of gDNA for a final volume of 25 µl. All PCR reactions (18S rDNA and 28S rDNA) were run in an Axygen® MaxyGene™ II thermocycler as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1 min, and finally an extension at 72 °C for 10 min. The Polymerase Chain Reaction (PCR) products were analyzed on 1% agarose gel electrophoresis in TAE 1X buffer, stained with RedGel (Biotium, San Francisco, USA), and visualized under UV light using the QIAxcel® Advanced System. The PCR products were forward and reverse sequenced with the same pair of primers previously used for amplification. To confirm the middle of the sequence, the internal pair of primers 503 (5′–CCTTGGTCCGTGTTTCAAGACG–3′) (Stock et al., 2001) and 504 (5′–CGTCTTGAAACACGGACTAAGG–3′) (García-Varela and Nadler, 2005) were used for 28S rDNA, whereas the internal primers 300R (5′–TCAGGCTCCCTCTCCGGA–3′) and 600R (5′–ACCGCGGCKGC TGGCACC–3′) (Littlewood and Olson, 2001) were used to amplify the 18S rDNA. Purification and sequencing of the PCR products were carried out by the Sanger sequencing method at Genewiz, South Plainfield, NJ, USA (https://www.genewiz.com/).

2.4. Phylogenetic analysis

The new sequences were edited in Geneious Pro 4.8.4 software (Biomatters Ltd., Auckland, New Zealand) and a consensus sequence was obtained for each specimen, except for the 28S rDNA of *Nybelinia* sp. and 18S rDNA from Onchoproteocephalidea since no quality sequences were obtained to perform phylogenetic analysis. The consensus sequences of each specimen were deposited in the Genbank (accession numbers are shown in the phylogenetic trees). Paragenophore were deposited in the Colección Nacional de Helmintos, UNAM (catalog numbers Table 1). All the sequences were checked using the Basic Local

Table 1
Summary of infection parameters of cestodes found in *Octopus maya* at Yucatan Peninsula. Abbreviations: BM = buccal mass; C = caecum; DG = digestive glandule; E: esophagus; G = gills; IS = ink sac; I = intestine.

Cestode	Host infected (n)	Prevalence %	Mean Abundance (±SD)	Site of Infection	Catalog Number
Eutetrarhynchus sp. 1	38	63	3(±4)	DG, IS	CNHE 11698
Kotorella pronosoma	8	13	0.2(±0.8)	I, E	CNHE 11693-95
Nybelinia sp. 1	2	3	0.03(±0.2)	G, E	CNHE 11696
Prochristianella sp. 1	60	100	456 (±766)	BM	CNHE 11697
Acanthobothrium sp.	11	18	0.2(±0.3)	C	CNHE 11699
Phoreiobothrium sp.	1	2	0.03(±0.1)	IS	–
Prosobothrium sp.	4	7	0.2(±0.4)	IS	–

Alignment Search Tool (BLAST) (www.ncbi.nih.gov/BLAST/). Three datasets of the metacestodes were generated in Mesquite 3.62 (https://www.mesquiteproject.org/) to determine the phylogenetic relationship: two datasets (18S rDNA and 28S rDNA) for Trypanorhyncha and a single one (28S rDNA) for Onchoproteocephalidea (since the 18S rDNA could not be amplified). The sets of data were complemented with a total of 157 taxa available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) whose accession numbers can be viewed in the phylogenetic trees. Based on previous phylogenetic assays, *Vittirhynchus squali* was chosen as the outgroup for Trypanorhyncha (Olson et al., 2010) and *Yorkeria hilli* as the outgroup for Onchoproteocephalidea (Caira et al., 2014). Each dataset was subsequently aligned with ClustalW (Thompson et al., 1994) and implemented on the website http://www.genome.jp/tools/clustalw/with the approach "SLOW/ACCURATE" and weight matrix "CLUSTALW" (for DNA). Long sequences from GenBank were trimmed to match the maximum length of the sequences of this study using Mesquite3.62. Likewise, sequences with more than 50% of the total length of the alignment of each dataset were considered in the analyses. The nucleotide substitution model was estimated for each dataset with the program jModelTest v2 (Darriba et al., 2012). For the phylogenetic analysis, the Maximum Likelihood (ML) method was implemented with the RAxML v. 7.0.4 software (Stamatakis, 2006). Phylogenetic analysis was performed with 1000 repetitions of Bootstrap (Bt) to obtain the best phylogenetic tree of each dataset. The ML trees were visualized in FigTree v.1.4.3 (Rambaut, 2006). and the genetic distances were calculated with MEGA v.6 (Tamura et al., 2013).

2.5. Infection parameters

The number of each cestode morphotype per host was recorded for further estimation of the prevalence (P) and mean abundance (MA) according to Bush et al. (1997). Prevalence (P) is the number of hosts infected with one or more individuals of a particular parasite species divided by the number of hosts examined for that parasite species. It is commonly expressed as a percentage. Mean abundance (MA), on the other hand, is the total number of individuals of a particular parasite species in a sample of a particular host species divided by the total number of hosts of that species examined (including those uninfected), ±SD.

3. Results

3.1. Infection in Octopus maya

A total of 60 specimens of *Octopus maya* (DML = 112.5 mm, weight = 451.1 g) were examined and 100% were found infected by plerocercus stages of Trypanorhyncha (four morphospecies) and/or plerocercoid stages of Onchoproteocephalidea (three morphospecies) (Table 1).

3.2. Morphological description

3.2.1. *Eutetrarhynchus sp. (Trypanorhyncha: Eutetrarhynchidae) (n = 5, Fig. 2A, Fig. 3A–C) (GenBank number: OP035616, OP035647, OP035648)*

Body elongated, 1100–6000 μm in total length (Fig. 3A). Scolex slender, acraspedote, 1050–3000 μm in length, 770–1100 μm in width (Fig. 2A). Two oval bothria, from 140 to 270 μm. Pars vaginalis longer than pars bothrialis. Pars bulbosa from 319 to 789 μm. Long and slender tentacles emerge from long bulbs (Fig. 2A). Metabasal armature heteroacanthous, typically heteromorphous. Spiniform and falciform hooks (Fig. 2A).

3.2.2. *Kotorella pronosoma (Trypanorhyncha: Tentacullariidae) (n = 3, Figs. 2B, Fig. 3D–F) (GenBank number: OP035617, OP035618, OP035619, OP035644, OP035645)*

Small larvae, 500–1000 μm in total length, 90–110 μm in width (Fig. 2B). Scolex elongated, craspedote, velum (Fig. 2B). Four elongated bothria, 158–174 μm. Pars post bulbosa absent (Fig. 2B). Oval bulbs, 49–53 μm. Short tentacles, retractor muscles originate at the base of bulbs (Fig. 2B). Tentacular armature homeocanthous, heteromorphous. Solid hooks arranged in quincunxes are all almost the same size along all tentacles. Small and spiniform hooks (Fig. 3D–F).

