Bruna Trevisan Souza

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Prof. Dr. Fernando P. L. Marques

Orientador

Dedico esta dissertação aos meu pais, João e Vera, por serem meu chão, meu ar e meu mar.

“The profit from our study is to have become better and wiser by it.”

Michel de Montaigne

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AVISO DO AUTOR

Todas as ações taxonômicas neste estudo são renunciadas para fins de nomenclatura, como recomendado no Artigo 8 do Código Zoológico Internacional de Nomenclatura (Ride *et al.*, 1999)

AUTHOR'S DISCLAIMER

All taxonomic actions in this work are hereby disclaimed for nomenclatural purposes, as recommended in Article 8 of the International Code of Zoological Nomenclature (Ride *et al.*, 1999).

Apresentação

O parasitismo é considerado um dos modos de vida mais bem sucedidos dentre os organismos viventes, visto que quase todos os metazoários de vida livre convivem com pelo menos um organismo associado (*i.e.*, parasita) (Poulin & Morand, 2000). Dentre os filos que apresentam formas de vida parasitária, podemos citar o filo Platyhelminthes, o qual possui mais de 40 mil espécies parasitas e de vida livre (Brooks & McLennan, 1993a; Rohde, 1996). Quanto aos platelmintos parasitas há um grupo muito peculiar formado por espécies que são endoparasitas obrigatórios do trato digestivo de vertebrados quando adultos (Stunkard, 1962). Conhecidos popularmente como solitárias, os Cestoda têm aproximadamente 6 mil espécies reconhecidas nas 18 ordens existentes (Caira *et al.*, 2012; Caira *et al*., 2014). Dentre suas estratégias de vida, 7 ordens habitam obrigatoriamente o intestino espiral de elasmobrânquios quando adultas (Tetraphyllidea, Rhinebothriidea Trypanorhyncha, Lecanicephalidea, Diphyllidea, Litobothridea e Cathetocephalidea) e apresentam uma longa história evolutiva junto a este grupo, podendo servir como instrumento para estudos evolutivos (Diesing, 1863; Carus, 1863; Wardle & McLeod, 1952; Dailey, & Overstreet, 1973; Brooks *et al.*, 1981b; Caira, 1990; Page & Charleston, 1998; Palm, 2004; Jensen, 2005; Tyler, 2006; Ruhnke *et al.*, 2011; Eyring *et al.*, 2012; Marques & Caira, 2016).

Estudos evolutivos que utilizam o sistema parasita-hospedeiro como ferramenta assumem que existe uma associação histórica entre eles. Associação histórica é definida como o processo pelo qual um associado (*e.g.*, organismos) interage com um hospedeiro (*e.g.*, área ou outros organismos) ao longo do tempo evolutivo. O termo “hospedeiro” é definido como uma entidade que de alguma forma aloja outra entidade, que por sua vez é o “associado” (Page, 1994). Em cada associação, o associado segue o hospedeiro com um certo grau

de fidelidade, que depende do balanço entre eventos de co-divergência, o qual assegura fidelidade, e três outros processos que, de certa forma, introduzem desvios da hipótese nula dentro do sistema associado/hospedeiro (*e.g.*, duplicação, transferência horizontal, e eventos de triagem; veja Page & Charleston, 1998). Espera-se, portanto, que grupos-irmãos de hospedeiros abriguem grupos-irmãos de organismos associados, o que pode ser testado por uma extensa e rigorosa base taxonômica e hipóteses filogenéticas para as linhagens participantes (Caira & Jensen, 2001; Paterson & Banks, 2001). Neste sentido, estudos de associação histórica são promissores para a compreensão do sistema parasita-hospedeiro, pois possibilitam esclarecer os processos responsáveis pela diversificação dos grupos envolvidos na longa relação evolutiva entre hospedeiros e associados (Brooks *et al.*, 1981a; Klassen, 1992; Brooks & McLennan, 1993; Page & Charleston, 1998; Ronquist & Huelsenbeck, 2003).

Um dos exemplos clássicos de associação histórica entre parasitas e hospedeiros refere-se à história de derivação das arraias da família Potamotrygonidae de seus ancestrais marinhos e suas linhagens parasitas (Brooks *et al.*, 1981b; Blair, 1994; Lovejoy *et al.*, 1998; Marques, 2000). A família Potamotrygonidae é representada por elasmobrânquios estenohalinos endêmicos dos sistemas fluviais da região Neotropical da América do Sul e são encontrados na maioria das bacias hidrográficas desta região (Thorson, 1970; Thorson *et al.*, 1978, 1983; Rosa, 1985; Nishida, 1990; Lovejoy, 1996; de Carvalho *et al.*, 2003; Rosa *et al.*, 2008). Atualmente, ~30 espécies são reconhecidas para este grupo que estão divididas em 4 gêneros: *Paratrygon* Duméril; *Potamotrygon* Garman; *Plesiotrygon* Rosa, Castello & Thorson; e *Heliotrygon* de Carvalho & Lovejoy (Marques, 2000; de Carvalho *et al.*, 2003; Rosa *et al.*, 2010; de Carvalho *et al.*, 2011; de Carvalho & Ragno, 2011; de Carvalho & Lovejoy, 2011). A fauna de endoparasitas dos potamotrigonídeos é

composta por elementos que refletem a origem desses hospedeiros (Brooks *et al*. 1981a; Marques, 2000; Reyda & Olson, 2003; Reyda, 2008; Reyda & Marques, 2011). Nela encontramos linhagens que apresentam uma relação mais próxima com os parasitas que habitam os elasmobrânquios marinhos, do que com os que habitam outras linhagens de água doce dos rios da América do Sul. Sendo assim, a fauna de cestóideos das arraias de água doce possui gêneros endêmicos do sistema neotropical (*e.g., Potamotrygonocestus* Brooks & Thorson, 1976, e *Nandocestus* Reyda, 2008), e outros compartilhados com espécies de elasmobrânquios marinhos (*e.g., Acanthobothrium* Blanchard, 1848, *Rhinebothrium* Linton, 1890 e *Anindobothrium* Marques, Brooks & Lasso, 2001).

Devido a endemicidade das arraias de água doce somada ao fato dos seus parasitas estarem proximamente relacionados aos parasitas das arraias marinhas, a origem dos potamotrigonídeos e sua diversificação têm sido alvo de grande interesse da comunidade científica (Brooks *et al.*, 1981a, b; Brooks, 1992, 1995; Lovejoy, 1996, 1997; Lovejoy *et al.*, 1998; Hoberg *et al.*, 1998; Zamparo *et al.*, 1999; Marques, 2000; de Carvalho *et al.*, 2004; Domingues & Marques, 2011; Aschliman, 2011; Marques & Caira, 2016). Brooks *et al.* (1981b) foram os primeiros a criar um cenário para explicar a origem da família Potamotrygonidae. Os autores consideraram que seria possível utilizar dados parasitários para inferir a história evolutiva de seu hospedeiro. Sendo assim, a partir de dados morfológicos dos parasitas, Brooks *et al.* (1981b) criaram um cenário em que o ancestral dos potamotrigonídeos seria um urofílídeo (atualmente Urotrygonidae, Naylor *et al.*, 2012), habitante do Oceano Pacifico que colonizou os sistemas fluviais da América do Sul no período do Cretáceo, antes do soerguimento dos Andes. Apesar da falta de dados parasitológicos robustos e do uso de métodos de inferência questionáveis (Lovejoy, 1997) é imprescindível creditar aos autores a responsabilidade por terem sido os

pioneiros em buscar explicar a origem desse intrigante grupo de elasmobrânquios.

Dados morfológicos e moleculares posteriores para os hospedeiros resultam em um outro cenário (Lovejoy, 1996; Lovejoy *et al.*, 1998; Lovejoy, 1999; Aschliman, 2011; Naylor *et al.*, 2012). Filogenias provenientes destes dados sugerem que o ancestral dos potamotrigonídeos teria invadido o sistema fluvial sul-americano durante as ingressões marinhas entre o Eoceno e Mioceno (~40 a 20 Ma) no norte da América do Sul, e se isolado durante as alterações nos padrões de drenagens do paleo-Orinoco e eventos orogênicos subseqüentes. Estes estudos sugerem ainda que o clado formado pelas espécies anfi-Americanas de *Himantura* (*H. pacifica* Beebe & Tee-Van do Pacifico Oriental e *H. schmardae* Werner do Caribe) é grupo irmão dos potamotrigonídeos (Lovejoy *et al.*, 1998; Lovejoy, 1999, de Carvalho *et al.*, 2004). Acredita-se, assim, que o Pacífico oriental e o Mar do Caribe sejam as áreas hipotéticas de derivação dos potamotrigonídeos.

O fato de a hipótese de Brooks *et al.* (1981b) não ter sido corroborada por estudos posteriores, que avaliaram os dados dos hospedeiros ao invés dos parasitas, reforçou o questionamento em relação aos métodos utilizados pelos primeiros autores. Lovejoy (1997) sugeriu que Brooks *et al.* (1981b), além de não terem utilizado uma base de dados morfológicos robusta, não consideraram que existem eventos que pudessem obscurecer a relação parasita-hospedeiro em uma associação histórica (*e.g.*, transferência horizontal, extinção e erro amostral), o que pode ter influenciado diretamente os resultados de suas análises. Fato é que uma análise mais robusta de dados parasitológicos para nematódeos do gênero *Echinocephalus* Molin, 1858 parasitas de *H. pacifica* chegou ao mesmo cenário baseado nos dados dos batóideos (Hoberg *et al.*, 1998). Ante o exposto, fica claro que é possível a utilização dos dados parasitológicos desde que seja considerado tanto a robustez dos dados

morfológicos quanto dos dados filogenéticos, os quais irão garantir uma análise fidedigna à história evolutiva das linhagens participantes (Caira & Jensen, 2001; Paterson & Banks, 2001).

Apesar deste estudo de *Echinocephalus* corroborar a atual hipótese de origem dos potamotrigonídeos (Hoberg *et al.*, 1998), a utilização dos dados parasitários para o entendimento da história biogeográfica das arraias de água doce ainda enfrenta grandes desafios. Desconhecemos grande parte da fauna parasitária de elasmobrânquios que residem na área de derivação hipotética dos potamotrigonídeos, principalmente para linhagens de cestóideos que são compartilhadas por hospedeiros marinhos e de água doce (veja abaixo). Uma das formas de utilizar os dados parasitários para inferir dados dos hospedeiros é por meio de estudos de associação histórica. Em associações históricas, para as quais assume-se que há um certo grau de fidelidade entre os hospedeiros e seus parasitas através de eventos de co-divergência (veja Page & Charleston, 1998), pode-se esperar que grupos-irmãos de hospedeiros abriguem grupos-irmãos de associados. Ao considerarmos que o grupo-irmão dos potamotrígonídeos possa ser de fato um clado formado pelas espécies anfi-Americanas de *Himantura*, investigar a sua fauna parasitária, focando nos grupos compartilhados com os potamotrigonídeos, a partir de bases taxonômicas e filogenéticas robustas pode gerar informações sobre as relações filogenéticas e história biogeográfica desses hospedeiros. No entanto, a diversidade destes grupos, notadamente para o Mar do Caribe e Pacífico oriental, e o relacionamento filogenético desta fauna em relação aos seus congêneres de água doce ainda é muito pouco conhecida.

O primeiro estudo a abordar a fauna parasitológica de cestóideos de espécies anfi-Americanas de *Himantura* foi realizado por Brooks (1977). Neste estudo, o autor descreveu seis novas espécies de cestóideos, dentre elas duas espécies de *Acantobothrium* (*A. tasajerasi* Brooks, 1977 e *A. Himanturi* Brooks, 1977), uma de *Rhinebothrium* (*R. Tetralobatum* Brooks, 1977) e *Caulobothrium*

anacolum Brooks, 1977, que seria transferida mais tarde para *Anindobothrium* como *Anindobothrium anacolum* (Brooks, 1977) Marques, Brooks & Lasso, 2001. Marques *et al*. (1996) descreveram duas espécies, também de cestóideos (*Acanthobothroides pacificus* Marques, Brooks & Lasso, 1996 e *Rhinebothrium geminum* Marques, Brooks & Lasso, 1996 [atualmente como *Scalithrium geminum* (Marques, Brooks & Lasso, 1996) Ball, Neifar & Euzet 2003]) de um único exemplar de *Himantura pacifica* proveniente da Costa Rica. Entretanto, as descrições destas espécies foram feitas com um número restrito de exemplares e não contemplam todas as informações encontradas em descrições recentes para estes grupos taxonômicos (*e.g.*, dados histológicos, microscopia eletrônica de varredura, entre outros), sugerindo a necessidade de que essas espécies sejam re-descritas. Adicionalmente, é evidente que se conhece muito mais da fauna parasitária de *H. schmardae* do que de *H. pacifica.* Tal conhecimento gera a expectativa de que *H. pacifica* deva hospedar linhagens de *Acanthobothrium*, *Rhinebothrium* e *Anindobothrium* uma vez que é comum encontrarmos "*geminate species*" (senso Jordan, 1908) em ambas as costas do istmo do Panamá.

O refinamento taxonômico precede qualquer estudo mais aprofundado sobre a evolução do sistema parasita-hospedeiro. Assim, para que possamos incorporar dados parasitológicos em estudos que visem elucidar a evolução dos potamotrigonídeos e seus parasitas é preciso identificar de forma mais precisa os componentes desse sistema. Nesse contexto, é importante conhecer a fauna de cestóideos parasitas de espécies anfi-Americanas de *Himantura*. Primeiro porque essas espécies residem na área de derivação hipotética do ancestral da linhagem dos elasmobrânquios de água doce e, segundo, porque estão filogeneticamente associadas aos potamotrigonídeos. Ambas características geram expectativas de que as linhagens marinhas mais próximas de linhagens dulcícolas sejam encontradas nesses hospedeiros. Ante o exposto, o presente

estudo inventariou as espécies de *Acanthobothrium*, *Rhinebothrium* e *Anindobothrium* parasitas de espécies anfi-Americanas de *Himantura*. Para tanto, a estruturação do presente estudo será organizada em três capítulos apresentados no formato de artigos científicos.

O primeiro, intitulado "***Rhinebothrium* Linton, 1890 (Eucestoda: Rhinebothriidea) from amphi-American species of *Himantura* (Myliobatiformes: Dasyatidae), including the description of one new species**", consiste na avaliação da fauna de *Rhinebothrium* das espécies anfi-americanas de *Himantura*, a qual resultou na redescrição de *R. tetralobatum* com a adição de uma nova localidade e no reconhecimento de uma nova espécie (*R. reydai* n. sp.). A partir dessas informações, este capítulo discute os padrões de infecção e de distribuição biogeográfica para as espécies deste gênero, discutindo a importância da representatividade biogeográfica na documentação da fauna parasitária de linhagens de hospedeiros. O capítulo 1 será submetido para o periódico *Zootaxa*, por sua relevância, que abrange não só a comunidade helmintológica, mas todos os grupos biológicos que possam ter histórias naturais similares, e por ser especializada em aspectos de sistemática zoológica.

O segundo capítulo, intitulado "**Systematics and diversification of *Anindobothrium* Marques, Brooks & Lasso, 2001 (Eucestoda: Rhinebothriidea)**" faz uma avaliação do status taxonômico do gênero *Anindobothrium* para o qual, devido às análises moleculares, foi proposta uma uma nova família para Rhinebothriidea, Anindobotriidae n. fam. Além disso, a diversidade do gênero aumentou de 2 para 4 espécies reconhecidas, das quais *A. anacolum* e *A. lisae* foram redescritas e duas novas espécies foram propostas. Este artigo será submetido para a *PlosOne*, por ser uma revista de grande visibilidade e que tem sido alvo de publicações na área de parasitologia de elasmobrânquios.

Por último, o capítulo 3, intitulado "**Species diversity of**

***Acanthobothrium* Blanchard, 1848 (Eucestoda: Oncoproteocephalidea) from amphi-American species of *Himantura* (Myliobatiformes: Dasyatidae)**" descreve 8 novas espécies de parasitas desses hospedeiros, e redescreve *Acanthobothrium himanturi* com adição de duas novas localidades. Este artigo inclui uma análise filogenética na qual estas novas linhagens são posicionadas entre as demais linhagens do gênero residentes em diversas regiões biogeográficas e infectando uma ampla diversidade de batóideos. Os resultados desta análise demonstram a associação filogenética de linhagens marinhas e de água doce, replicando o padrão de divergência dos hospedeiros. O artigo se encerra com a discussão das implicações destas novas evidências no debate sobre as evidências parasitológicas nas hipóteses de origem das linhagens de arraias Neotropicais. Este artigo será submetido à *Zootaxa* pelos mesmos motivos supracitados.

As referências de cada capítulo serão apresentadas no formato estipulado por cada periódico ao qual serão submetidos. Por questões estéticas, as formatações como margem e tipo da letra seguirão o padrão sugerido pelo modelo de dissertações do Departamento de Zoologia do Instituto de Biociências da Universidade de São Paulo.

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Capítulo 1

Rhinebothrium Linton, 1890 (Eucestoda: Rhinebothriidea) from amphi-American species of *Himantura* (Myliobatiformes: Dasyatidae), including the description of one new species

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Abstract

Taxonomic refinement is the foundation of co-evolutionary studies, since it provides the information required to establish robust relationships among lineages within coevolving systems. Here, we aim to improve our knowledge on the fauna of cestode parasites of amphi-American species of *Himantura* Müller & Henle, the putative sister taxon of potamotrygonids - a unique group of batoids restricted to Neotropical freshwater systems. Our motivation is to establish the sound taxonomic framework that would allow us to explore the historical associations among marine and freshwater batoids and their cestode parasites in the near future. Toward this goal, we document the fauna of *Rhinebothrium* Linton, 1890 of amphi-American species of *Himantura* as a result of the examination of newly collected specimens from 5 different localities representing the eastern Pacific Ocean and the Caribbean Sea. Overall, we examined 33 spiral intestines, 11 from the eastern Pacific species *Himantura pacifica* (Beebe & Tee-Van) and 22 from the Caribbean species *H. schmardae* (Werner). However, only samples from the Caribbean were infected with members of *Rhinebothrium*. *Rhinebothrium tetralobatum* Brooks, 1977, originally described from *H. schmardae* based on 6 specimens, is herein redescribed. For the first time, our redescription provides data on the microthrix morphology and internal anatomy as well as a better understanding of the morphometric variability of this taxon. We discovered a new species of *Rhinebothrium* in *H. schmardae* collected off the Caribbean coast of Panama. *Rhinebothrium reydai* n. sp. is diagnosed by possessing 4 testes per proglottid, acraspedote proglottids, anterior and porteriormost loculus single, and a lower number of loculi (34–44), which distinguishes it from all the 41 species of *Rhinebothrium* currently recognized with the exception of *R. chollaensis* Friggens & Duszynski, 2005. The latter species is a parasite of *Urobatis halleri* (Cooper) from the eastern Pacific Ocean and it can be distinguished from *Rhinebothrium reydai* n. sp. by possessing leaf-like instead of bilobed-stalked bothridia and by the absence of a cephalic peduncle present in *R. reydai* n. sp. Moreover, we discuss the patterns of infection and biogeographical distribution for species of *Rhinebothrium* in amphi-American species of *Himantura*. The apparent disjunctive distribution of *R. tetralobatum* and *R. reydai* n. sp. in the Caribbean Sea throughout their host distribution, *H. schmardae*, and the absence of species of *Rhinebothrium* in the eastern Pacific sister-host, *H. pacifica*, reveal the importance of sample size and biogeographical representation for documenting the parasite fauna of host lineages.

Keywords: Cestodes, taxonomy, historical association, geminate species, Caribbean Sea, eastern Pacific Ocean.

Introduction

Studies on historical associations and evolution of any host-parasite system might provide information, which could be used to elucidate both origins (Page and Charleston, 1998; Caira and Jensen, 2001). A classic example of historical association studies is the derivation of Neotropical freshwater stingrays of the family Potamotrygonidae from marine ancestors and the supposed codivergency among cestode parasites and their hosts (Brooks *et al.*, 1981b; Blair, 1994; Lovejoy *et al.*, 1998; Marques, 2000). Brooks *et al.* (1981b) pioneered the study of this system and based on parasitological data suggested that potamotrygonids derived from a Pacific dwelling urolophid-like ancestor (presently of the family Urotrygonidae, see Marques and Caira, 2016) before the uplift of the Andes in the Cretaceous era. This seminal study sparkled great debate in the literature (Straney, 1982; Caira, 1990, 1994; Lovejoy, 1997), most of which questioning the lack of robust parasitological data and methods of inference. In recent years, however, a body of evidence has surfaced corroborating an alternative hypothesis suggesting that the clade formed by amphi-American species of *Himantura* Müller & Henle is the sister of potamotrygonids (Aschliman, 2011; Naylor *et al.*, 2012) and that the divergence between these two lineages was a result of the marine incursion events during the Paleogene period, between the early Miocene and mid-Eocene (*i.e.*, 22.5–46 mya; see Lovejoy *et al.*, 1998; Marques, 2000; de Carvalho *et al.*, 2004), and that the tropical eastern Pacific Ocean and Caribbean Sea represents the hypothetical area of derivation. Hence, the study of the cestode fauna of amphi-American species of *Himantura* can potentially reveal the historical associations among these related batoid lineages and associated helminth parasites.

The fauna of cestode parasites of amphi-American species of *Himantura* is poorly known despite its relevance for the understanding of this classic biogeographical puzzle. The lack of knowledge is mainly due to the fact that only two studies reported cestodes for these hosts. Brooks (1977) examined 3 specimens of *Himantura schmardae* (Werner) off the Caribbean coast of Colombia from which he described 6 species of cestodes, namely *Acanthobothroides thorsoni* Brooks, 1977; *Acanthobothrium himanturi* Brooks, 1977; *A. tasajerasi* Brooks, 1977; *Anindobothrium anacolum* (Brooks, 1977) Marques, Brooks & Lasso, 2001; *Scalithrium magniphallum* (Brooks, 1977) Ball, Neifar & Euzet, 2003 and *Rhinebothrium tetralobatum* Brooks, 1977. Decades latter, Marques *et al.* (1996) examined a single specimen of *H. pacifica* (Beebe & Tee-Van) off the coast of Costa Rica from which they described *Acanthobothroides pacificus* Marques, Brooks & Ureña, 1996 and *Scalithrium geminum* (Marques, Brooks & Ureña, 1996) Ball, Neifar & Euzet, 2003. Both studies relied on very few host specimens and a restricted biogeographical representation. In addition, most taxonomic descriptions were based on a small sample size of cestode specimens, compromising an adequate account of morphological variability for these taxa.

Members of *Rhinebothrium* Linton, 1890 can be found in marine and freshwater (*i.e.*, potamotrygonids) batoids alike. The genus is composed of 41 valid species, of which 7 are restricted to freshwater potamotrygonids (Marques and Reyda, 2015) and only a single species, *R. tetralobatum*, has been reported for a member of its sister clade, *Himatura schmardae*. To date, there is no species of *Rhinebothrium* reported from *Himantura pacifica*, which is unexpected since two pairs of putative geminate species (sensu Jordan, 1908) of cestodes have been reported for these sister-species of hosts (see Marques *et al.*, 1996). Here, we present the results of our most recent survey of *Rhinebothrium* parasites of amphi-American species of *Himantura* which includes the redescription of *R. tetralobatum*, the discovery of a second species infecting *H. schmardae*, and a discussion on the patterns of distribution of these parasite lineages. We furthermore address the role of sample size and

biogeographical representation on the accuracy of the documentation of the parasite fauna of host lineages, which leads us to the prediction that at least one species of *Rhinebothrium* is yet to be found infecting *H. pacifica* in the eastern Pacific Ocean.

Material and Methods

We examined a total of 33 spiral intestines of amphi-American species of *Himantura*, 22 of *H. schmardae* and 11 of *H. pacifica*. Specimens of *H. pacifica* from the Eastern Pacific were collected from Panama, Montijo, Veráguas province (07°29'37.9"N, 81°13'21.9"W) during January 2015. Specimens of *H. schmardae* were collected from: Colombia (Tasajeras, Magdalena (10°58'46.84”N,74°19'32.19"W and 11°0'49.71”N, 74°16'19.32”W), during May 1989 - 5 individuals; Belize (Head Caye in Punta Gorda, Toledo, 16°13'20.8"N, 88°35'38.3"W; North of Southwater Caye and Tobacco Caye in Dangriga, Stann Creek, 16°49'43.1"N, 88°04'48.1"W and 16°54'15.2"N, 88°03'38.2"W, respectively), during May 2012 - 5 individuals; Trinidad and Tobago (Maracas, San Juan-Laventille, 10°45'46.8"N, 61°26'34.8"W), during January 2014 - 1 individual; and Panama (Atlantic Ocean, Almirante, Bocas del Toro province, 9°17'39.1"N, 82°20'42.0"W and 9°17'20.3"N, 82°21'18.9"W), during January 2015 - 11 individuals. The specimens were collected following the guidelines of the permit issued to Janine Caira by INDERENA - Instituto de Pesca y Fauna Terrestre de Bogotá in Colombia and by the Ministry of Forest, fisheries and sustainable development (Belize Fisheries Department - Proc. No 000016-12) in Belize; and to Fernando P. L. Marques by the Ministry of Food Production - Fisheries Division in Trinidad and Tobago (issued on September 30th, 2014) and by ANAM - Autoridad Nacional del Ambiente in Panama (SE/A101-14).

After capture, stingrays were euthanized and their spiral intestines removed, opened with a mid-ventral incision, washed with seawater and fixed in a 4% seawater-buffered formaline solution. After a few days, both wash and spiral intestines were transferred to 70% ethanol for long-term storage and subsequent examination. Cestodes recovered from the spiral intestines and its contents were sorted under the stereomicroscope specimens of *Rhinebothrium* were selected for light and scanning electron microscopy, as well as histology.

Whole worms selected for light microscopy were hydrated in a regressive alcoholic series, stained with Delafield`s hematoxylin (9:1 solution), destained in a 1% acid (HCl) ethanol solution, followed by a 1% basic (NaOH) ethanol solution, dehydrated in a graded ethanol series, cleared in methyl salicylate and mounted in Canada balsam on glass slides under coverslips. Morphometric data and photographic documentation were obtained using an Olympus SC30 camera and the Analysis 5.0 software (Olympus Soft Images Solutions) attached to an Olympus BX51 microscope. The packages Fiji/ImageJ (Schindelin *et al.*, 2012) and WormBox (Vellutini and Marques, 2014) were used to process images and compute morphometric data, respectively. Only complete specimens with mature (*i.e.*, with open genital pores) or further developed proglottids (*e.g.*, with atrophied testes or vas deferens filled) were examined and measured in this study. All measurements for reproductive structures were taken from terminal proglottids, unless in cases where terminal proglottids presented atrophied testes. In these instances, testes data was obtained from subterminal mature proglottids. All measurements are in micrometers unless otherwise stated, and are presented as ranges followed in parentheses by the number of specimens from which the variable was taken. Repeated measurements for the number and dimensions of testes and for the dimensions of vitelline follicles were averaged for individuals. Terminology for the shape of the bothridia follows Clopton (2004).

Specimens selected for histological sections were embedded in paraffin and sectioned at 7µm intervals using a LEICA RM 2025 retracting rotary microtome. Sections were mounted on glass slides flooded with distilled water and initially dried on a slide warmer for 5 minutes and later transferred to an oven for 30 minutes at 60° C. Cross sections of mature proglottids were stained with Mayer's hematoxylin and counterstained with eosin, dehydrated in a graded ethanol series, cleared in xylene, and mounted in Entellan (Merck). The anterior portion of each worm sectioned was prepared as a whole mount as described above and kept as a voucher.

Scoleces selected for scanning electron microscopy (SEM) were carefully cleaned with brushes to remove the host tissue and mucus, hydrated in a graded ethanol series, transferred to 1% osmium tetroxide overnight, dehydrated in a graded ethanol series, and placed in hexamethyldisilizane (HMDS). They were allowed to air-dry overnight and were subsequently mounted on carbon tape on an aluminum stub, sputter-coated with gold/palladium and examined with a Zeiss DCM 940 and FEI Quanta 600 FEG scanning electron microscope. The strobila of the worm used for SEM was prepared as a whole mount voucher as described above. Microthrix terminology follows Chervy (2009).

Museum abbreviations are as follows: **CHIOC**, Coleção Helmintológica do Instituto Oswaldo Cruz, Rio de Janeiro, RJ, Brazil; **HWML**, Harold W. Manter Laboratory, University of Nebraska, Lincoln, Nebraska, U.S.A.; **LRP**, Lawrence R. Penner Parasitology Collection, Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut, U.S.A; **MZUSP**, Museu de Zoologia da Universidade de São Paulo, SP, Brazil; **MIUP**, Museu de Invertebrados G. B. Fairchild, Estafeta Universitaria, Universidad de Panamá, Veráguas, Panama; and **USNM**, National Museum of Natural History, Smithsonian Institution, Washington, D.C, U.S.A.

Results

Rhinebothrium tetralobatum Brooks, 1977
(Figs. 1,2,3)

Type-host: *Himantura schmardae* (Werner) (Myliobatiformes: Dasyatidae).
Type-locality: Caribbean Sea, 15 km west of La Cienaga, Magdalena, Colombia (11°01'N, 74°15'W).
Additional locality: Tasajeras, Magdalena, Colombia (10°58'46.84”N, 74°19'32.19''W and 11° 0'49.71”N, 74°16'19.32”W).
Site of infection: Spiral intestine.
Type material: Holotype (USNPC 73967) and 6 paratypes (HWML 20253, 20266).
Specimens deposited: #######.

Redescription. [Based on the type series comprised of holotype (USNPC 73967) and 6 paratypes (HWML 20253, 20266), and 58 additional mature specimens, which included 53 whole mounts, 2 worms observed with SEM, and 3 used for cross sections]. Worms craspedote (Fig. 1A), apolytic, 4.1–19.0 mm (n = 42) long, composed of 80–206 (n = 42) proglottids. Scolex 278–801 (n = 48) in maximum width, composed of 4 bilobed-stalked bothridia (Fig. 1B, 3A). Bothridia lomeniform-shaped constricted at center, with muscular rims, 487–1,094 (n = 48) long by 122–328 (n = 48) wide divided by 23–34 (n = 48) transverse septa and 1 medial longitudinal septum into 47–69 (n = 32) loculi, with anteriormost loculus single, 26–50 (n = 31) long by 31–59 (n = 31) wide and posteriormost loculi double. Medial

longitudinal septum extending from posterior margin of anteriormost loculus to posterior margin of bothridia. Cephalic peduncle present; neck varying in length. Entire proximal and distal surface of bothridia covered with acicular filitriches and gladiate spinitriches (Fig. 3B-G). Neck covered with capilliform filitriches (Fig. 3H).