3.2.3. *Nybelinia sp. (Trypanorhyncha: Tentacullariidae) (n = 2, Figs. 2C, Fig. 3G–I) (GenBank number: OP035641)*

Small larvae from 800 to 1000 μm in total length, 800–900 μm in width (Fig. 2C; Fig. 3G). Scolex small, craspedote, velum, 300–315 μm. Four bothria in opposite arrangements, 190–225 μm. Oval bulbs, 60–80 μm. Short tentacles, retractor muscles originate at the base of bulbs (Fig. 2C). Metabasal armature homeocanthous. Large, falciform, and solid hooks (Fig. 2C).

3.2.4. *Prochristianella sp. 1 (Trypanorhyncha: Eutetrarhynchidae) (n = 4, Figs. 2D, Fig. 3J–L) (GenBank number: OP035620, OP035621, OP035642, OP035643)*

Elongated larvae, 600–920 μm in total length, 60–150 μm in width (Fig. 3J). Scolex small, acraspedote. Two quadrangular bothria, 61–127 μm (Fig. 2D). Pars post bulbosa absent. Long tentacles, retractor muscles with origin at the posterior part of bulbs (Fig. 2D). Pars bulbosa, 90 μm. Metabasal armature heteroacanthous typical. The tentacular armature consists of ascending spirals of solid and spiniform hooks (Fig. 2D).

3.2.5. *Acanthobothrium sp. (Onchoproteocephalidea: Onchobothriidae) (n = 3, Figs. 2E, Fig. 3M–N) (GenBank number: OP035622)*

Elongated larvae, 4000–5000 μm in total length, 700–800 μm in width (Fig. 2E; Fig. 3M). Scolex small, with apical sucker, 115–130 μm. Four elongated and sessile bothridia, 65–72 μm. Each bothridia is divided into three loculi with two transversal septa (Fig. 2E). Strobila is large with calcareous corpuscles (Fig. 2E).

3.2.6. *Phoreiobothrium sp. (Onchoproteocephalidea: Onchobothriidae) (n = 1, Figs. 2F, Fig. 3O–P) (GenBank number: OP035623)*

Small larvae, 250 μm in total length, 120 μm in width (Fig. 2F;

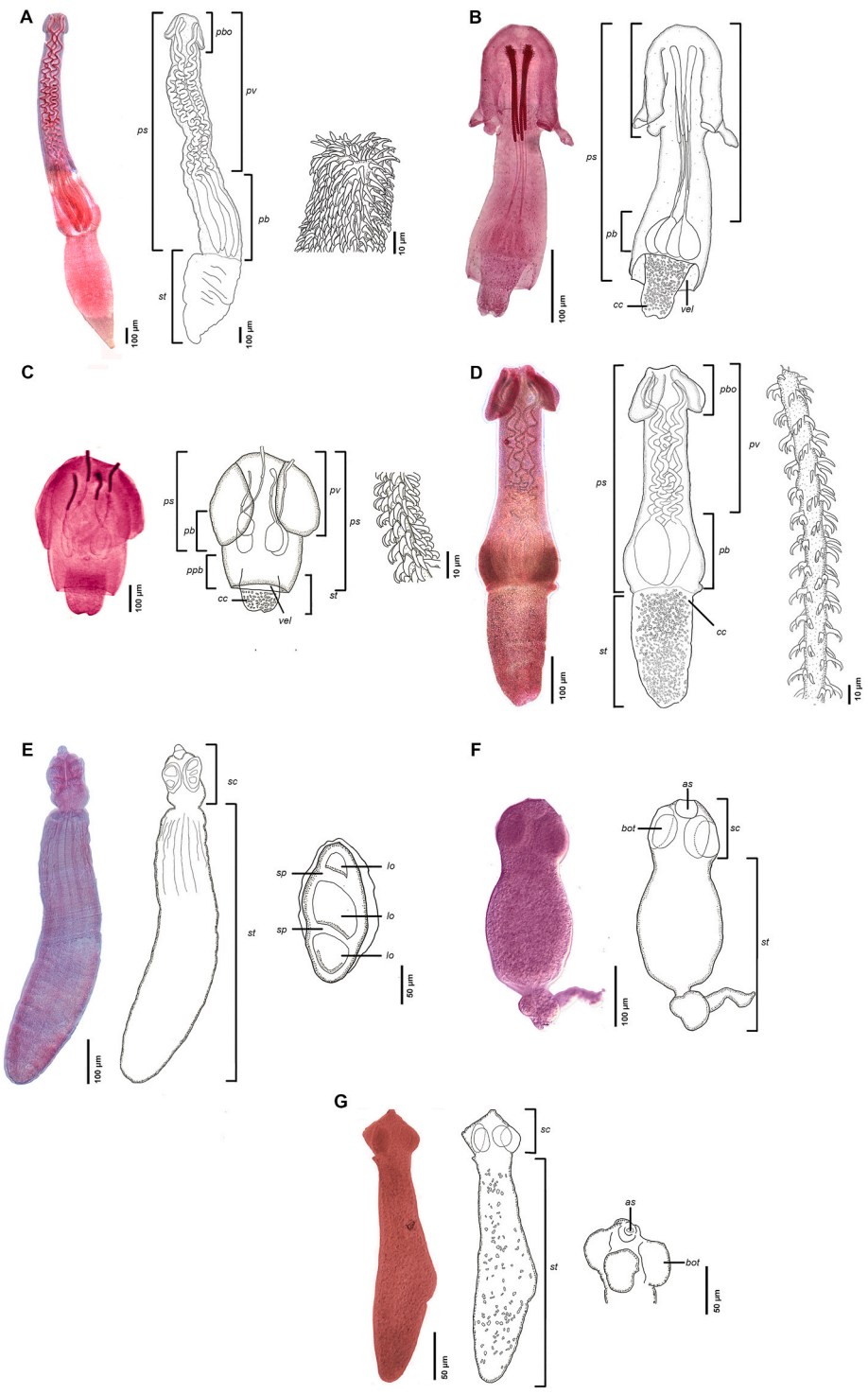

Fig. 2. Schematic drawings and photographs of the stained specimens of cestode parasitizing *Octopus maya*. **Trypanorhyncha** (A–D): **A-** *Eutetrarhynchus* sp.; **B.** *Kotorella pronosoma*; **C-** *Nybelinia* sp.; **D-** *Prochristianella* sp. **Onchoproteocephalidea** (E–G): **E-** *Acanthobothrium* sp.; **F-** *Phoreiobothrium* sp.; **G-** *Prosobothrium* sp.- Abbreviations: as = apical sucker; bot = bothridia cc = calcareous corpuscles; lo = loculi; pb = pars bulbosa; pbo = pars bothrialis; ppb = pars postbulbosa; ps = pedunculus scolecis; pv = pars vaginalis; sc = scolex; sp = septa; st = strobilo; vel = velum.

Fig. 3O). Scolex with apical sucker. Four bothridia with quadrangular shape, 45–60 µm (Fig. 2F). No other structure was observed.

3.2.7. Prosobothrium sp. (Onchoproteocephalidea: Prosobothriidae) (n = 1, Figs. 2G, Fig. 3Q–R)

Elongated larvae, 400–600 µm in total length, 100–200 µm in width (Fig. 2G; Fig. 3Q). Four small and plate bothridia 60 to 40 µm. Apical sucker, and calcareous corpuscles in all strobila (Fig. 2G).