Immature proglottids wider than long, becoming longer than wide with maturity, 68–178 (n = 42) in number. Mature proglottids 173–900 (n = 41) long by 73–220 (n = 42) wide (Fig. 1D), 6–62 (n = 42) in number. Some terminal proglottids with sperm-filled vas deferens and atrophied testes. Absence of gravid proglottids. Testes in anterior ⅓ to ½ of proglottid, testes oval in shape, 15–44 (n = 37) long by 10–32 (n = 37) wide (Fig. 1C, 2A), 2 (n = 38) in number, 1 (n = 38) pre-poral and 1 (n = 38) anti-poral testis. Cirrus sac in anterior ½ of proglottid, round, 25–78 (n = 38) long by 28–69 (n = 38) wide, contains spined, eversible cirrus (Fig. 1C). Genital atrium indistinct. Genital pore 18–33% (n = 41) of proglottid length from anterior end, irregularly alternating. Vagina anterior to cirrus sac. Vaginal sphincter not present. Ovary near posterior end of proglottid, composed of 4 lobes paired anteriorly and posteriorly in frontal view and paired dorsally and ventrally in cross-sections (Fig. 2B), symmetrical, 68–348 (n = 42) long by 40–112 (n = 42) in maximum width. Vitelline follicles extending length of proglottid, oval, 7–24 (n = 15) long by 4–19 (n = 15) wide. Free gravid proglottids and eggs not observed.

Remarks. The acquisition of additional specimens from Colombia provided a better understanding of the morphology of this species. We were able to examine 5 additional spiral intestines of the type host from the type locality, of which 4 (80%) were infected with *R. tetralobatum*. Interestingly, this species was not found in any other locality we sampled in the Caribbean. Therefore, this species seem to be restricted to the Atlantic coast of Colombia.

The original description was based on 6 specimens and did not include information on internal anatomy and/or tegumental structures (*i.e.*, microtriches). In addition, we also detected some discrepancies between the original description and re-examination of the type series. Brooks (1977) reported that *R. tetralobatum* was 15–30 mm in length, with a genital pore position between 44 and 48% from anterior end of proglottid and an ovary measuring 198–300 in length. These accounts differ from our measurements of the type series (*i.e.*, 5.4–9.7 mm, 23–33% and 86–159, respectively). Furthermore, the examination of additional material shows that *R. tetralobatum* ranges from 4.2 to 19 mm in length, possesses genital pores that are located 18 to 33% from the posterior end, and that the ovary is 68–348 in length.

Among 41 valid species of *Rhinebothrium*, *R. tetralobatum* shares the presence of 2 testes per proglottid with 5 other members of the genus (Table 1). This restricted group consists of the freshwater species *R. fulbrighti* Reyda & Marques, 2011 infecting *Potamotrygon orbignyi* Castelnau from the Bay of Marajó, Brazil; the marine Atlantic species *R. biorchidum* Huber & Schmidt, 1985 of *Urobatis jamaicensis* Cuvier from Jamaica, *R. spinicephalum* Campbell, 1970 of *Dasyatis americana* Hildebrand & Schroeder from the coast of Virginia, U.S.A.; the eastern Pacific species *R. ditesticulum* Appy & Dailey, 1977 of *Urobatis halleri* Cooper from California, U.S.A., and *R. rhinobati* Dailey & Carvajal, 1976 of *Rhinobatos planiceps* Garman from the coast Antofagasta, Chile. Biogeographical distribution aside, *R. biorchidum*, *R. rhinobati*, and *R. spinicephalum* differ from *R. tetralobatum* by having fewer segments (15–26, 18–33, 36–49 vs. 80–206, respectively) and a smaller number of bothridial loculi (22–30, 22, 32–34 vs. 47–69, respectively). In addition, *R. biorchidum* and *R. rhinobati* can be further distinguished from *R. tetralobatum* by their smaller size (1.2–2.5, 1.8–2.8 mm vs. 4.1–19.0 mm, respectively).

Rhinebothrium distesticum and *R. fulbrighti* most closely resemble *R. tetralobatum* in their total length (9.6–28.7 and 3.1–18.0 mm vs. 4.1–19.0, respectively) and the number of

proglottids (160–276 and 40–168 vs. 80–206, respectively). However, they can be distinguished from *R. tetralobatum* by other morphological characters. For instance, *R. fulbrighti* possesses less bothridial loculi in comparison to *R. tetralobatum* (43–53 vs. 47–69, respectively). In addition, the former has been described as having only 2 ovarian lobes whereas the latter has 4; also, *R. fulbrighti* is a freshwater lineage that inhabits a different biogeographical region (*i.e.*, freshwater systems of South America). *Rhinebothrium ditesticulum* can be distinguished from *R. tetralobatum* by the morphology of the bothridia. It has a single posteriormost loculus on the bothridia and the anterior and posterior bothridial surfaces are completely separated, whereas, *R. tetralobatum* possess 2 posteriormost loculi and the anterior and posterior bothridial surfaces are not completely separated, which have a constriction at the center.

***Rhinebothrium reydai* n. sp.**
(Figs. 4,5,6)

Type-host: *Himantura schmardae* (Werner) (Myliobatiformes: Dasyatidae).
Type-locality: Caribbean Sea off Almirante, Bocas Del Toro Province, Panama (09°17'39.1"N, 82°20'42.0"W and 09°17'20.3"N, 82°21'18.9"W).
Site of infection: Spiral intestine.
Specimens deposited: #######.
Etymology: The species is named in honor of Dr. Florian B. Reyda, who took part in the sampling trip to Panama and who has contributed to the knowledge of this group of cestodes for many years.

Description. [Based on 50 mature specimens: 47 whole mounts, 1 worm observed with SEM, and 2 used for cross-sections]. Worms acraspedote (Fig. 4A), apolytic, 2.2–7.4 mm (n = 47) long, composed of 26–68 (n = 47) proglottids. Scolex 237–376 (n = 8) in maximum width composed of 4 bilobed-stalked bothridia (Fig. 4B, 6A). Bothridia lomeniform-shaped, 280–461 (n = 9) long by 111–202 (n = 9) wide, divided by 17–22 (n = 15) transverse septa and 1 medial longitudinal septum into 34–44 (n = 15) loculi, with anteriormost and posteriormost loculus single. Medial longitudinal septum extending from posterior margin of anteriormost loculus to anterior margin of the posteriormost loculus. Anteriormost loculus 18–26 (n = 10) long by 29–41 (n = 10) wide. Cephalic peduncle present; neck varying in length. Entire proximal and distal bothridial surface covered with acicular filitriches and gladiate spinitriches, except for anterior loculus with acicular filitriches only (Fig. 6B-G). Neck covered with capilliform filitriches (Fig. 6H).

Immature proglottids wider than long, becoming longer than wide with maturity, 19–56 (n = 47) in number. Mature proglottids (Fig. 4C,D) 218–554 (n = 42) long by 67–146 (n = 43) wide, 26–68 (n = 47) in number. Some terminal proglottids with sperm-filled vas deferens and atrophied testes. Gravid proglottids absent. Testes in 2 irregular columns; in anterior ¾ of proglottid, somewhat oval, 20–43 (n = 27) long by 17–34 (n = 27) wide (Fig. 5A), 4 (n = 47) in number, 2 (n = 47) poral and 2 (n = 47) aporal. Cirrus sac in anterior ½ of proglottid, wider than long, round to pyriform shaped, 33–59 (n = 39) long by 37–83 (n = 39) wide, containing spined eversible cirrus. Genital atrium prominent. Genital pores 31–49% (n = 42) of proglottid length from posterior end, irregularly alternating. Vagina anterior to cirrus sac, thick-walled, sinuous, antero-medial portion of vagina adjacent to cirrus sac directed laterally to common genital atrium. Vaginal sphincter not present. Ovary near posterior end of proglottid, inverted A-shaped in frontal view and tetra-lobed in cross-section (Fig. 5B), symmetrical, 89–279 (n = 40) long by 39–79 (n = 40) wide at isthmus. Isthmus indistinct.

Vitelline follicles extending length of proglottid, oval, 5–15 (n = 14) long by 4–12 (n = 14) wide. Free gravid proglottids and eggs not observed.

Remarks. *Rhinebothrium reydai* n. sp. was only found in 5 out of 11 specimens of *H. schmardae* we collected in the northern Caribbean coast of Panama. Interestingly, despite sharing the same host as *R. tetralobatum* in adjacent waters, there is no evidence that these two species overlap in distribution.

This new taxon is unique among *Rhinebothrium* spp. due to a combination of some morphological characters, including the presence of only 4 testes per proglottid and a single posterior bothridial loculus. Compared to other 10 species that share the presence of 4 testes per proglottid (see Table 1) only *R. chollaensis* Friggens & Duszynski, 2005 possesses a single posterior bothridial loculus. The latter species was originally described from *Urobatis halleri* from the eastern Pacific coast of Mexico. *Rhinebothrium reydai* n. sp. resembles *R. chollaensis* in total length (2.2–8.4 vs. 1.3–5.1, respectively), number of proglottids (26–70 vs. 32–84, respectively) and number of bothridial loculi (34–44 vs. 40–49, respectively). Despite these similarities, *R. chollaensis* can be distinguished from *R. reydai* n. sp. by possessing leaf-like instead of bilobed-stalked bothridia and by the absence of a cephalic peduncle, which is present in *R. reydai* n. sp. The resemblance between these two species and their biogeographical distributions might suggest a close phylogenetic association, which is yet to be tested within the context of a phylogenetic study.

Discussion

Studies on co-evolution require accurate taxonomic information to establish precise associations between parasites and hosts (Caira and Jensen, 2001; Paterson and Banks, 2001). Within this context, the main goal of the present study was to document the fauna of *Rhinebothrium* from amphi-American species of *Himantura* with the expectation that in future we will be able to identify the marine lineages closely related to the freshwater lineages of *Rhinebothrium* found in potamotrygonids.

Prior to the present study, our understanding of the richness of cestode parasites of amphi-American species of *Himantura* was a result of two contributions. Brooks (1977) examined three specimens of *H. schmardae* off the coast of Colombia (see Thorson *et al.*, 1983), which enabled him to describe 6 species of "tetraphyllideans", including *R. tetralobatum*. Marques *et al.* (1996) examined a single specimen of *H. pacifica* off the coast of Costa Rica from which they found two new species of cestodes, *Acanthobothroides pacificus* and *Scalithrium geminum*, which were considered by the authors to represent putative geminate species of *A. thorsoni* and *S. magniphalum*, both found in the host sister-species *H. schmardae*. Therefore, our study started with the premise that at least a new species of *Rhinebothrium* would be present in the eastern Pacific coast of Panama.

The absence of members of *Rhinebothrium* in *H. pacifica* could be accounted for as an artifact of sample size and/or biogeographic representation. Members of this genus are common in batoids (Linton, 1890; Brooks, 1977; Friggens and Duszynski, 2005; Reyda and Marques, 2011; Marques and Reyda, 2015). The presence of two species of this genus occurring in the sister host species *H. schmardae* raises the expectation that geminate species should be found in *H. pacifica*, which would mirror the reports on *Acanthobothroides* (Marques *et al.*, 1996), *Anindobothrium* (Trevisan *et al.* in prep. – Cap. 2) and *Scalithrium* (Marques *et al.*, 1996). The distributional pattern for the species of *Rhinebothrium* parasitizing *H. schmardae*, including the absence of members of the genus in the host

population in Belize, revealed that the parasite diversity is heterogenically distributed throughout the range of the host.

The pattern of distribution of the Caribbean species of *Rhinebothrium* found in *H. schmardae* deserves further comments. A total of 5 specimens of *H. schmardae* from Belize were examined, which included 3 reasonably large specimens (*e.g.*, with a disk diameter larger than 75 cm) highly infected with cestodes, and yet no specimens of *Rhinebothrium* could be found. Although the number of hosts sampled in Belize (n = 5) should to be considered suboptimal, we would expect to detect the presence of species of *Rhinebothorium* with a confidence of 95%, if the prevalence of member of this genus is above 45% (see Post and Millest 1991). Although this estimate is based on a series of assumptions related with sampling design and parasite distribution among host individuals (most likely not met for our samples), it might help to understand the apparent absence of species of *Rhinebothrium* in that population. The prevalence of the new species *R. reydai* in the host population of Panama was 45.5% (*i.e.*, 5 out of 11 specimens infected), whereas for *R. tetralobatum* it was 80% (*i.e.*, 4 out of 5 infected) in Colombia. These values suggest that the prevalence of species of *Rhinebothrium* in some populations of *H. schmardae* are above the levels that would allow the detection of members of this genus (if present) in Belize, assuming that the prevalence of *Rhinebothrium* in this population followed the same trend observed. On the other hand, it is true that theoretically, given the number of hosts we examined from Belize, it would be unlikely that we would find any species of cestodes with prevalence below 45%. Although all these estimates are based on small number of samples and simplified models, both not intended to be used to evaluate community ecology parameters in the first place, the data at hand suggests a considerable heterogeneity in cestode composition in amphi-American species of *Himantura*.

Heterogeneity in parasite composition among hosts is known to radically alter the shape of species accumulation curves (Dove and Cribb, 2006) imposing constraints to properly access the parasite richness of host lineages. Species accumulation curves plot species discovery as a function of sampling effort. This concept is well known by community ecologists within and outside the parasitological literature (Goteli and Colwell, 2001; Dove and Cribb 2006; Kamiya *et al.* 2014; Poulin, 2014). However, we are unaware of studies that considered species accumulation curves in which the main goal was to determine the richness of parasite lineages to address historical associations. This should be as relevant in co-evolutionary studies as it is in community ecology of parasites, since the observation that species accumulation curves approached an asymptote would indicate that sample effort was adequate to characterize the parasite lineages associated with a given host.

How species richness is distributed among sampling units and between scales of organization is generated by heterogeneity in parasite composition (Dove and Cribb, 2006). As such, if richness is concentrated at the individual sample level, that is higher α-richness, the parasite composition would be less heterogenic and species accumulation curves would approach the asymptote faster compared to communities dominated by β-richness. In the latter, individual samples, most of the time considered as hosts, tend to be dissimilar and total richness is only incremented by a regional pool of species. For β-richness communities, species accumulation curves tend to be characterized by gradual slopes and late asymptotes and hence depend on a greater sampling effort. This might be the case for the cestode richness of amphi-American species of *Himantura*.

The only example we are aware that reports heterogeneity in parasite composition along batoid host range was provided by Mojica *et al.* (2014). These authors addressed the richness of lecanicephalids of the genus *Hornellobothrium* Shipley and Hornell, 1906 parasites of *Aetobatus ocellatus* Kuhl from Borneo and Northern Australia. They found this

host species to house only one species of the genus in each locality surveyed (e.g., *H. extensivum* Jensen, 2005 from the Timor Sea; *H. gerdaae* Mojica, Jensen and Caira, 2014 from the Gulf of Carpentaria; *H. najaforme* Mojica, Jensen & Caira, 2014 from the Arafura Sea). Mojica *et al.* (2014) credit the pattern to be an artifact of sampling across the Indo-Pacific localities, since for each of them only a single host specimen was examined. In theory, only parasites with prevalence higher than 95% would be detected with 95% confidence with the examination of a single host specimen (Post and Millest, 1991). We are unaware of any other example within batoids in which a widespread host has been examined for cestodes along its entire range. Therefore, we are yet to understand how α- and β-richness shape the total species richness of cestodes in batoid fishes. Nonetheless, it is obvious that a well designed study, accounting for sample size bias and ideal biogeographical representation would be important to understand the patterns have been observed for lecanicephalids in *Aetobatus ocellatus* from the Indo-West Pacific and species of *Rhinebothrium* from *H. schmardae* in the Caribbean.

We think that species accumulation curves should be considered beyond parasite ecology studies. Its relevance to co-evolutionary studies resides on the importance of sample size and biogeographical representation to document the lineages associated with a given host. We have to keep in mind that there are additional components of the hierarchical structure of communities that might influence the shape of species accumulation curves, which might even require additional sampling effort. In general, we tend to assume that individual hosts are the main unitary measure of sampling effort. This uncritical assumption ignores that hosts in a population differ in any number of ways including size, age, diet and behavior. Those differences might influence local richness and diversity. For instance, there is some evidence that elasmobranchs in different ages can host a different cestode fauna once due to their mouth size the diet changes (Caira, 1990; Caira and Euzet, 2001).

The results of our study not only show that our initial goal is yet to be completed but we also have to rethink the how we approach sampling design for co-evolutionary studies by incorporating the role of α- and β-richness on the composition cestode fauna target hosts. We predict that the patterns of parasite distribution we observed in amphi-American species of *Himantura* are likely to be replicated for other batoids as we compile information from different host lineages. If β-richness is a relevant component of total richness, as it seems to be, we will have to allocate additional time and resources to recognize the parasite lineages associated with host lineages. Within this framework, we predict that as we increase our sample size along the distribution of both species of amphi-American *Himantura*, we will certainly find additional species of cestodes, especially in the tropical eastern Pacific.

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TABLE 1. Morphometric characters from species of *Rhinebothrium* reported from the Western Atlantic Ocean and Neotropical frashwater rivers of South America. **TL**, total length; **# prog**, number of proglottids; *# loculi*, number of loculi; **# testes**, number of testes. Ecoregions provided according to Spalding *et al.* (2007).

Species	**TL (mm)**	**# prog**	**# loculi**	**# testes**	**Type Host**	**Ecoregion**
Freshwater species						
R. corbatai Menoret & Ivanov, 2011	3.3–7.5	96–190	71–75	3–5	*Potamotrygon motoro*	Neotropial freshwater/La Plata basin
R. fulbrighti Reyda & Marques, 2011	3.1–18	40–168	43–53	2–3	*Potamotrygon orbignyi*	Neotropical freshwater/Amazon
R. mistyae Menoret & Ivanov, 2011	20–59.9	353–974	75–79	4–7	*Potamotrygon motoro*	Neotropical freshwater/La Plata basin
R. paratrygoni Rego & Dias, 1976	8–80	266–1,060	63–71	4–9	*Paratrygon sp.*	Neotropical freshwater/La Plata basin
Marine species						
R. biorchidum Huber & Schmidt, 1985	1.2–2.5	15–26	22–30	2	*Urobatis jamaicensis*	Tropical Northwestern Atlantic/Greater Antilles
R. chollaensis Friggens & Duszynski, 2005	1.3–5.1	32–84	40–49	4	*Urobatis halleri*	Warm Temperate Northeast Pacific/Cortezian
R. ditesticulum Appy & Dailey, 1977	9.6–28.7	160–276	48–54	2	*Urobatis halleri*	Tropical Eastern Pacific/Revillagigedos
R. kruppi Golestaninasab & Malek, 2015	1.5–2.4	12–17	42–46	4–5	*Glaucostegus granulatua*	Somali,Arabian/Gulf of Oman
R. maccallumi Linton, 1924	6–28	66–211	29–31	3–6	*Dasyatis centroura*	Cold Temperate Northwest Atlantic/Scotian Shelf
R. margaritense Mayes & Brooks, 1981	< 5.7	75–100	53–55	3–6	*Dasyatis guttata*	Tropical Northwestern Atlantic/Southern Caribbean
***R. reydai* n. sp.**	**2.2–8.4**	**26–70**	**34–44**	**4**	***Himantura schamardae***	**Tropical Eastern Pacific/Nicoya**
R. rhinobati Dailey & Carvajal, 1976	1.8–2.8	18–33	23	2	*Rhinobatos planiceps*	Warm Temperate Southeastern Pacific/Humboldtian
R. spinicephalum Campbell, 1970	1.7–4.4	36–49	32–34	2	*Dasyatis americana*	Cold Temperate Northwest Atlantic/Virginian
R. taeniuri Ramadan, 1984	5.1–5.7	29–30	8–22	4–8	*Taeniura lymma*	Red Sea and Gulf of Aden /Northern and Central Red Sea
R. tetralobatum Brooks, 1977	4.1–19	80–206	47–69	2	*Himantura schmardae*	Tropical Northwestern Atlantic/Southern Caribbean
R. walga (Shipley & Hornell, 1906) Euzet, 1953	NA	15–25	42	4–6	*Himantura walga*	West and South Indian Shelf /South India and Sri Lanka

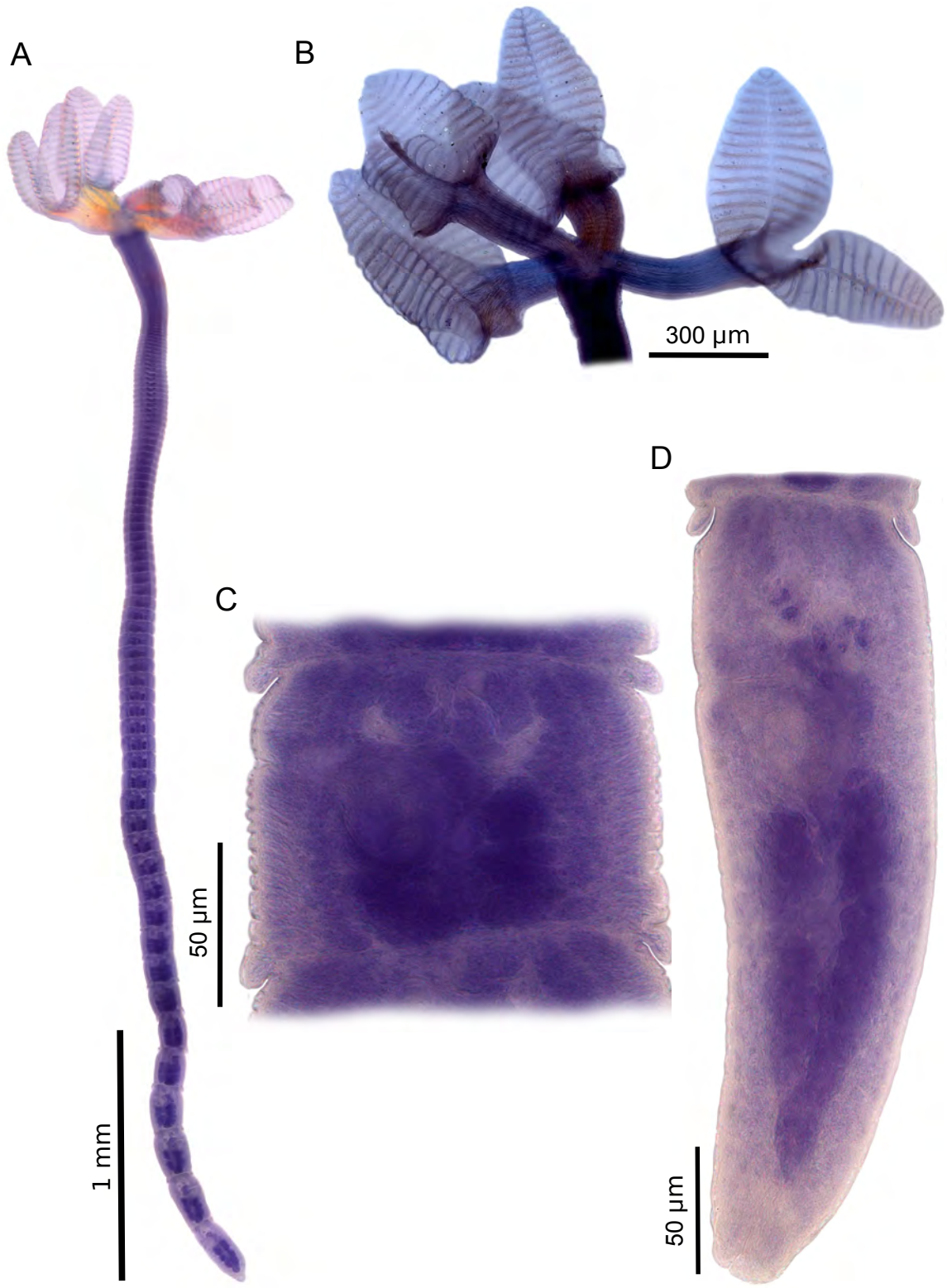

FIGURE 1. Light micrographs of *Rhinebothrium tetralobatum* Brooks, 1977 from *Himantura schmardae* from the type locality. **A.** whole worm; **B.** scolex; **C.** subterminal proglottid; **D.** terminal proglottid.

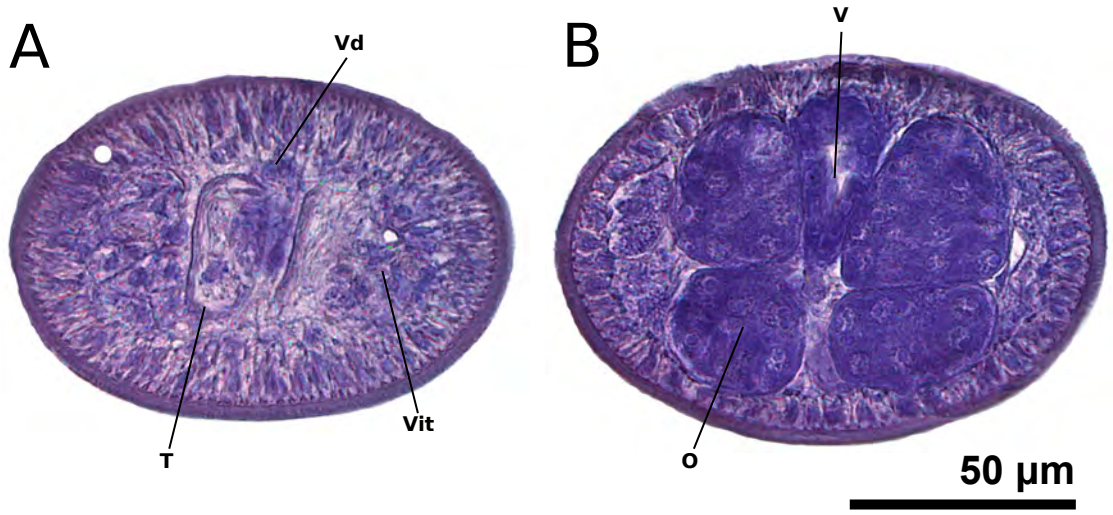

FIGURE 2. Micrographs of transversal histological sections of *Rhinebothrium tetralobatum*. **A**. section at level of testes; **B**. section at level of ovary. *Abbreviations*: **O**. ovary; **T**. testes; **V.** vagina; **Vd.** deferens vas; **Vit.** vitelline follicles.

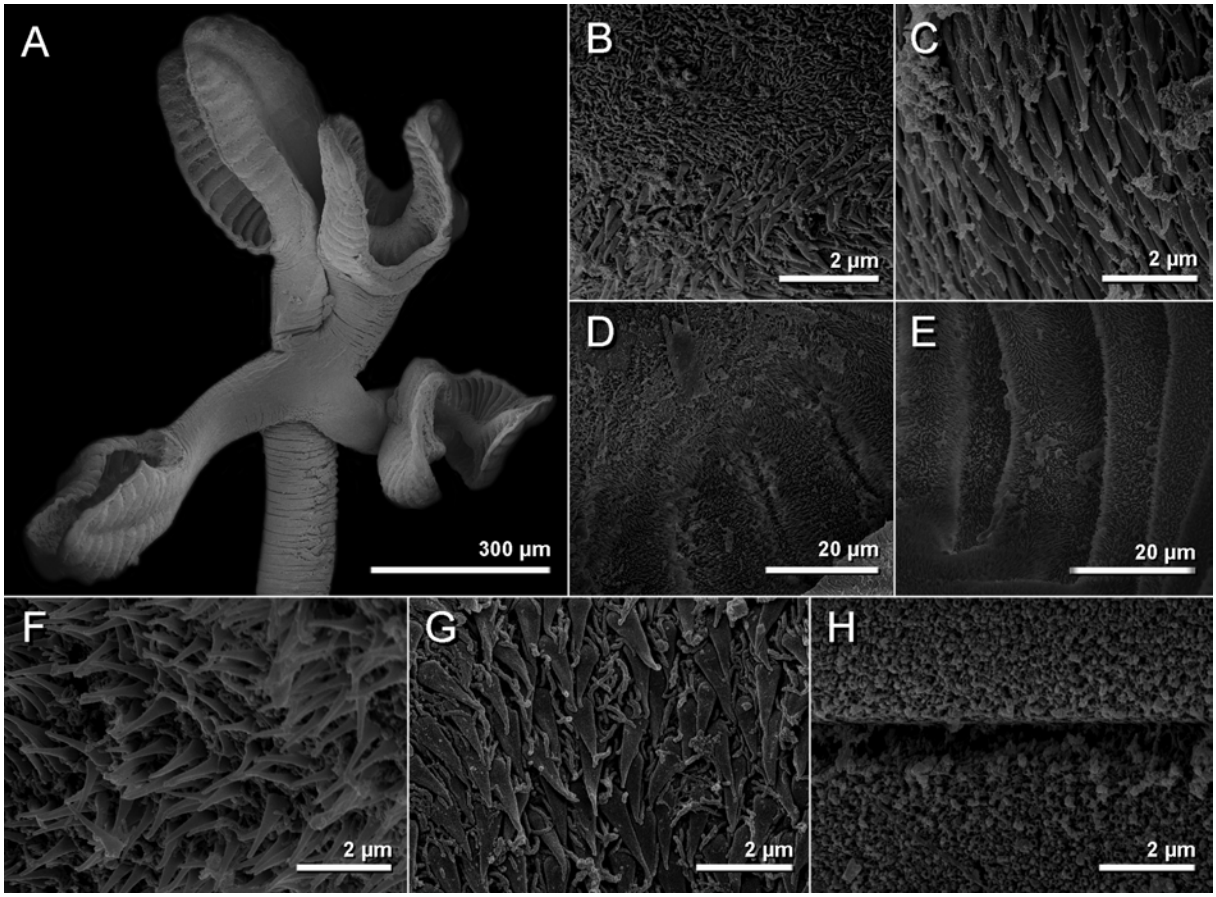

FIGURE 3. Scanning electron micrographs of *Rhinebothrium tetralobatum*. **A**. scolex; **B**. proximal surface of anterior loculus; **C**. proximal surface of middle loculus; **D**. distal bothridial surface near centre of bothridium; **E**. distal surface of transverse septa; **F.** distal bothridial surface near anterior longitudinal septa; **G**. surface posterior proximal bothridial surface; **H**. cephalic peduncle.