3.3. Phylogenetic relationship

3.3.1. Trypanorhyncha

For the 28S rDNA, three partial sequences (995–1445 bp in length) from *Kotorella pronosoma*, two (1411 and 1453 bp in length) from *Prochristianella* sp., and a single sequence from *Eutetrarhynchus* sp. (1460 bp in length) were included in an aligned dataset of 1631 bp in length, with nucleotide frequencies of A = 0.214604, C = 0.21835, G = 0.322620, and T = 0.244423. The maximum likelihood (ML) value of the tree obtained with 28S rDNA sequences was ln = −15209.189111. The topology showed two main clades (Fig. 4). The Trypanorhyncha (*Kotorella*

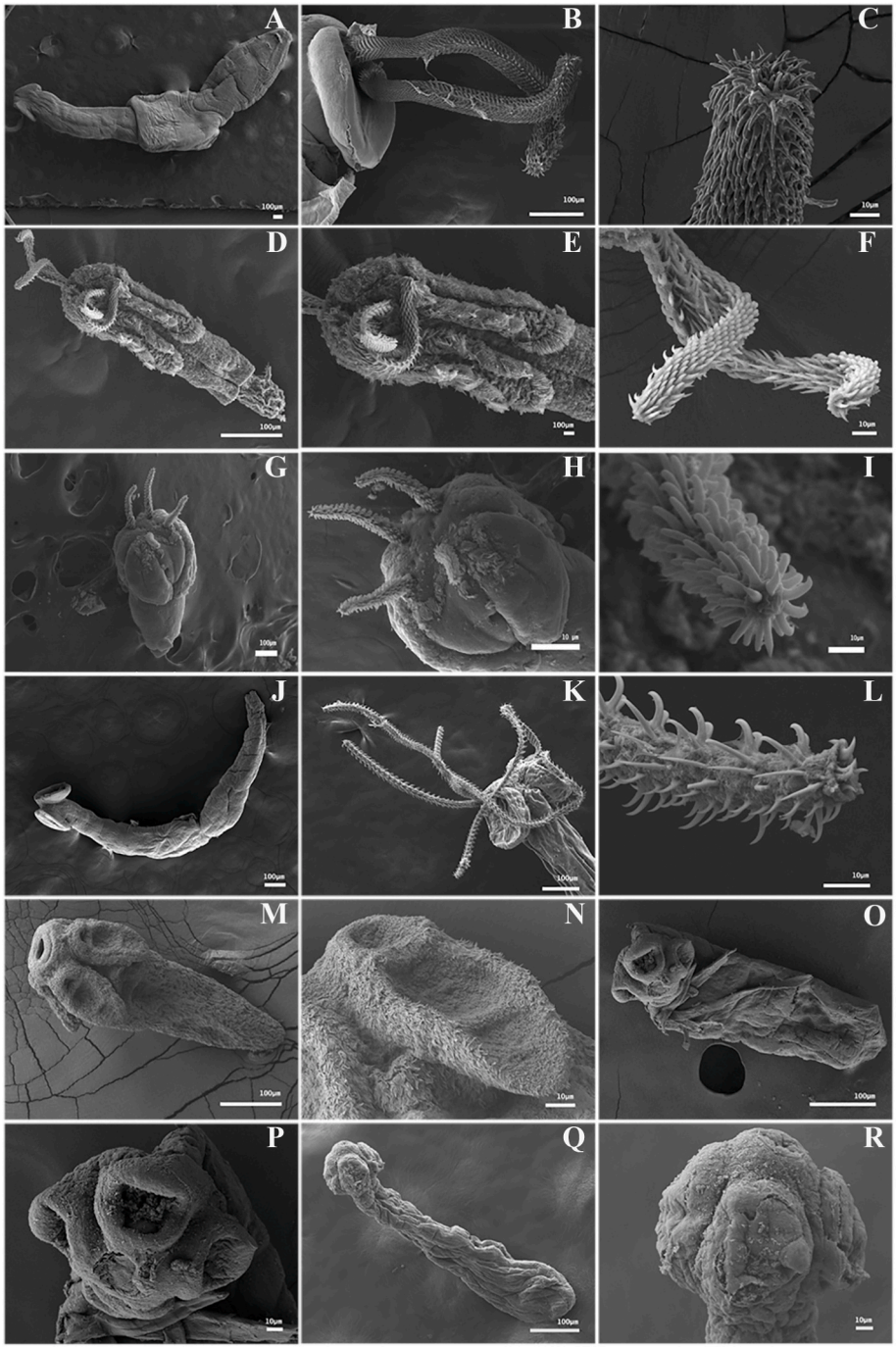

Fig. 3. Scanning electron microscopy of whole specimens and the detail of scolecis of the cestodes found as parasites of *Octopus maya*. **Trypanorhyncha** (A–L): **A-C** *Eutetrarhynchus* sp.; **D-F** *Kotorella pronosoma*; **G-I** *Nybelinia* sp.; **J-L** *Prochristianella* sp. **Onchoproteocephalidea** (M–R): **M-N** *Acanthobothrium* sp. **O–P** *Phoreiobothrium* sp.; **Q-R** *Prosobothrium* sp.

pronosoma, *Eutetrarhynchus* sp., and *Prochristianella* sp.) from *O. maya* are grouped in the same subclade (Fig. 4). *Kotorella pronosoma* formed a non-monophyletic group with the genera *Tentacularia*, *Nybelinia*, *Heteronybelinia*, and *Mixonybelinia* (Bt = 100; Fig. 4). The *Kotorella* genus was non-monophyletic in our analysis since two independent groups were formed. In one group, *K. pronosoma* ex *O. maya* nested with specimens ex *Syacium papillosum* (Pleuronectiformes) and *Hypanus say* (Myliobatiformes) (Bt = 100; Fig. 4) without genetic differences among them; whereas *Kotorella* sp. and *K. pronosoma* ex *Taeniura lymma* (Myliobatiformes) and *Bathytoshia lata* (Myliobatiformes), respectively, nested in an independent clade (Bt = 89; Fig. 4). The genetic divergence between *K. pronosoma* from *O. maya* and *K. pronosoma* ex *T. lymma* and *D. thetidis* was 2% and 3.5%, respectively. Concerning *Eutetrarhynchus* sp. ex *O. maya*, it formed a non-monophyletic group with species of *Eutetrarhynchus, Dollfusiella*, *Paroncomegas*, and *Tetrarhynchobothrium* (Bt = 100); *Eutetrarhynchus* also did not form a monophyletic clade since the species *Eutetrarhynchus pacificus* is grouped with *Dollfusiella martini* and not with the species found in the octopus. Genetic distances ranging from 10.5% to 18.2% between *Eutetrarhynchus* sp. and the rest of the species in the clade. Particularly *Eutetrarhynchus* sp. ex *O. maya* differed genetically from *E. pacificus* by 18.2%. *Prochristianella* sp. formed a non-monophyletic group with *Parachristianella*, *Mecistobothrium*, *Tetrarhynchobothrium*, and *Oncomegoides* (Bt = 91; Fig. 4). *Prochristianella* sp. from *O. maya* grouped with *Prochristianella* sp. 1 ex *Sphyrna tiburo*