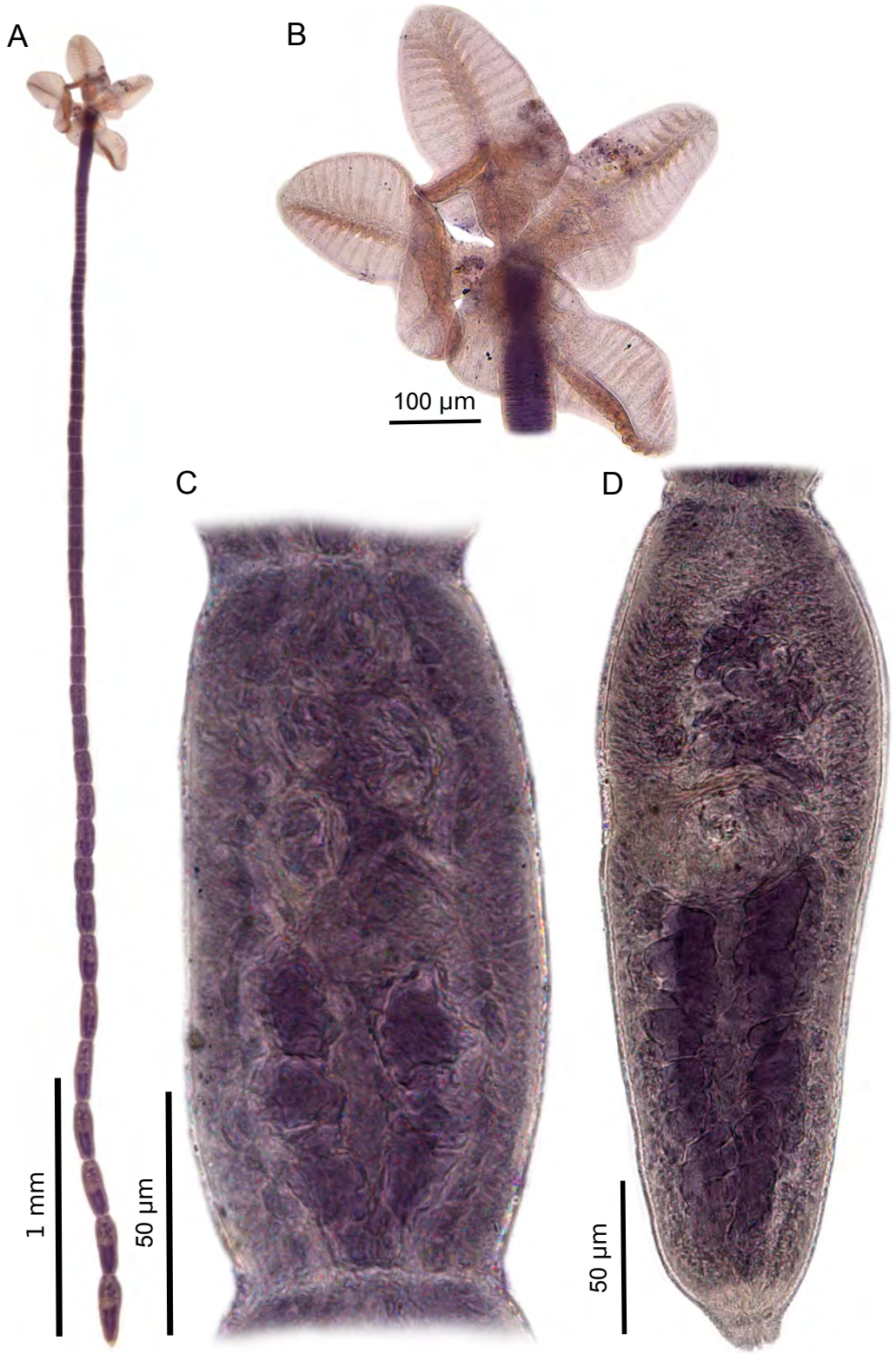

FIGURE 4. Light micrograph of *Rhinebothrium reydai* n. sp. from *Himantura schmardae* from the type locality. **A.** whole worm; **B.** scolex; **C.** subterminal proglottid; **D.** terminal proglottid.

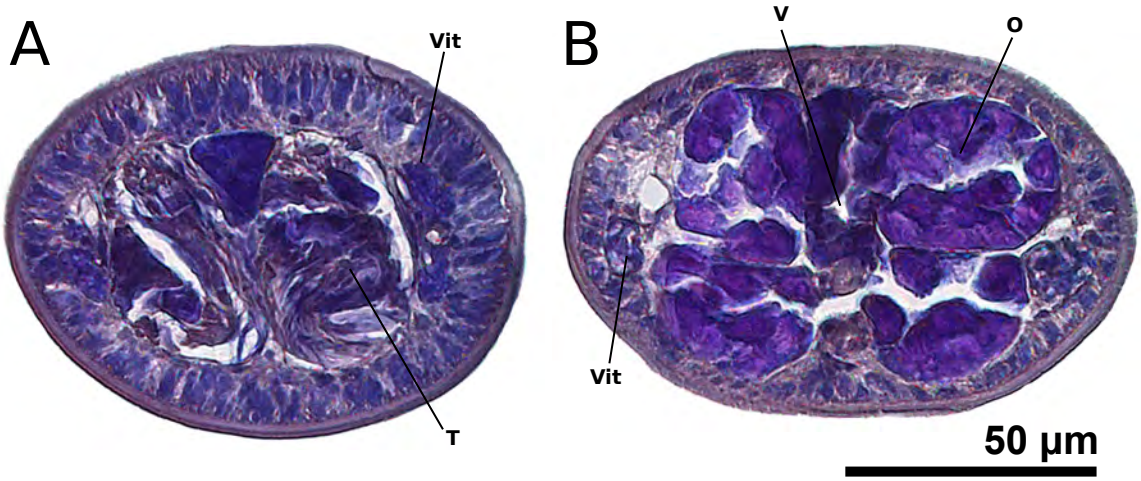

FIGURE 5. Micrographs of transversal histological sections of *Rhinebothrium reydai* n. sp. **A**. section at level of testes; **B.** section at level of ovary. *Abbreviations*: **O.** ovary; **T**. testes; **V**. vagina; **Vit.** vitelline follicles.

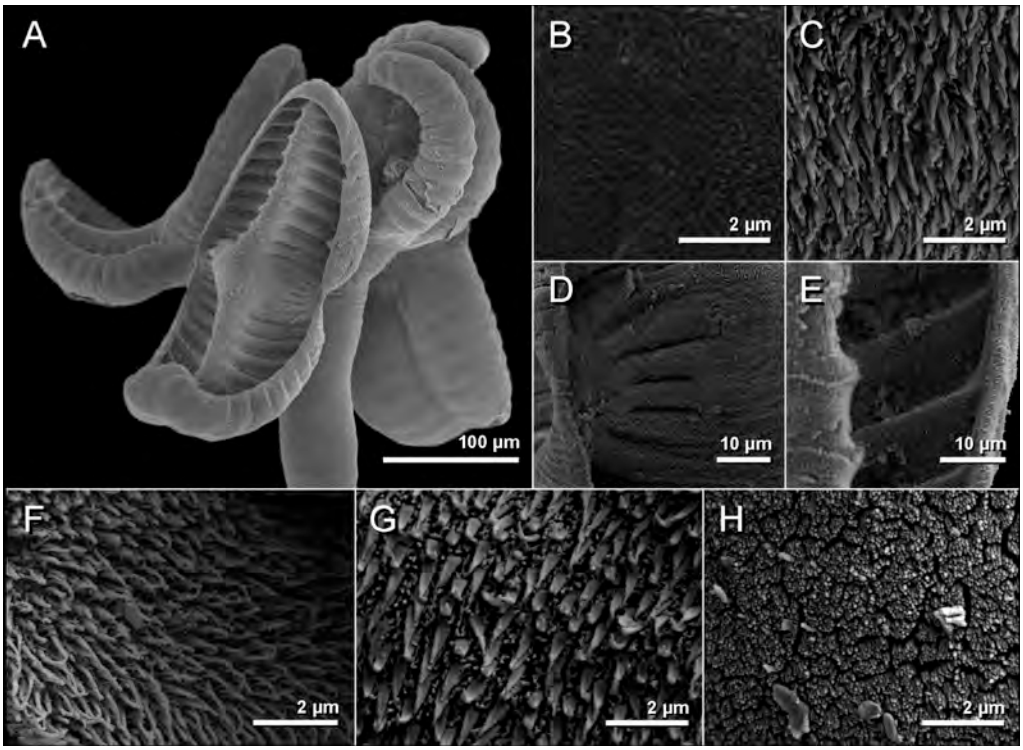

FIGURE 6. Scanning electron micrographs of *Rhinebothrium reydai* n. sp. **A**. scolex; **B**. proximal surface of anterior loculus; **C**. proximal surface of middle loculus; **D**. distal bothridial surface near centre of bothridium; **E**. distal surface of transverse septa; **F**. distal bothridial surface near anterior longitudinal septa; **G**. posterior proximal bothridial surface; **H**. cephalic peduncle.

Capítulo 2

Systematics and diversification of *Anindobothrium* Marques, Brooks & Lasso, 2001 (Eucestoda: Rhinebothriidea)

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Abstract

The genus *Anindobothrium* Marques, Brooks & Lasso, 2001 is found in both marine and Neotropical freshwater stingrays from the family Potamotrygonidae. The patterns of host association within the genus support the most recent hypothesis about the origin of potamotrygonids, which suggests that the ancestor of the Potamotrygonidae colonized South American river systems through marine incursion events during the Paleogene period and that the clade composed by amphi-American species of *Himantura* Müller & Henle [*H. schmardae* (Werner) and *H. pacifica* (Beebe & Tee-Van)] is their sister-group. However, despite the importance of the genus *Anindothothrium* to understand the diversification of potamotrygonids, no additional efforts were done to better investigate their diversity and phylogenetic relationships. Also, the existence of only two species reported for this genus [*A. anacolum* (Brooks, 1977) Marques, Brooks & Lasso, 2001 and *A. lisae* Marques, Brooks & Lasso, 2001] generates expectations that the diversity within *Anindobothrium* is underestimated. Recent collecting efforts enabled the most extensive documentation of the fauna of *Anindobothrium* parasitizing amphi-American species of *Himantura*, *Potamotrygon schroederi* Fernández-Yépez, *P. orbignyi* (Castelnau) and *P. yepezi* Castex & Castello from 6 different countries, representing the eastern Pacific Ocean, Caribbean Sea, and three river basins in South America (Rio Negro, Orinoco, and Maracaibo). Overall, we examined 152 potamotrygonids and 33 specimens of amphi-American species of *Himantura*. This unprecedented number of host specimens enabled us to provide the redescription of *A. anacolum* and *A. lisae*, and the description of two new species, one from the eastern Pacific Ocean and the other from the Caribbean Sea (*A. carioni* n. sp. and *A. inexpectatum* n. sp., respectively). For the first time, our redescription provides data on the microthrix morphology and internal anatomy as well as a better understanding on the morphological variability of species of *Anindobothrium*. In addition to the improvement on the knowledge of the diversity of this group, our results provided a robust phylogenetic analysis to test the phylogenetic position of the genus among selected lineages of cestodes. Based on our results, we propose a new family, the Anindobothriidae n. fam., to accommodate the genus *Anindobothrium* in the order Rhinebothriidea Healy, Caira, Jensen, Webster & Littlewood, 2009.

Key words: Discriminant analysis, phylogenetic analysis, integrative taxonomy, Potamotrygonidae, Dasyatidae, *Himantura*, eastern Pacific Ocean, Caribbean Sea, Amazonas.

Introduction

Members of *Anindobothrium* Marques, Brooks & Lasso, 2001 are found in marine batoids and Neotropical freshwater stingrays of the family Potamotrygonidae, mirroring the distribution patterns of some other cestode genera such as *Acanthobothrium* Blanchard 1848, and *Rhinebothrium* Linton, 1890. The genus *Anindobothrium* was erected to accommodate the marine species *Caulobothrium anacolum* Brooks, 1977, a parasite of *Himantura schmardae* (Werner) collected off the Caribbean coast of Colombia, and two species found in freshwater potamotrygonids [(*A. lisae* Marques, Brooks & Lasso, 2001 and *A. guariticus* (Marques, Brooks & Lasso, 2001)]. *Anindobothrium lisae* was first recorded from *Potamotrygon orbignyi* (Castelnau) (Potamotrygonidae) from the Rio Negro, near Barcelos (Brazil), whereas, *A. guariticus* was found in *Paratrygon aiereba* Müller & Henle from Caño Guarítico, a tributary of the Rio Apure/Orinoco in Venezuela. The latter species, however, was subsequently transferred to *Nandocestus* Reyda, 2008. Therefore, *Anindobothrium* is now comprised of two, valid species: its type, *A. anacolum* (Brooks, 1977) Marques, Brooks & Lasso, 2001 and *A. lisae*.

The observation that *Anindobothrium* is comprised by one member from Neotropical freshwater batoid and *H. schmardae* from the Caribbean Sea supports the most recent hypothesis about the origin of freshwater stingrays [1,2,3,4,5,6]. There are evidences to suggest that the ancestor of the Potamotrygonidae colonized South America through marine incursion events during the Paleogene Period, between the early Miocene and mid-Eocene (*i.e.*, 22.5–46 mya) [1,3,4]. According to this hypothesis the current tropical eastern Pacific Ocean and the Caribbean Sea are hypothetical areas of derivation of potamotrygonids. This biogeographic scenario is corroborated by phylogenetic studies on batoids based on morphological and molecular data, suggesting that amphi-American species of *Himantura* Müller & Henle [*H. schmardae* and *H. pacifica* (Beebe & Tee-Van)] form a clade sister to potamotrygonids [2,5,6].

In historical associations between hosts and parasites, if events of co-divergence prevail, one would expect to find congruence between patterns of diversification between host and parasite phylogenies [7]. The same would apply for historical associations between areas and organisms in which vicariance events would render sister taxa inhabiting sister areas. Within this framework, it is expected that the freshwater lineages of *Anindobothrium* would be closely related to those found in amphi-American species of *Himantura*. As such, the genus *Anindobothrium* might serve as a good indicator to track the historical association between potamotrygonids and marine hosts. Despite the relevance of this genus to understand the evolution of this host-parasite system, no efforts have been carried out to explore the diversity of *Anindobothrium* and its phylogenetic position among cestode genera.

The existence of two species of amphi-American species of *Himantura* also generates some expectation towards the diversity of the marine lineages of *Anindobothrium*. Thus far, only the Caribbean host species, *H. schmardae*, is known to house one species of *Anindobothrium*. Its sister-host, *H. pacifica*, is known to be infected by members of *Acanthobothrium* and *Scalithrium* Ball, Neifar & Euzet, 2003, two genera commonly found in marine batoids [8,9,10,11]. In fact, these hosts are believed to share putative geminate species [12] for both genera [13]. In addition, the information on the diversity of cestodes parasitizing amphi-American species of *Himantura* is a result of the examination of very few hosts from two isolated localities, one specimen of *H. pacifica* from the eastern Pacific coast of Costa Rica [13] and 3 specimens of *H. schmardae* off the Caribbean coast of Colombia [14]. Therefore, it is expected that a more extensive survey of *Anindobothrium* parasitizing amphi-American species of *Himantura* would reveal the hidden diversity of this genus.

At present, the systematic position of *Anindobothrium* is considered uncertain [15]. Marques et al. [16] erected the genus as a member of the Phyllobothriidae, a family of the notoriously polyphyletic Tetraphyllidea Carus, 1863. In the past decade, the concept of the Phyllobothriidae has suffered extensive modifications [15,17,18], whereas the Tetraphyllidea has been disassembled [19]. Throughout the recent rearrangement of former tetraphyllidean taxa and redefinition of the Phyllobothriidae, Ruhnke [15] suggested that *Anindobothrium* is likely to be a member of the Rhinebothriidea Healy, Caira, Jensen, Webster and Littlewood, 2009, since its members possess stalked bothridia – a putative morphological synapomorphy for the order, which is supported mainly by molecular data [17,18,20). Since, Ruhnke's [15] proposition, no effort has been made to address the phylogenetic position of *Anindobothrium* and test the hypothesis that the genus is a member of the Rhinebothriidea.

Here, we address the phylogenetic position of *Anindobothrium* and the diversity of the genus in amphi-American species of *Himantura* as well as potamotrygonid hosts. The phylogenetic position of the genus will be discussed in light of a phylogenetic analysis based on molecular data. The taxonomic representation of this dataset includes representatives of the genus from both marine and freshwater systems in addition to selected members of cestode genera representing all major lineages now recognized as members of the Rhinebothriidea as well as other orders of cestodes. The diversity of the genus is the result of molecular and morphological data from an unprecedented number of specimens after examining over 152 potamotrygonid hosts as well as 33 specimens of amphi-American species of *Himantura*.

Materials and Methods

Biological Material

A total of 92 specimens of *P. orbignyi* (Castelnau) and 39 specimens of *P. schroederi* Fernández-Yépez from the Rio Negro and Orinoco basin, 21 specimens of *P. yepezi* Castex & Castello from the Maracaibo basin and 33 specimens of amphi-American species of *Himantura* from the eastern Pacific Ocean and the Caribbean Sea were examined in this study. Collecting activities took place in six different countries and followed the guidelines of collecting permits issued to F.P.L. Marques by IBAMA no. 006/96–DIFAS of January, 1996; no. 015/ 2004 of January, 2004; 083/05–DIFAS of July, 2005; and 24451–1 of July, 2010) in Brazil; by the Instituto Socialista de la Pesca y Agricultura – INSOPESCA AMAZONAS no. 038 in Venezuela, by the Ministery of Food Production – Fisheries Division (issued in September, 2014) in Trinidad & Tobago and by the Autoridad Nacional del Ambiente – ANAM (SE/A-101–14, issued in December, 2014) in Panama; and to J.N. Caira by INDERENA Institute de Pesca y Fauna Terrestre from Bogotá in Colombia and by the Ministry of Forest, Fisheries and Sustainable Development (Belize Fisheries Department - Proc. No 000016-12, issued in 2012) in Belize.

For each individual host, images and more detailed collection data can be accessed at http://www.elasmobranchs.tapewormdb.uconn.edu by entering its assigned Collection Code and Collection Number (*e.g.*, PN15-09). The hosts and their assigned collection codes and numbers are as follows: 65 specimens of *P. orbignyi* from the Rio Negro, Barcelos, Amazonas, Brazil [(0°98'S, 62°92'W) (FMBR96–149 to 153, 155) (0°42'42.83"S, 62°59'35.87"W) (RN04–04, 16), (0°45'53.63"S, 62°56'35.51"W) (RN04–13, 15), (0°46'31.8"S, 62°56'14.28"W) (RN04–18, 21, 28, 31, 34, 39), (0°45'29.87"S, 62°59'18.23"W) (RN04–23, 24), (0°58'38.64"S, 62°54'46.44"W) (RN04–54), (0°52'11.28"S, 62°46'37.92"W)

(RN04–69, 72 to 77), (0°54'24.11"S, 62°58'21.72"W) (RN05–03, 08, 09, 11, 13 to 17, 20, 24, 26, 27, 32, 41, 44, 46, 47, 49 to 51, 53 to 60), (0°46'41.88"S, 63°8'10.32"W) (RN05–70), (0°58'29.60"S, 62°56'14.70"W) (RN11–12, 25 to 29, 60, 62)]; one specimen of *P. schroederi* from Ilha do Catalão, Manaus, Amazonas, Brazil [(0°58'38.64" S, 62°54'46.44" W) (FMBR96–145)]; 31 specimens of *P. schroederi* from Rio Negro, near Barcelos, Amazonas, Brazil [(0°45'29.87"S, 62°55'14.51"W) (RN04–08), (0°58'38.64"S, 62°54'46.44"W) (RN04–51, 53, 93, 94, 97, 98), (0°54'24.11"S, 62°58'21.72"W) (RN05–01, 12, 18, 19, 21 to 23, 25, 36, 43, 48, 61), (0°58'29.60"S, 62°56'14.70"W) (RN11–14, 23, 24, 58, 59, 61, 63), (0°58'29.60"S, 63°2'33.80"W) (RN11–34, 35), (0°50'0.90"S, 62°55'38.10"W) (RN11–42), (0°40'11.40"S, 63°1'0.80"W) (RN11–48, 50)]; 27 specimens of *P. orbignyi* from Orinoco basin [five (VZ11–26, 27, 29, 34, 35) from Rio Apure, Munoz, Apure, Venezuela (7°53'32.50"N, 68°52'49.80"W), three (VZ13–71, 74, 85) from Rio Grande, Antonio Diaz, Delta Amacuro, Venezuela (08°35'50.0"NN, 60°56'04.6"W), 12 (VZ13–01 to 04, 06, 07, 10, 12 to 16) from Agua Mena, Puerto Ayacucho, Amazonas, Venezuela (05°37'28.7"N, 67°16'29.3"W), seven (VZ13–27 to 33) from Rio Apure, San Fernando de Apure, Apure, Venezuela (09°04"32.7"N, 67°32'18.6"W)]; seven specimens of *P. schroederi* from the Orinoco basin [one (VZ13–05 from Ca-o Mesetas, Puerto Carre-o, Vichada, Colombia, 05°37'28.7"N, 67°40'02.8"W), (six from Ca-o Tomo, Cumaribo, Vichada, Colombia – VZ13–18 to 20, 24 from 07°54'33.3"N, 67°50'11.0"W and VZ13–25, 26 from 09°04"32.7"N, 67°50'11.0"W)]; 21 specimens of *P. yepezi* from the Maracaibo basin [five (VZ11–01 to 05) from Lake Maracaibo, Maracaibo, Zulia, Venezuela (10°56'14.40"N, 71°42'46.70"W), 16 (VZ11–06 to 21) from Laguna, Sinacaica, Guarija/Sinamaica, Venezuela (11°2'39.0"N, 71°51'42.50"W)]; five specimens of *H. schmardae* from Ciénaga Grande near Rapelón, Colombia [Tasajeras, Magdalena (10°58'46.84”N,74°19'32.19"W and 11°0'49.71”N, 74°16'19.32”W)] (C–43, 44, 46, 47, 49), five specimens from Head Caye, Toledo, Punta Gorda (16°13'20.8"N, 88°35'38.3"W) (BE–2, 3), from north of Southwater Caye, Dangriga, Stann Creek (16°49'43.1"N, 88°04'48.1"W) (BE–4, 5), and from Tobacco Caye, Dangriga, Stann Creek (16°54'15.2"N, 88°03'38.2"W) (BE–9), Belize, one specimen from Maracas Beach, Maracas Bay Village, San Juan-Laventille (10°45'46.8"N, 61°26'34.8"W), Trinidad & Tobago (TT14–6); 11 specimens from Almirante, Bocas del Toro (9°17'39.1N, 82°20'42.0"W and 9°17'20.3"N, 82°21'18.9"W), Panama (PN15–51 to 61); and 11 specimens of *H. pacifica* from Playa Caleta, Isla Cebaco, Golfo de Montijo, Montijo, (7°29'37.9"N, 81°13'21.9"W), Panama (PN15–9, 12 to 16, 18, 19, 23 to 25). The spiral intestines of hosts were removed and opened with a longitudinal incision. They were fixed individually in 10 % formalin buffered in seawater (1:9) or 95 % ethanol and shaken for approximately two minutes. Samples fixed in ethanol were stored at –20° C; those fixed in formalin were transferred to 70 % ethanol after approximately one week for long-term storage. Parasite specimens included in this study were selected from the spiral intestines using a stereomicroscope.

Morphological data

Cestode specimens prepared as whole mounts for light microscopy were hydrated in a regressive ethanol series, stained with Delafield's hematoxylin (9:1 solution), destained in a 1 % acid (HCl) ethanol solution, followed by a 1 % basic (NaOH) ethanol solution, dehydrated in a graded ethanol series, cleared in methyl salicylate and mounted in Canada balsam on glass slides under coverslips. Selected specimens were fully dehydrated in a graded ethanol series, stained with Acetocarmine, dehydrated in 100 % ethanol, and cleared and mounted as described above.

Histological sections were prepared from terminal proglottids of at least one specimen per population (*i.e.*, from each host and locality). Anterior parts of the worms were removed and prepared as a whole mounts as described above to serve as a voucher. Posterior parts of the strobila were embedded in paraffin according to conventional techniques. Sections were prepared at 7 µm intervals using an LEICA RM 2025 retracting rotary microtome. Sections were placed on glass slides, allowed to dry on a slide warmer for 5 min and then in an oven for 30 min, stained in Mayer's hematoxylin, counterstained in eosin, dehydrated in a graded ethanol series, cleared in xylene, and mounted in Canada balsam on glass slides under coverslips.

Scoleces selected for scanning electron microscopy (SEM) were carefully cleaned with brushes to remove the host tissue and mucus, hydrated in a graded ethanol series, transferred to 1 % osmium tetroxide overnight, dehydrated in a graded ethanol series, and placed in hexamethyldisilizane (HMDS). Subsequently, they were allowed to air-dry overnight and mounted on carbon tape on an aluminum stub. The stubs were sputter-coated with gold/palladium and examined with a Zeiss DCM 940 and FEI Quanta 600 FEG scanning electron microscope. The strobila of the worm used for SEM was prepared as a whole mount voucher as described above.

Line drawings were prepared with the aid of a drawing tube attached to a Zeiss Axioscope 2. Whole mounts were photographed using either a Zeiss Axioskope 2 equipped with a SPOT digital camera or an Olympus BX51 equipped with an Olympus SC30 camera. Fiji/ImageJ [21] was used to process the images; morphometric data were compiled with WormBox [22] and further summarized using the software R [23]. Only complete specimens with mature (*i.e.*, with open genital pores) or further developed proglottids (*e.g.*, with atrophied testes or filled vas deferens) were examined and measured in this study. All measurements for reproductive structures were taken from terminal proglottids, unless in cases those terminal proglottids presented atrophied testes, in which case those measurements were taken from subterminal mature proglottids. All measurements are in micrometers unless otherwise stated, and are presented as ranges followed by the number of specimens from which each variable was taken in parentheses. Repeated measurements for the number and dimensions of testes and for the dimensions of vitelline follicles were averaged for individuals. Terminology for the shape of the bothridia follows Clopton [24]. Microthrix terminology follows Chervy [25].

Museum abbreviations are as follows: **CHIOC**, Coleção Helmintológica do Insituto Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro, Brazil; **HWML**, Harold W. Manter Laboratory of Parasitology Collection, University of Nebrasca, Lincoln, Nebraska, U.S.A.; **INPA**, Instituto Nacional de Pesquisas da Amazônia, Manaus, Amazonas, Brazil; **LRP**, Lawrence R. Penner Parasitology Collection, Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, Connecticut, U.S.A.; **MZUSP**, Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil; **USNM**, National Museum of Natural History, Smithsonian Institution, Washington, D.C., U.S.A.; and **USNPC**, United States National Parasite Collection, Beltsville, Maryland, U.S.A. (now available at the **USNM**).

Molecular data acquisition

Scolices and posterior portions of strobila from specimens used in molecular analyses were prepared as whole mounts as described above. The hologenophores [26] were deposited at the MZUSP. The middle portion of the strobila of each specimen was removed and allowed to air dry for about 5 minutes at room temperature. Total genomic DNA was extracted using Agencourt DNAdvance - Nucleic Acid Isolation Kit (Beckman Coulter) following

manufacturer's instructions. Genomic DNA was quantified using a micro-volume spectrophotometer, NanoDrop 2000 (Thermo Scientific). Polymerase Chain Reaction (PCR) was used to amplify partial sequences of nuclear regions: 18S rDNA, 28S rDNA (D1-D3), Calmoduline (**Cal**), and Internal Transcribed Spacer 1 (**ITS-1**), and the mitochondrial region of Cytochrome Oxidadase I (**COI**). Amplifications were performed in a 25 µl volume containing 1 µl of DNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 200 lM dNTPs, 1.0–3.0 mM MgCl2, 0.4 lM of each primer, and 1 U of Taq DNA polymerase recombinant (Fermentas, Thermo Scientific). General PCR conditions included initial denaturation for 5 min at 95° C, 35 cycles of denaturation for 30 sec at 95° C, annealing for 30 sec at specific temperatures (see below), extension for 1 min to 1 min and 10 sec at 72° C, and a final extension for 7 min at 72° C. Amplifications and sequencing were performed with following primer sets: 18S rDNA with 300F 5' – AGGGTTCGATTCCGGAG – 3' and WormB 5' – CTTGTTACGACTTTTACTTCC – 3' at 55° C; 28S rDNA (D1-D3) with LSU-5F 5' – TAGGTCGACCCGCTGAAYTTAAGCA – 3' and LSU-1500R 5' – GCTATCCTGAGGGAAACTTCG – 3' at 58° C; Cal with Cal F 5' – GARCARATTGCIGARTTYAARGARGC – 3' and Cal 5' – R1 TTCTTCRTARTTIACYTGICCRTC – 3' with 5 cycles at 55° C followed by 5 cycles at 50° C; ITS-1 with CAS-18S F1 5' – TACACACCGCCCGTCGCTACTA – 3' and CAS-5,8S B1d 5' – TTCTTTTCCTCCSCTTAYTRATATGCTTAACAS – 3' at 58° C; and COI with sean1 5' – TTTACTTTGGATCATAAGCG – 3' and HCO 2198 5' – TAAACTTCAGGGTGACCAAAAAATCA – 3' at 45° C. PCR products were purified using an Agencourt AMPure XP DNA Purification and Cleanup kit (Beckman Coulter Inc.). Products were subsequently cycle-sequenced directly from both forward and reverse directions using ABI Big-Dye Sequence Terminator (v. 3.1), cleaned with ethanol precipitation, and sequenced on an ABI Prism Genetic Analyzer (3131XL) automated sequencer (Applied Biosystems/ThermoFisher).