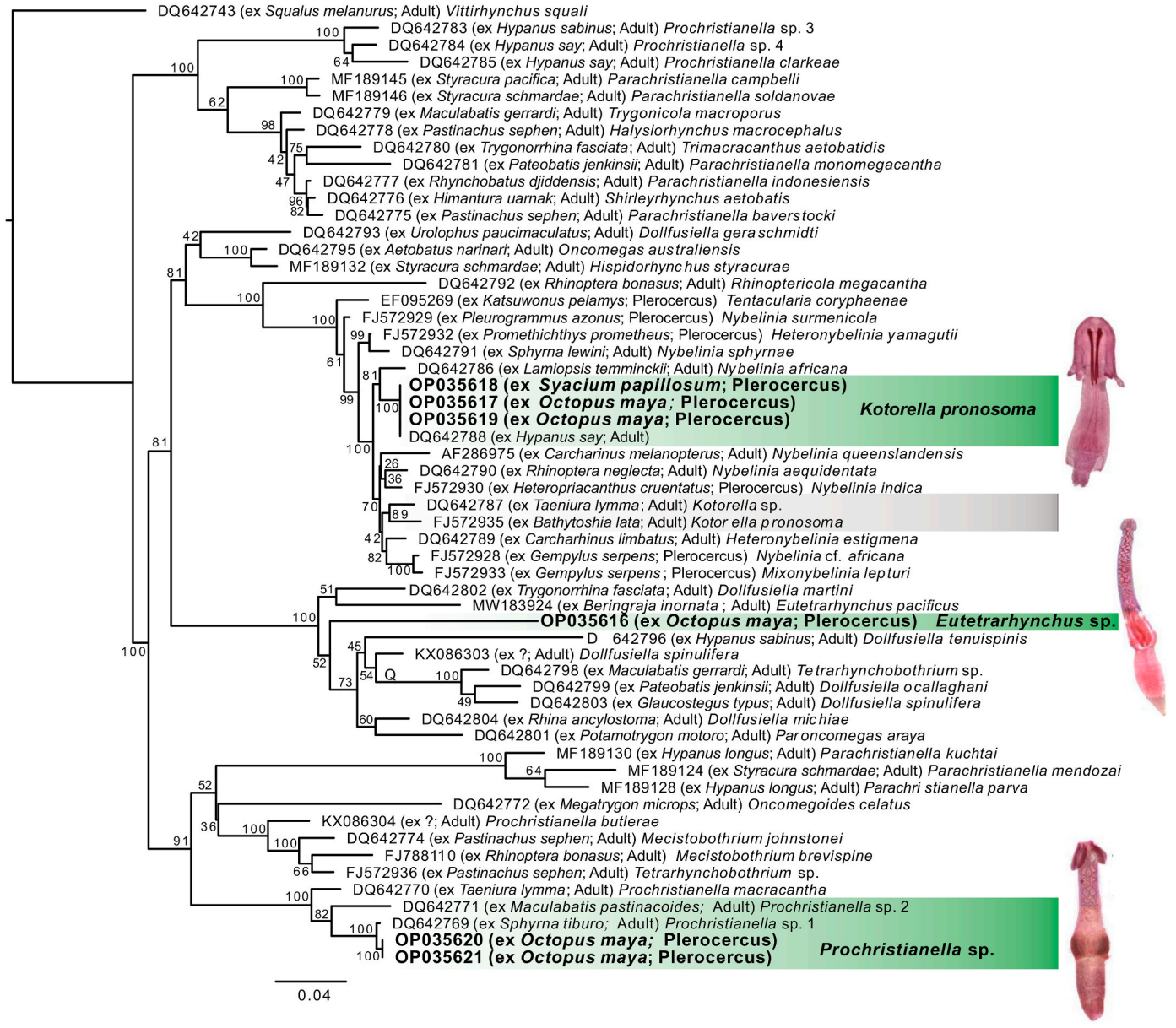

Fig. 4. Phylogenetic tree based on the Maximum Likelihood (ML) analysis of the species of the Order Trypanorhyncha found in *Octopus maya* constructed on partial large subunit ribosomal gene (28S) (likelihood = −15209.189111). Bootstrap support values for ML are provided at the nodes; ex = host; the stage of development of the cestode in parentheses.

(Carcharhiniformes), showing 1% of genetic distance (Fig. 4).

Regarding the 18S rDNA, three partial sequences (545–552 bp in length) from *Prochristianella* sp., two (558 bp in length) from *Eutetrarhynchus* sp., two (545 and 553 bp in length) from *Kotorella pronosoma*, and a single sequence (555 bp in length) from *Nybelinia* sp. were aligned in a dataset of 1524 bp with 59 additional sequences of Trypanorhyncha (nucleotide frequencies A = 0.237116, C = 0.216641, G = 0.284535, and T = 0.261707). The ML value of the 18S rDNA tree was ln = −7987.787948. The resulting phylogenetic tree showed that octopus cestodes grouped in four different clades (Fig. 5). Plerocercoids of *Nybelinia* sp. formed a non-monophyletic group with *Tentacularia*, *Heteronybelinia*, *Kotorella*, and *Mixonybelinia* (Bt = 100; Fig. 5). Particularly, *Nybelinia* sp. ex *O. maya* grouped with *Nybelinia africana* (GenBank ID: DQ642948) (Bt = 43) showing a 3% of genetic distance (Fig. 5). *Kotorella pronosoma* ex *O. maya* grouped with adult stages of the same species found in *H. say* and *B. lata* (0% and 2.7% of interspecific genetic distance, respectively), but did not cluster with *Kotorella* sp. ex *T. lymma*, which showed 2.8% of interspecific genetic divergence with respect to *K. pronosoma* ex *O. maya*. Concerning *Eutetrarhynchus* ex *O. maya*, this parasite grouped with *Dollfusiella*, *Paroncomegas*, and *Tetrarhynchobothrium* with a high support value (Bt = 90), showing an interspecific genetic distance ranging from 8.9% to 12.9% among these species (Fig. 5). Finally, *Prochristianella* sp. ex *O. maya* formed a highly supported group (Bt = 100) with specimens of the same genus infecting *S. tiburo*, *Maculabatis pastinacoides* (Myliobatiformes), and *Prochristianella macracantha* ex *T. lymma*, showing an interspecific genetic distance of 2.4% and 0.7%, respectively, among these species (Fig. 5).