Contiguous sequences were assembled using the package Consed/PhredPhrap [27,28,29,30]. Sequences were aligned using MAFFT [31] and visualized and edited in BioEdit (v. 7.1.3.0; [32]) to remove leading and trailing regions that varied in length.

Phylogenetic analyses

Two major analytical protocols were applied according to the main goals of this study. The first set of analyses addressed the phylogenetic position of *Anindobothrium* within major lineages of allied cestodes, since the genus was erected within the polyphyletic Phyllobothriidae, whose concept has been modified in the past years [17,18,20]. The second analysis used molecular data as a tool for species discovery within *Anindobothrium*. All methods used in both approaches are listed below.

Phylogenetic position of *Anindobothrium*

Nucleotide sequences of 18S rDNA and 28S rDNA (D1-D3 regions) were first submitted to phylogenetic analysis by direct optimization (**DO**; [33]) using POY (version 5.1.1; [34]) under parsimony as optimality criteria. Initial tree searches included 10 iterations of two independent searches for 1 h 30 min using the command search [*i.e.*, search(max_time:0:01:30)] assuming equal weights for all character transformations. This search was conducted in a 10 X 2.83 GHz Intel® Core™2 Quad Processor Q9550 computer cluster. A DO sensitivity search [35] was performed using nine alignment parameter sets in which gap extension costs varied from one to eight and transformation costs (transversions

and transitions) from one to four with an opening gap cost twice that of gap extension cost rendering the following alignment cost ratios for opening gaps, extension gaps, transversions, and transitions, respectively: 0:1:1:1, 2:1:1:1, 2:1:1:2, 2:1:2:1, 2:2:1:1, 2:2:1:2, 2:2:2:1, 2:4:1:1, 2:4:1:2, and 2:4:2:1. For each parameter set, tree space was explored by two independent searches of 2 h [*i.e.*, search(max_time:0:02:00)] in the same computer cluster environment as the previous analysis but using five nodes. After compiling candidate trees by DO, we submitted unique topologies to tree refinement by tree-fusing algorithm [36] and re-diagnosis by iterative pass alignment (**DO/IP+Fuse**; [37]). Our final analytical step under this optimality criterion was to verify the results obtained with DO/IP+Fuse by performing a phylogenetic analysis of the implied alignment (sensu [38]) generated by the previous step in TNT [39] using its New Technology searches [36,40] with following parameters: rep 100, ratchet 50, fuse 20, hold 10. We evaluated nodal support by using Goodman-Bremer values (GBS, [41,42,43]; see [44]). To obtain this metric, we considered the shortest tree found by TNT based on the implied alignment above and executed a modified version of the script BREMER.RUN distributed with TNT. This script considered 1,000 replicates with 10 repetitions of ratchet and drift [36,40] in constrained searches and the remaining default parameters. Finally, putative transformations for selected branches were compiled using the consensus tree obtained by TNT with YBYRÁ [45].

We also analyzed the previous datasets using maximum likelihood (**ML**) to identify nodes sensitive to a different optimality criterion. We started by submitting the implied alignment generated by DO/IP+Fuse to model selection in jModeltest (version 2.1.6; [46,47]) considering 88 candidate models ranked by AICc scores. Following, tree searches were performed using the parallel implementation of GARLI (version 2.0; [48]) applying 1,000 independent search replicates and remaining default parameters of GARLI configuration file. This search was conducted by implementing 20 searches replicates in 50 X 2.83 GHz Intel® Core™2 Quad Processor Q9550 computer cluster. Log-likelihood difference support (LLD; sensu [49]; see [50,51]) was calculated for selected nodes using constrained negative searches for particular clades in GARLI under the same configuration settings as the initial tree search for ML.

Species discovery within *Anindobothrium*

We started with simultaneous phylogenetic analyses of nuclear regions 18S rDNA, 28S rDNA (D1-D3), Cal, and ITS-1, and the mitochondrial region of COI for three major populations of species of *Anindobothrium* (Eastern Pacific, Caribbean Sea, and Neotropical freshwater systems). We submitted unaligned nucleotide sequences to phylogenetic inference by DO using POY (version 5.1.1; [52]) under parsimony as the optimality criterion. Tree search was performed by three independent searches for 30 min using the command search (i.e., search(max_time:0:00:30)) assuming equal weights for character transformations in the same computer cluster mentioned above. All trees compiled by DO were re-diagnosed by IP and the implied alignment submitted to TNT to verify the results using xmu algorithm for 1,000 replicates and holding at the most 10 trees per replicate. A similar analysis was conducted partitioning the dataset into nuclear and mitochondrial regions. The first partition was analyzed in POY as described above and COI was only analyzed in TNT with the same settings as before. Selected clades were diagnosed using YBYRÁ [45] within each partition.

Molecular data was analyzed using ML as optimality criterion based on the implied alignments resulted from previous analyses. Model selection for the concatenated dataset and for each nuclear region was performed in jModeltest considering 88 candidate models ranked by AICc scores. Under this optimality criterion, phylogenetic analyses were performed for

each gene region separately, as well as for two concatenated datasets: one considering only nuclear genes and the other including all regions. We analyzed each concatenated dataset using two different partition models. One considered a single substitution model for all regions and the other considered individual substitution models. Independently of dataset or partition model, tree searches were performed using parallel implementation of GARLI applying a total of 1,000 independent search replicates in 10 X 2.83 GHz Intel® Core™2 Quad Processor Q9550 computer cluster. The best partition model was selected based on AICc information criterion.

Congruence between phylogenetic patterns and morphological data was observed by compiling morphometric and meristic data for marine specimens of *Anindobothrium*. The dataset included the newly collected material as well as the type series of *A. anacolum*. A total of 29 variables were selected, most of which are traditionally used in taxonomy of the group (see below) and missing entries were filled with the mean within each putative species. All statistical analyses were performed in R as follows. The first step was to identify and exclude all highly correlated variables (*i.e.*, $r > 0.75$). Then, a Principal Component Analysis (PCA) was performed to identify whether or not there were any morphological patterns in our data congruent with phylogenetic patterns. Putative groups suggested by phylogenetic analyses and PCA were tested by Linear Discriminant Analysis (LDA; [53,54]). The error rate of the discriminant function was evaluated by 1,000 iterations of 10-fold cross validation procedure. This dataset and R scripts are available upon request to the authors.

Results

Phylogenetic analyses

Phylogenetic position of *Anindobothrium*

To address the phylogenetic position of *Anindobothrium* within the selected lineages of cestodes, 18S and 28S nucleotide data was generated for 27 terminals (Table 1). These terminals included two haplotypes of *Caulobothrium* sp. found in *Potamotrygon* sp. from the Delta of Orinoco, two members of *Rhinebothroides* sp. collected from *Potamotrygon wallacei* de Carvalho, Rosa & de Araujo from the Rio Negro, three specimens of *A. lisae* found in potamotrygonids from the Rio Negro and Orinoco river basins, and 20 other haplotypes of *Anindobothrium*, most of which collected from amphi-American species of *Himantura*. To this dataset, 67 selected terminals from Healy et al. [17], Caira et al. [19], Ruhnke et al. [18], and Marques and Caira [20] were included. These additional sequence data included 16 out-group terminals representing members of the Litobothriidea (three), Cathetocephalidea (two), Lecanicephalidea (four), Onchoproteocephalidea (one), Phyllobothriidea (one), and "Tetraphyllidea" (five); and 51 rhinebothriideans, which included members of all families according to Ruhnke et al. [18] and Marques and Caira [20].

Tree search using direct optimization under equal weights for all transformation was based on 1,279 builds followed by TBR (Tree-bisectioning and redraft, [55]), 22,032 cycles of tree fusing [36], and 622 iterations of ratchet [40]. Sensitivity search included 186 builds followed by TRB, 2,437 cycles of tree fusing, and 88 iterations of ratchet. Combined, the tree search under direct optimization found 72 unique topologies ranging from 6,407 to 6,663 steps in length. The re-diagnosis of these 72 unique topologies under Iterative Pass algorithm followed by tree fusing rendered a single topology with 6,350 transformations. The implied alignment submitted to TNT resulted in two topologies 6,346 steps in length. These two trees

differed on internal arrangements of apical terminals with near-zero branch lengths. Figure 1A displays the summary results of this analysis, including Goodman-Bremer support for selected nodes. A topology with all terminals is provided in supplementary results (Fig. S1)

Implied alignment resulted from the analyses above was submitted to ML phylogenetic inference assuming GTR+Γ+I as the substitution model. This analysis resulted in a topology with -lnL 28523.9385, which summary is presented in Figure 1B along with values of Likelihood Length Difference as a measure of support for selected clades. The detailed sister-group relationships hypothesized by ML analysis is presented in supplementary results (Fig. S2).

Both optimality criteria supported the monophyly of families but suggested different sets of sister-group relationships (Fig. 1A, B). These differences involve clades with relatively lower support. According to the parsimony analysis, *Anindobothrium* is sister to the Anthocephalidae, whereas ML topology suggested it is sister to the clade Anthocephalidae + Escherbothriidae.

Although the internal branches supporting sister-group relationships among families and genera, such as *Anindobothrium* and the "New Genus 11", have relatively lower support, all families have a relative high support (Fig. 1A, B). Also, branches leading to clades for recognized families, representatives of *Anindobothrium* nested, and for the New Genus 11 possess sets of molecular synapomorphies that could be used as putative diagnostic nucleotides for each of them (Fig. S1). For instance, *Anindobothrium* is supported by 30 transformations from the 18S region, three of which are observable (non-gap), unique, non-ambiguous synapomorphies, and 44 from 28S region, four of which are observable (non-gap), unique, non-ambiguous synapomorphies. The amount of transformations inferred for *Anindobothrium* (74) is smaller than what was recovered for the Echeneibothriidae Healy et al. [17] (86) but larger when compared to all other families.

Species discovery within *Anindobothrium*

Molecular data: Our first step towards recognizing independent lineages within this genus was to provide a phylogenetic analysis for 23 haplotypes of *Anindobothrium* based on nucleotide sequences of the nuclear regions 18S rDNA, 28S rDNA (D1-D3), Cal, ITS-1, and COI (Table 1). Our biogeografical representation included three individuals identified as *A. lisae* (one) from *Potamotrygon schroederi* from the Rio Negro and (two) *P. orbignyi* from the Rio Negro and Mid-Orinoco river basins, three terminals identified as *A. anacolum* from Trinidad and Tobago parasitizing *H. schmardae* and one from the Lago Maracaibo infecting *P. yepezi*. We also included 15 unidentified members of *Anindobothrium*, among which 10 individuals were from Belize and one from the Caribbean coast of Panama recovered from *H. schmardae*, and four worms parasitizing *H. pacifica* from the eastern Pacific coast of Panama (Table 1). In addition to these 23 haplotypes of *Anindobothrium*, the dataset included two individuals of *Anthocephalum hobergi* (Zamparo, Brooks & Barriga, 1999) from *Urobatis tumbesensis* (Chirichigno F. & McEachran) off the coast of Ecuador, which were used as out-group taxa.

Tree search using direct optimization under equal weights for all transformations was based on 126 builds followed by TRB, 13,465 cycles of tree fusing, and 79 iterations of ratchet, found 144 trees, 1,249 steps long. The re-diagnosis of all trees using iterative pass found all of them to have 1,245 steps. The phylogenetic analysis of the implied alignment resulted in 11 trees equally parsimonious with 1,245 steps. The consensus tree of these 11 topologies (Fig. 2A) suggested the monophyly of *A. lisae*, the haplotypes of *Anindobothrium* from eastern Pacific, and a clade comprised by members of the genus from Belize and

Panama (Caribbean). However, *A. anacolum* resulted as paraphyletic. The same phylogenetic pattern was observed when the partition for COI was analyzed separately in TNT. This analysis found 12 topologies at a cost of 371 steps, whose consensus tree is presented in Figure 2B. Our analysis based on direct optimization of nuclear genes resulted in 184 builds, 22,826 cycles with tree fusing, and 156 iterations of ratchet to find 215 trees with 868 steps. The re-diagnosis of these trees by iterative pass found all of them to have 863 steps and the implied alignment analyzed in TNT rendered two topologies with same cost. Contrary to COI, the nuclear genes recognized four monophyletic groups within *Anindobothrium.* Among those not recognized by COI, *A. anacolum* resulted as a monophyletic group (Fig. 2C).

The ML analysis rendered similar results. Model selection suggested that the best fitting model for the concatenated dataset was TVM+Γ+I, whereas for 18S, 28S, Cal COI, and ITS-1 it was TPM3uf+ Γ, TIM2+Γ, TIM1+ Γ, TPM1uf+I, and TPM1uf+ Γ (respectively). For the simultaneous analysis of all data we considered two partition models. One analysis considered the model TVM+Γ+I for all concatenated partitions and the other assumed distinct substitution models for each gene regions separately. The AICc favored the partition model in which different substitution models were assigned to each region (Table 2). This analysis recovered the same phylogenetic pattern as obtained by parsimony analysis (Fig. 2A). The phylogenetic analysis of COI using ML corresponded to most of the nodes recovered by parsimony analysis (see Fig. 2B). For nuclear genes, the partition model also favored different substitution models for each partition (Table 2). The ML analysis of nuclear regions displayed the same topology as obtained for the parsimony analysis (see Fig. 2B). However, ML analysis of individual partitions did not recover some nodes.

Morphological data: The 29 morphological variables were used to evaluate whether there was congruence between phylogenetic patterns and morphology. We obtained morphological data for 64 specimens of *A. anacolum*, including five individuals from the type series (USNPC 73969 – holotype, and HMWL 20265a-d – paratypes), 24 individuals from the type locality and 35 from Trinidad and Tobago. We included 41 worms from the Caribbean representing the clade formed by specimens of *Anindobothrium* n. sp. c. from Belize (31) and Panama (10). Additionally, we included 32 specimens of *Anindobothrium* n. sp. p. collected from *H. pacifica* from the eastern Pacific coast of Panama. The correlation analysis of the 29 variables revealed that some are highly correlated ($r > 0.70$, see Table 3). Therefore we excluded eight variables from further analyses.

The PCA suggested that PCA1 explains 23 % whereas PCA2 explains 15 % of variance (Fig. 3A). The centroid around the means (95 % confidence) suggested that individuals assigned to *A. anacolum* clustered together with most of the specimens from its type series and worms collected in the type locality as well as from Trinidad and Tobago. The results of this analysis found great overlap between members of this genus collected in the eastern Pacific Ocean and from the Caribbean coast of Panama/Belize. These two populations overlapped at the edge of the centroid of *A. anacolum*. The loadings of PCA1 indicate that total length, bothridial length and scolex width have greater influence in that component, whereas for PCA2, most of the variance is due to the length of poral testes and cirrus sac dimensions (Table 4).