3.3.2. Onchoproteocephalidea

A single sequence of the 28S rDNA gene was amplified for *Phoreiobothrium* sp. (360 bp in length) and *Acanthobothrium* sp. (1060 bp in length). Both sequences were aligned with 60 sequences of Onchoproteocephalidea providing an aligned dataset of 1155 bp with nucleotide frequencies of A = 0.217083, C = 0.224792, G = 0.325049, T =

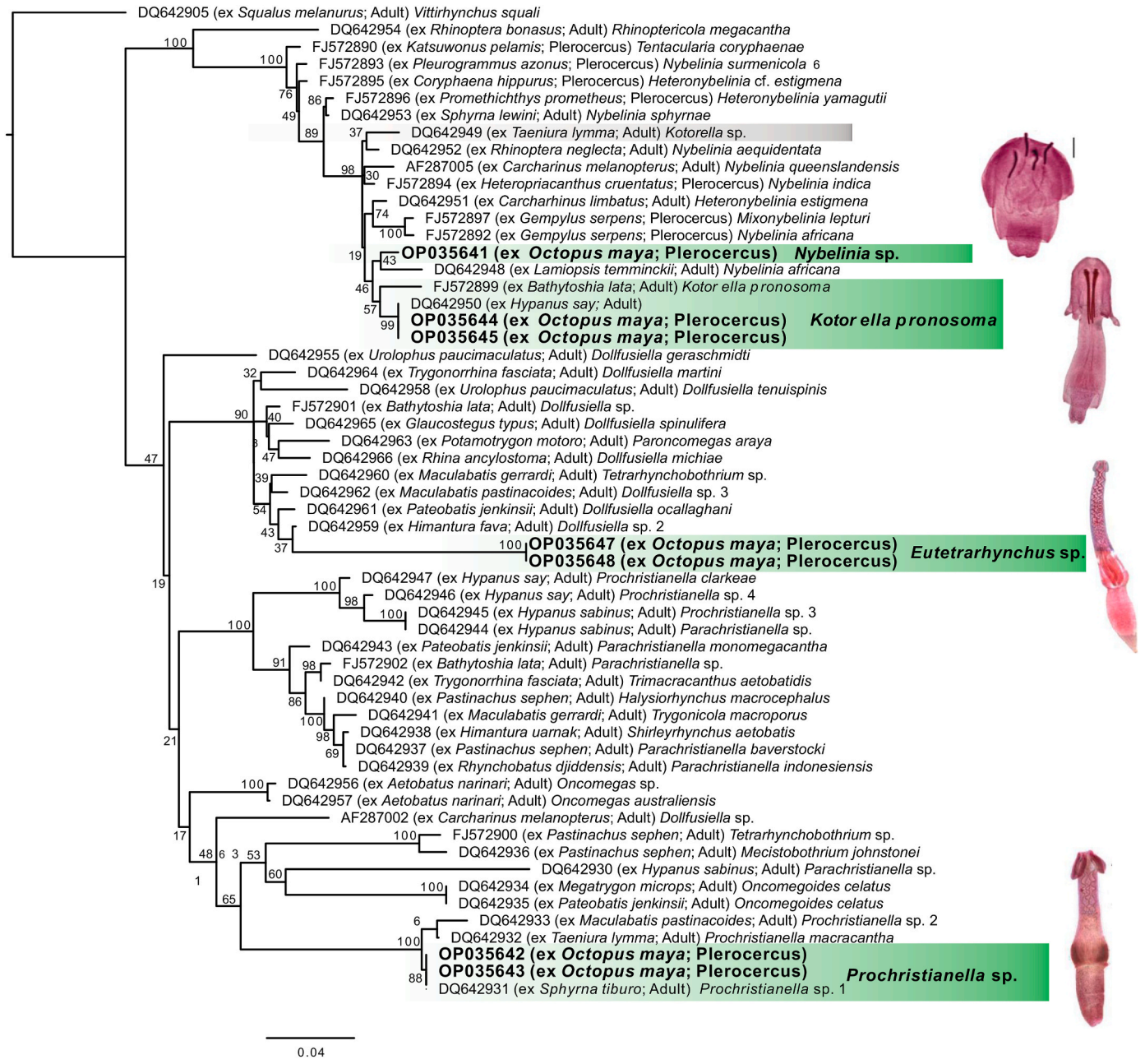

Fig. 5. Phylogenetic tree based on the Maximum Likelihood analysis of the species of the Order Trypanorhyncha found in *Octopus maya* constructed on partial small subunit ribosomal gene (18S) (likelihood = −7987.787948). Bootstrap support values for ML are provided at the nodes; ex = host; the stage of development of the cestode in parentheses.

0.233076, and a ML value of ln = - 9067.566753 (Fig. 6). The cestodes phenotypically identified as *Acanthobothrium* and *Phoreiobothrium* grouped into non-monophyletic clades with their respective congeners (Fig. 6). *Acanthobothrium* sp. ex *O. maya* formed a highly supported group (Bt = 83) with *Onchobothrium*, *Uncibilocularis*, and *Potamotrygonocestus* (Fig. 6). In addition, *Acanthobothrium* sp. ex *O. maya* nested with specimens of *Acanthobothrium* sp. ex *Diplectrum formosum* (Perciformes) (Bt = 100) and *Octopus vulgaris* (Octopodiformes) (Bt = 100). No genetic variation was found between *Acanthobothrium* sp. ex *O. maya* and ex *D. formosum*, whereas the genetic distance between *Acanthobothrium* sp. ex *O. maya* and ex *O. vulgaris* varied between 2.7% and 2.9%; while it ranged from 4.1% to 12.2% for the remaining species (Fig. 6).

The plerocercoids *Phoreiobothrium* sp. ex *O. maya* formed a clade with the genera *Phyllobothrium*, *Platybothrium*, *Prosobothrium*, and *Triloculatum* (Bt = 92). In addition, plerocercoids of *Phoreiobothrium* sp. ex *O. maya* nested with those found in *Opsanus beta* (Batrachoidiformes) without intraspecific genetic variability. The interspecific genetic distance with other species of *Phoreiobothrium* ranged from 5.1% to 15.5%.

3.4. Infection parameters

The overall prevalence and abundance of each cestode species are shown in Table 1. *Prochristianiella* sp. 1 was found infecting the buccal mass of 100% of octopuses examined, with an overall mean abundance of 456 (±766) cestodes per host, varying from 106 in Sisal to 1327 Rio Lagartos (Table 2). The second most prevalent species (63% overall) was *Eutetrarhynchus* sp., collected in the digestive gland and ink sac of the host from all localities with a mean abundance of 3 (±4) cestodes per host. The prevalence values of this species varied between 33% in

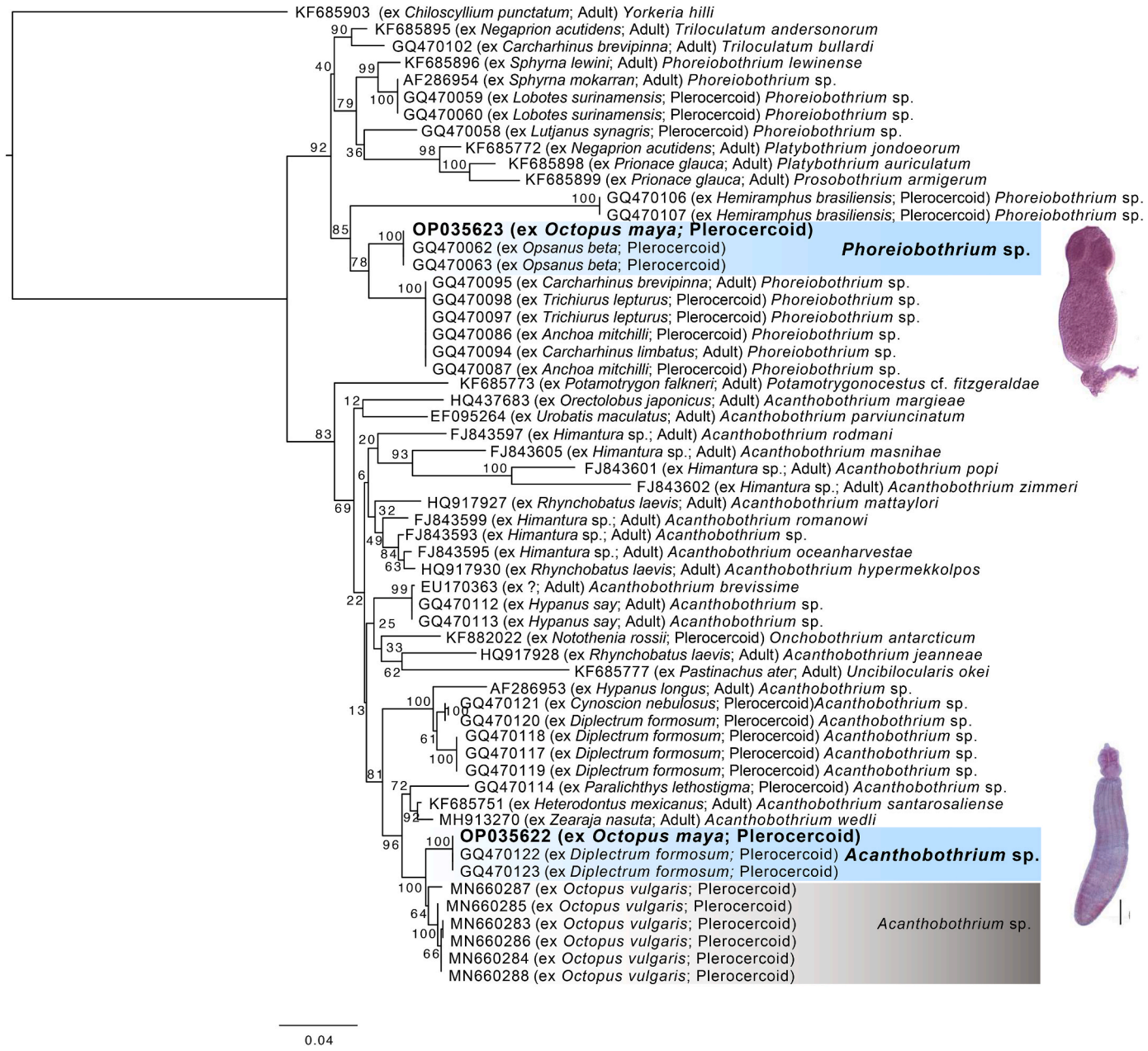

Fig. 6. Phylogenetic tree based on the Maximum Likelihood analysis of the species of the Order Onchoproteocephalidea found in *Octopus maya* constructed on partial large subunit ribosomal gene (28S) (likelihood = −9067.566753). Bootstrap support values for ML are provided at the nodes; ex = host; the stage of development of the cestode in parentheses.

Dzilam and 93% in Progreso (Table 2). From the rest of the species, the prevalence and mean abundance values were lower than 20% and one specimen per host, respectively; except for individuals of *Acantobotrium* sp. that reached a prevalence between 7% and 33%, respectively. The least frequent species was *Phoreiobothrium* sp. Collected from 7% of octopus ink sacs, and only from Rio Lagartos (Table 2).

4. Discussion

Seven taxa of Cestoda class previously reported by Guillén-Hernández et al. (2018a) were found in the host *Octopus maya*. Based on phenotypic characters and molecular data four of the identified metacestodes belong to Trypanorhyncha order: *Eutetrarhynchus* sp., *Kotorella pronosoma*, *Nybelinia* sp., *Prochristianella* sp. 1; and the last three to Onchoproteocephalidea order: *Acanthobothrium* sp., *Phoreiobothrium* sp., and *Prosobothrium* sp.

4.1. *Eutetrarhynchus sp.*

Currently five species comprehend the genus *Eutetrarhynchus*: *Eutetrarhynchus beveridgei* (Schaeffner, 2014), *Eutetrarhynchus leucomelanus* (Shipley and Hornell, 1906), *Eutetrarhynchus pacificus*, *Eutetrarhynchus platycephali* (Palm, 2004) and *Eutetrarhynchus ruficollis* (Eysenhardt, 1829) (Beveridge et al., 2021). All these species are characterized by a slender acraspedote scolex and two oval bothria in opposite arrangement. Tentacle sheaths spirally coiled, heteroacanthous armature with homeo- or heteromorphous, hollow hooks, the absence of basal swelling as well as the possession of either, a craspedote or acraspedote strobila (Campbell and Beveridge, 1994; Palm, 2004). However, an accurate identification of *Eutetrarhynchus* species is complicated due to the morphological similarities with the species of the genus *Dollfusiella* like a slender acraspedote scolex, two oval bothria, heteroacanthous armature with homeo- or heteromorphous and acraspedote strobila (Palm, 2004;

Table 2
Summary of infection parameters values of cestodes found in *Octopus maya* at each sampled locality.

Cestode	Sampled Locality							
	Sisal		Progreso		Dzilam		Rio Lagartos	
	Prevalence %	Mean Abundance (±SD)	Prevalence %	Mean Abundance (±SD)	Prevalence %	Mean Abundance (±SD)	Prevalence %	Mean Abundance (±SD)
Eutetrarhynchus sp.	93	6(±6)	47	1(±7)	33	0.6(±1)	73	2(±2)
Kotorella pronosoma	7	0.1(±0.3)	13	0.1(±0.3)	0	0	33	1(±1)
Nybelinia sp. 1	0	0	0	0	7	0.1(±0.3)	7	0.1(±0.3)
Prochristianella sp. 1	100	107(±145)	100	167(±107)	100	166(±107)	100	1328(±1123)
Acanthobothrium sp.	27	0.3(±0.4)	7	0.1(±0.3)	7	0.1(±0.3)	33	0.3(±0.6)
Phoreiobothrium sp.	0	0	0	0	0	0	7	0.3(±0.6)
Prosobothrium sp.	13	0.5(±1.3)	0	0	0	0	13	0.5(±1.3)

Schaeffner, 2014).

According to Palm (2004), Beveridge et al. (2004) a taxonomic character that allows to differentiate between *Eutetrarhynchus* and *Dollfusiella* is the basal swelling on the tentacles reported in the latter. Unfortunately, in this study, it was not possible to obtain individuals with the tentacles completely everted, which in turn made impossible to observe such characteristics. Concerning the molecular identification of the parasites, our data showed that *Eutetrarhynchus* sp. found in *O. maya* grouped with *Dollfusiella* (Figs. 4 and 5). However, the genetic distance, given by the length of the branch between our specimen and the *Dollfusiella* genus, is rather large (Figs. 4 and 5). To date, the sequence of *E. pacificus* is the only available in the GenBank database and it does not group with that from the *O. maya* specimen. However, this could be explained by the size of the *E. pacificus* sequence (420 bp) that is shorter than *Eutetrarhynchus* from *O. maya* (1463 bp). Therefore, the first one does not provide enough data to make an adequate clustering and phylogenetic analysis. Consequently, we reserve ourselves to change the actual classification given by Guillén-Hernández et al. (2018a) until new material is examined.

4.2. *Kotorella pronosoma*

The genus *Kotorella* (Euzet and Radujkovic, 1989) was previously assigned to the genus *Otobothrium* or *Nybelinia* in the Tentacullariidae family. However, at present, it is considered a monotypic genus with a single recognized species *Kotorella pronosoma* (Stossich, 1901) (Euzet and Radujkovic, 1989). Beveridge et al. (2017) stated that this species is distributed around the world. The adult stages are found in the stomach and spiral valve, while larval stages infect the stomach and mesentery of teleost fish (Palm and Overstreet, 2000). The specimens recorded in *O. maya* belong to the genus *Kotorella* because they exhibited typical morphological characteristics of the genus, such as scolex elongated, craspedote, and velum, four elongated bothria, pars post bulbosa absent, bulbs oval, and short tentacles (Fig. 2D and E; Fig. 3B). In this study, three sequences (995, 1444, and 1445 bp) were obtained from the 18S rDNA and two more (545 and 553 bp) from 28S rDNA, respectively. After comparing all the sequences of *K. pronosoma* infecting *O. maya* with those available in the public database, 100% of the similarity was obtained with larvae and adults of *K. pronosoma* from the Gulf of Mexico infecting *Syacium papillosum* and *Hypanus say* (Olson et al., 2010). Both fishes act as intermediate and definitive hosts, respectively, for *K. pronosoma* (Palm and Overstreet, 2000; Olson et al., 2010; Vidal-Martínez et al., 2019). An unexpected result showed that our sequence had a similarity percentage of 94.67–95.05% in 28S and 97.25% in 18S rDNA with *Kotorella* specimens found in the Indian Ocean. The phylogenetic analyses showed that the *K. pronosoma* from *O. maya* grouped without genetic differences with the *K. pronosoma* from the Gulf of Mexico. In contrast, they do not have a close phylogenetic relationship with *Kotorella* from the Indian Ocean. This phylogenetic result has two implications: 1) as the representatives of the genus were not grouped in a single clade, then *Kotorella* could be a non-monophyletic genus, result that had also been reported in the phylogeny of Olson et al. (2010); 2) the genetic and phylogenetic differences suggest that *Kotorella* is not a cosmopolitan genus and species. Therefore, a detailed review of the diagnostic characteristics of the genus is encouraged to avoid ambiguities in its identification.

4.3. *Nybelinia sp.*

The genus *Nybelinia*, belonging to the Tentacullariidae family, is characterized by having a compact scolex, craspedota, four sessile bothria arranged oppositely, and four short tentacles. This genus is distributed worldwide and includes many cosmopolitan species found even in deep waters that exceed 200 m. In its adult form, it is found in the digestive system of a wide variety of species of rays, and sharks. In its larval form, it has been recorded in teleosts fish and frequently in cephalopods that act as paratenic hosts (Palm, 2004; Tedesco et al., 2020). The characters such as compact scolex, craspedote, four triangular and sessile bothria, arranged oppositely, without scolex peduncle, four short and elongated tentacles emerging from short bulbs identify the specimens found in *O. maya* as belonging to the genus *Nybelinia* (Fig. 2F–H, Fig. 3C). Due to the larval stage of this cestode, other characteristics that are relevant for the identification at the species level could not be observed, therefore, the determination of this larva remained at the genus level. Concerning molecular data, unfortunately, only sequences of the 18S rDNA were successfully obtained. Olson et al. (2010) confirmed through their phylogenetic analysis that *Nybelinia* is not monophyletic either, which is consistent with what was observed in our trees, where the analyzed *Nybelina* species are not grouped into a single clade; according to the phylogenetic analyses, the *Nybelinia* sp. ex *O. maya* from this study was related to a specimen identified as *N. africana*, however, it has no morphological similarities with that reported by Palm (2004). Both *Nybelinia* (*N. africana* and *Nybelinia* sp.) mentioned have been reported as cosmopolitan species found in waters ranging from Australia, the African coast, and Brazil (Palm, 1997). Following the above, and based on the morphological characteristics, it can be said that the individuals found in *O. maya* do not belong to *N. africana*. For this reason, the specimens found in *O. maya* will remain classified at the genus level. We expect that molecular data will soon increase to complement morphological data available to date.

4.4. *Prochristianella sp. 1*

Although the genus *Prochristianella* is globally distributed, some species are endemic or locally distributed such as *Prochristianella* sp. 1 which infects elasmobranchs and shrimps in the Gulf of Mexico (Palm, 2004). Currently, *Prochristianella penaei* (syn., *P. hispida*), and *Prochristianella tenuispine* are the only species recorded in the Gulf of Mexico

(Jensen, 2009).

Based on the molecular data of 28S rRNA, the parasites of *Prochristianella* that infect *O. maya* are 99% similar to *Prochristianella* sp. 1, isolated from the Bonnethead shark *Sphyrna tiburo* collected in the Gulf of Mexico (Olson et al., 2010), and *Prochristianella macracantha* isolated from *Taeniura lymma* collected in Malaysia (Haseli et al., 2017). The latter is ruled out as conspecific species since its morphological characteristics differ from the parasites found in *O. maya* (e.g., in terms of size, basal swelling, and shape and size of the bothria). In addition, *P. macracantha* is easily distinguished by 2–3 macro hooks at the base of each tentacle. In contrast, macro hooks are absent in *Prochristianella* sp. 1, isolated from *O. maya* (Palm, 2004) (Fig. 2I–K; Fig. 3D). Although the genus *Prochristianella* is cosmopolitan, some species are endemic or locally distributed (Palm, 2004). In this work, since the geographical distribution of *Prochristianella* sp. 1 is restricted to species of elasmobranchs and shrimps in the Gulf of Mexico, we think that the parasites found in *Octopus maya* belong to *Prochristianella* sp. 1 species like that reported by Olson et al. (2010).

4.5. *Acanthobothrium sp.*

Specimens of *Acanthobothrium* sp. infecting *O. maya* from the Yucatán Peninsula were originally misidentified as *Phyllobothrium* sp. in prospecting studies (Aguirre-Macedo, com. pers.). The collection of fresh material in this study allowed a better observation of the characteristics of the parasites. The new material matched those previous records and none of them showed folds, curvatures, and ripples in the bothridal part as in *Phyllobothrium* (Khalil et al., 1994). Therefore, it is dismissed that the parasites found in *O. maya* (Fig. 2L–O; Fig. 3E) belong to the genus *Phyllobothrium* yet were recorded in *Octopus vulgaris* (Gestal et al., 1998). In contrast, the parasites infecting *O. maya* showed a scolex with an apical sucker, four bothria, each one divided into three loculi with two transverse septa, large strobila with calcareous corpuscles, and no hooks observed; these structures are in accordance with genus *Acanthobothrium* (Khalil et al., 1994). Similar plerocercoids were recorded in the common octopus (*Octopus vulgaris*) from the Tyrrhenian Sea (Southern Italy, Central Mediterranean) with 97.95% identity match and 98% coverage of *Acanthobothrium* sp. from *O. maya* (Tedesco et al., 2020).

On the other hand, 94.38% identity match and 100% coverage of *Acanthobothrium* sp. from *O. maya* and *Acanthobothrium* sp., larvae from the spiral intestine of *Dasyatis say* that was collected in Florida, USA was found (Jensen and Bullard, 2010). Molecular data available in public databases (e. g. GenBank) showed records of the genus *Acanthobothrium* from the northern part of the Gulf of Mexico (Florida, Mississippi), most of them as adults and others as plerocercoid stages (Holland et al., 2009; Jensen and Bullard, 2010). Since the parasites found in *O. maya* did not present genetic variation (Bt = 100) with the *Acanthobothrium* sp. infecting *Diplectrum formosum* from Mississippi (Jensen and Bullard, 2010), it can be asserted that both specimens correspond to a similar undescribed species (As seen in Fig. 5).

4.6. *Phoreiobothrium sp.*

Individuals of this cestode morphotype species were previously identified as *Echeneibothrium* sp. (Guillén-Hernández et al., 2018a). Due to the poor morphological development of these plerocercoid larvae, a more detailed morphological characterization was not possible. Plerocercoids at this developmental stage lack visible structures that allow application of relevant taxonomic keys (Fig. 2P–Q; Fig. 3F) (Khalil et al., 1994). Likewise, there was the only specimen found in the entire sampling (n = 60 octopuses); however, this individual has two important characteristics: four quadrangular bothria and one oral sucker. According to Hochberg (1990), there are species of this genus that have been described in approximately 30 species of cephalopods around the world.

When searching for the resulting sequence of the individual found in *O. maya*, in the BLAST, the results yielded a 100% agreement in identity and coverage with *Phoreibothrium* sp. which suggests that the individual found in *O. maya* belongs to this genus. According to Palm and Caira (2008), the larvae of the order Onchoproteocephalidea are different from their adult counterparts since when they change hosts they can develop more complex structures than those presented in their previous host. This suggests that the individual found in *O. maya*, although morphologically very different from the adult, the results of the comparison in the BLAST in identity and coverage conclude a 100% compatibility.

The genus *Phoreibothrium* is part of the order Onchoproteocephalidea (Caira et al., 2017). Currently there are 14 reported species of *Phoreibothrium* (Caira and Jensen, 2017), of which seven of them are restricted to requiem sharks (Carcharhinidae) and five to hammerhead sharks (*Sphyrna*) (Owens, 2008), all of them present in waters of the Atlantic Ocean, Gulf of Mexico, Pacific Ocean, coasts of Australia, and the Indian Ocean (Caira and Reyda, 2005). This genus is mainly characterized by the shape of the scolex, which has four bothria, each of them divided into a loculi that contain a pre-hook and a posterior loculi that are separated by a pair of hooks that are often triptych (Caira and Jensen, 2015). Given the above, it is stated here that the individual found in *O. maya* belongs to a species of the genus *Phoreibothrium*.

The phylogenetic analyses showed that the *Phoreiobothrium* sp. of *O. maya* were nested without genetic differences with the adult stage of *Phoreiobothrium* sp. from *Opsanus beta* in the northern part of the Gulf of Mexico (Olson et al., 2010). This phylogenetic result can affirm that both specimens belong to the same organism.

4.7. *Prosobothrium sp.*

Four individuals of the *Prosobothrium* genus were recorded in *O. maya*. All the plerocercoid specimens were found poorly developed, which made it difficult to find structures that allowed for further identification. However, we certainly observed the main characteristics found in larvae that are the four discs present in the scolex (Fig. 2R–T; Fig. 3G).

Caira and Jensen (2017) established that only three species belong to genera *Prosobothrium*, they belong to the Prosobothriidae family, which is characterized by its scolex, with four sessile, circular, and concave (disk-shaped) bothria. In its adult form, it develops dense spines on the surface of the neck. Final hosts are usually sharks, blue sharks, and nurse sharks (Caira and Jensen, 2017).

No viable Prosobothrium sequence was obtained to perform the BLAST search. . It was impossible to complement the morphological observations with molecular data and thus, make a precise identification of this species. However, in accordance with the identification given by Guillén-Hernández et al. (2018a), we retained the parasite isolated from *O. maya* in the genus *Prosobothrium*.

4.8. *Infection parameters*

All cestodes found in this study have been previously reported from the Gulf of Mexico. Authors such as Gestal et al. (1998), Hochberg (1990), Pascual et al. (1996), Jensen and Bullard (2010), Guillén-Hernandez et al. (2018a), and Tedesco et al. (2020) have reported the presence of cestodes in the genus *Acanthobothrium*, *Eutetrarhynchus*, *Nybelinia*, and *Prochristianella* in some cephalopods.

Cestodes are the most dominant group of helminths that parasitize cephalopods and nurse a great diversity of larval stages of these parasites (Hochberg, 1990; Pascual et al., 2019; Tedesco et al., 2020). This diversity indicates that cephalopods are important as intermediate and paratenic hosts for cestodes that mature into elasmobranchs and teleost fishes; they are transferred from host to host through the food chain. The transmission of the cestodes to *Octopus maya* most likely occurred through the intake of shrimp, copepods, and other crustaceans available

in the sampling area (Hochberg, 1990; Gestal et al., 1998; Pascual and Guerra, 2007; Jensen and Bullard, 2010). Of the seven taxa found in this study, *Prochristianella* sp. 1 presented the highest infection parameters in all localities. This species has been previously reported by Guillén Hernández et al. (2018a), as the species with the highest values of infection, dominance, abundance and intensity, which reflects the high availability of food (shrimp) and, therefore, infect larvae that parasitize this cephalopod. López-Struck (2011) established shrimp as the first intermediate host of *Prochristianella* sp. 1, and part of the diet of *O. maya* from an early age. This indicated that shrimp are an important part of the food chain for this octopus during its life, and part of the constant reinfection and therefore accumulation of this species in *O. maya*. For all the above, it can be established that the infection of this cestode is the most important for *O. maya* in the Yucatan Peninsula.

In the case of *Nybelinia* sp. and *Phoreiobothrium* sp., which presented the lowest prevalence and abundance values, it may be related to the fact that *O. maya* might not be the main intermediate host, thus establishing *Octopus maya* as a paratenic or transport host or maybe even an accidental infection. For both species, there are reports of adult-staged organisms in actinopterygians, elasmobranchs, and marine mammals in the Gulf of Mexico (Felder and Camp, 2009).

Regarding the highest infection parameters found in Rio Lagartos, it suggests that the environmental characteristics of each locality could be promoting different communities of intermediate and definitive hosts, leading to differences in composition of the parasite community, recruitment, and therefore the spatial distribution of parasites in the area (Esch, 1983; Guillén Hernández et al., 2018a).

According to Herrera-Silveira et al. (2013), the Rio Lagartos fishing area is in an area where the sea water currents are highly influenced by the Caribbean upwelling; it contributes to the dynamics of nutrients and primary productivity. Consequently, Rio Lagartos has the necessary characteristics for the disposition of different and abundant organisms that can act as paratenic, intermediate, or definitive hosts not only for cestodes but other metazoan parasites as well.

In conclusion, this study showed that *O. maya* hosts the same species of parasites in the four sampled locations with differences in the infection parameters among localities. In addition, here we confirm, for the first time, the identity of larval stages of cestodes found in O. maya based on their morphological and molecular information. Additional sequences linked to a detailed morphological characterization need to be added to public databases to clarify the identity of those larvae unidentified to date.

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Declaration of competing interest

The authors declare no competing interest.

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