The LDA was performed to evaluate whether the recognized marine lineages based on molecular data could be discriminated by morphological data. Our analysis was able to discriminate representatives of marine clades (Fig. 3B). The proportion of traces (*i.e.*, the percentage separation achieved by each discriminant function) was 59 % for LDA1 and 41 % for LDA2. Loadings for each discriminant function suggested that terminal mature proglottid ratio and number of testes are the most important variables for LDA1, whereas the number of

mature proglottids and vitelline follicles length were most important variables for LDA2 (Table 5). The cross validation procedure indicated a discriminant function error rate of 3 %.

Our results indicate that the phylogenetic position of *Anindobothrium* is sensitive to optimality criteria, most likely due to the relative lower support found in internal nodes. However, we found that the amount of molecular divergence in the branch supporting the monophyly of *Anindobothrium* is as great as, if not greater than, the families recognized for the order Rhinebothriidea. Based on this observation we will recognize this clade as a distinct taxon for which we provide morphological and molecular diagnoses. Within *Anindobothrium*, we are able to recognize four independent lineages, two of them already described, *A. anacolum* and *A. lisae*, whereas the other two require formal description. Based on these results, we will erect a new family, the Anindobothriidae n. fam., to provide a coherent taxonomic scheme for the inclusion of *Anindobothrium* within the Rhinebothriidea, emend the diagnosis of the genus to accommodate re-evaluation of the morphological diversity of its lineages, redescribe the existing species of the genus, and describe the new species we recognized based on molecular and morphological characters. The proposed taxonomic actions are as follows.

Taxonomic actions

Anindobothriidae n. fam.

Diagnosis. Scolex with four bothridia; bothridia with marginal loculi, with or without longitudinal and transversal septa; bothridial facial loculi present or absent; bothridial apical sucker present; anterior to posterior orientation of bothridia conspicuous; myzorhynchus lacking in adult stage. Genital pore anterior. Postvaginal testes present. Vitelline follicles interrupted by ovary. Parasites of whiptail stingrays (Dasyatidae) and Neotropical freshwater stingrays (Potamotrygonidae).

Type- and only genus. *Anindobothrium* Marques, Brooks & Lasso, 2001.

Remarks. Our molecular data provided unequivocal evidence that *Anindobothrium* is a member of the Rhinebothriidea [17]. The results corroborate Ruhnke's [15] observation that the presence of stalked bothridia found in members of *Anindobothrium* would support that the genus should be transferred to this order. Although Ruhnke [15] considered this possibility, the most recent taxonomic account on the order by Ruhnke et al. [18] did not address the position of *Anindobothrium* within the order and its families, although they defined the families within Rhinebothriidea, their composition, and putative members. Our justification for a new family to accommodate *Anindobothrium* is not only based on the molecular divergence of clades assigned to particular families within the order, but also on the morphology of the genus that does not conform with the existing diagnoses of the families provided by Ruhnke et al. [18].

The family Anindobothriidae n. fam. can be distinguished from the Echeneibothriidae de Beauchamp, 1905 by the absence of a myzorhynchus in the adult stage and by the position of genital pore (anterior vs. mid-posterior). It differs from the Rhinebothriidae Euzet, 1953 by possessing a clear anteroposterior orientation of the bothridia characterized by a conspicuous apical sucker and by the interruption of the ovary by vitelline follicles. It closely resembles the Anthocephaliidae Ruhnke, Caira & Cox, 2015 and the Escherbothriidae Ruhnke, Caira & Cox, 2015 but can be easily distinguished by the possession of post-vaginal testes. Below we provide a revised key of the Rhinebothriidea to accommodate the Anindobothriidae n. fam.

Key to families of Rhinebothriidea (modified from Ruhnke et al. [18]):

1. Myzorhynchus present in the adult stage…….…….....…...Echeneibothriidae
- Myzorhynchus absent in the adult stage…….2
2. Bothridia lacking apical suckers and anteroposterior orientation; vitelline follicles usually not interrupted by ovary…..……..Rhinebotriidae
- Bothridia with apical suckers and conspicuous anteroposterior orientation; vitelline follicles usually interrupted by ovary...……...…3
3. Presence of post-vaginal testes……..……...Anindobothriidae
- Absence of post-vaginal testes……4
4. Genital pore in anterior 1/2 of proglottid..…….Escherbothriidae
- Genital pore in anterior 1/4 of proglottid……..Anthocephaliidae

***Anindobothrium* Marques Brooks & Lasso, 2001**

Amended Diagnosis. Rhinebothriidea, Anindobothriidae n. fam. Worms craspedote, euapolytic. Scolex with four bothridia; myzorhynchus absent. Bothridia stalked, typically longer than wide, with or without longitudinal septa, with apical sucker and marginal loculi, with or without two rows of facial loculi. Mature proglottids longer than wide. Testes numerous, arranged in multiple irregular columns; post-poral field present. Vas deferens extending anteriorly from mid-proglottid to enter to cirrus sac at anterior margin, more porally than anti-porally; external seminal vesicle absent. Genital pores marginal, in anterior 1/4 of proglottid, irregularly alternating; genital atrium shallow. Cirrus sac thin-walled, tilted posteriorly, containing coiled cirrus; cirrus armed with spinitriches. Vagina extending from ootype along midline of proglottid to anterior margin of cirrus sac and laterally, becoming sinuous, to open into genital atrium anterior to cirrus sac; vaginal sphincter present; seminal receptacle not present. Ovary H-shaped in frontal view, tetralobed in cross section; ovarian margins lobulate. Vitellarium follicular; in two lateral bands; bands extending length of proglottid, interrupted by terminal genitalia, may be partially interrupted by ovary. Uterus median, ventral, sacciform, with poorly differentiated lateral diverticula or lacking diverticula, extending from ovarian isthmus to anterior margin of proglottid. Excretory vessels four in number, arranged in dorsal and ventral pairs at lateral margins of proglottid. Parasites of the Dasyatidae and the Potamotrygonidae (Myliobatiformes).

Remarks. Marques et al. [16] considered the genus as a member of the Phyllobotriidae, then a family of Tetraphyllidea. Runhke [15] considered *Anindobothrium* as *incertae sedis* in his monograph on the Phyllobotriidae. This family was recently elevated to the status of an order by Caira et al. [19], whose composition did not include the genus. The amended diagnosis also makes several corrections to the previous one and includes modifications to accommodate the new findings on the morphology of this taxon. For instance, Marques et al. [16] described *Anindobothrium* as possessing bilobed bothridia, while in fact its members have elongated ones. Moreover, the absence of longitudinal septa became incongruent with the examination of additional specimens of *A. anacolum* and the presence of marginal loculi is found in all species we now recognize within the genus. Also, the vitelline follicles may be partially interrupted by ovary. Since *Anindobothrium* is the only member of the Anindobothriidae n. fam., the genus can be differentiated from all other genera within the Rhinebothriidae by the same characters that differentiate the families of this order (see the key to the families; above).

***Anindobothrium anacolum* (Brooks, 1977) Marques, Brooks and Lasso, 2001**
(Figs. 5, 6, 7, 8)

Type-host: *Himantura schmardae* (Werner) (Myliobatiformes: Dasyatidae).
Additional host: *Potamotrygon yepezi* Castex & Castello (Myliobatiformes: Potamotrygonidae).
Type-locality: Caribbean Sea, 15 km west of La Cienaga, Magdalena, Colombia (11°01'N, 74°15'W).
Additional localities: Caribbean Sea, Tasajeras, Magdalena, Colombia (10°58'N, 74°19'W and 11° 0'N, 74°16'W); Lake Maracaibo, Maracaibo, Zulia, Venezuela (10°56'N, 71°42'W) and Maracas bay, Maracas, San Juan-Laventille, Trinidad & Tobago (10°45'N, 61°26'W).
Site of infection: Spiral intestine.
Type material: Holotype (USNPC 73969) and 4 paratypes (HWML 20265).
Additional specimens deposited: #######.

Redescription. [Based on the type series comprised of holotype (USNPC 73969) and four paratypes (HWML 20265), and 66 additional mature specimens, which included 59 whole mounts, five worms observed with SEM, and two serially-sectioned]. Worms acraspedote, apolytic, 3.1–15.1 mm (n = 63) long, composed of 8–33 (n = 61) proglottids (Fig. 5, Table 6). Scolex with greatest width 366–1,041 (n = 45), composed of four stalked bothridia (Fig. 6A, 7A). Bothridia elliptoid-shaped, 356–889 (n = 62) long by 128–707 (n = 62) wide, divided by 19–27 (n = 50) transverse septa and one medial, longitudinal septum into 39–55 (n = 50) facial loculi. Medial longitudinal septum extending from posterior margin of apical sucker to posterior margin of bothridium. Apical sucker 27–70 (n = 51) long by 25–74 (n = 49) wide. Short cephalic peduncle; neck varying in length. Proximal surface of apical sucker covered by acicular filitriches (Fig. 7D) and proximal medial bothridia covered by gladiate spinitriches (Fig. 7E). Distal surfaces of bothridia covered by gladiate spinitriches and acicular filitriches (Fig. 7B, C, F, G). Neck covered by capilliform filitriches (Fig. 7H).

Immature proglottids wider than long. Mature proglottids 686–2,484 (n = 63) long by 176–425 (n = 63) wide, 1–7 (n = 61) in number. Terminal proglottids may present sperm-filled vas deferens (Fig. 6B). Gravid proglottids not observed. Testes in anterior ¾ of proglottid, in two irregular columns, extending from near anterior margin of proglottid to anterior margin of ovary, round to oval, 32–96 (n = 59) long by 23–79 (n = 59) wide; 24–50 (n = 59) in number; 2–8 (n = 59) pre-poral, 7–20 (n = 59) post-poral, 13–26 (n = 59) anti-poral (Fig. 8A). Cirrus sac in anterior ¼ of proglottid, round, 36–163 (n = 63) long by 61–180 (n = 63) wide, armed with spinitriches eversible cirrus (Fig. 6C). Genital atrium present. Genital pores 15–32 % (n = 63) of proglottid length from anterior end, irregularly alternating. The vagina runs anterior to the cirrus sac and then turns posteriorly towards the ootype. Ovary near posterior end of proglottid, bilobed in dorso-ventral view, tetra-lobed in cross-section (Fig. 8B), follicular, symmetrical, 85–613 (n = 63) long by 67–267 (n = 63) wide at isthmus; anteroventral lobes converging anteriorly to midline of proglottid, but not fusing. Vitelline follicles extending length of proglottid, 12–49 (n = 53) long by 8–35 (n = 53) wide. Eggs not observed.

Remarks. The acquisition of additional specimens from the type locality, other localities in the Caribbean Sea and adjacent waters (*i.e.*, Lake Maracaibo) allowed to include the description of the microthrix morphology for the first time and also provided a better understanding on the distribution and patterns of infection of *A. anacolum*. This species seems to have a restricted distribution in the Caribbean coast of Colombia, Trinidad and Tobago and

the Maracaibo basin. In the latter locality, it was unexpected to find this species infecting *Potamotrygon yepezi*, a freshwater species of the family Potamotrygonidae. This could represent an accidental infection of a potamotrygonid host due to a dispersal of marine intermediate host stages into the estuarine waters of the Maracaibo basin.

The re-examination of the type series and additional material revealed that the original description provided by Brooks [14] as well as the diagnosis of this species amended by Marques et al. [16] did not provide a fair account on the bothridial morphology of this taxon. Both previous studies provided a description of the bothridia based on an immature specimen (USNPC 73969, holotype). As such, the specimen did not possess a longitudinal septum and the marginal loculi are inconspicuous, structures we observed in newly collected specimens. Although Marques et al. [16] illustrated a poorly defined anterior marginal loculus (their figure 1), which we now consider as an apical sucker, they followed the original description of this species considering this structure as being absent.

The examination of additional material provided a better understanding of the morphological variability of this species. In addition to that, we hereby increase the range of some structures, which seem more variable than previously reported. Both previous studies found that the total length was 6.8–15.4 but we found it to be 3.1–15.1. Brooks [14] observed 23–24 loculi, while Marques et al. [16] found between 42 and 44. The present study revealed a number of loculi of 39–55, which closely corresponds to the number of Marques et al. [16]. For genital pore position, both studies reported it to be 19–29 % from anterior end, whereas, we found 15–32 %.

***Anindobothrium lisae* Marques, Brooks & Lasso, 2001**
(Figs. 9, 10, 11)

Type-host: *Potamotrygon orbignyi* (Castelnau) (Myliobatiformes: Potamotrygonidae).
Additional host: *Potamotrygon schoederi* (Fernández–Yépez) (Myliobatiformes: Potamotrygonidae).
Type locality: Rio Negro, near Barcelos, Amazonas, Brazil (00º59’S, 62º58’W).
Additional locality: Rio Negro, near Barcelos, Amazonas, Brazil (00°54’S, 62°58’W and 00°98’S, 62°92’W); Rio Apure, Munoz, Apure, Venezuela (7°53'N, 68°52'W).
Site of infection: Spiral valve.
Type material: Holotype (CHIOC 34375) and 3 paratypes (HWML 16379a and INPA 400a,b).
Additional specimens deposited: #######.

Resdescription. [Based on the type series comprised of holotype (CHIOC 34375) and three paratypes (HWML 16379a and INPA 400a,b), and 34 additional mature specimens, which included 29 whole mounts, three worms observed with SEM, and two serially-sectioned]: Worms acraspedote, apolytic, 2.5–11.7 mm (n = 25) long, composed of 7–24 (n = 26) proglottids (Fig. 9, Table 6). Scolex with greatest width 386–1,197 (n = 32), composed of four, stalked bothridia (Fig. 9). Bothridia orbicular-elliptoid shaped, 225–643 (n = 17) long by 297–838 (n = 12) wide, with 40–58 (n = 10) marginal loculi and an apical sucker, 20–79 (n = 8) long by 30–79 (n = 9) wide. Transverse and longitudinal septa absent. Short cephalic peduncle; neck varying in length. Proximal surface of apical sucker covered by papilliform and acicular filitriches (Fig. 10D); proximal medial bothridia covered by gladiate spinitriches

(Fig. 10E). Distal surfaces of bothridia covered by gladiate spinitriches and acicular filitriches (Fig. 10B, C, F, G). Neck covered by capilliform filitriches (Fig. 10H).

Immature proglottids wider than long. Mature proglottids 772–2,395 (n = 28) long by 257–643 (n = 28) wide, 1–4 (n = 26) in number. Vas deferens not sperm-filled in terminal proglottids. Gravid proglottids not observed. Testes in anterior ¾ of proglottid, in two irregular columns, extending from near anterior margin of proglottid to anterior margin of ovary, round to oval, 44–101 (n = 27) long by 19–61 (n = 27) wide; 30–72 (n = 27) in number; 3–15 (n=27) pre-poral, 10–21 (n = 27) post-poral and 15–43 (n = 27) anti-poral (Fig. 11A). Cirrus sac pyriform shaped in anterior ¼ of proglottid, 45–130 (n = 27) long by 65–212 (n = 27) wide, armed with spinitriches eversible cirrus. Genital atrium present. Genital pores 18–44 % (n = 27) of proglottid length from anterior end, irregularly alternating. The vagina runs anterior to the cirrus sac and then turns posteriorly towards the ootype. Ovary near posterior end of proglottid, billobed in dorso-ventral view, tetra-lobed in cross-section (Fig 11B), follicular, symmetrical, 55–159 (n = 26) long by 138–385 (n = 26) wide at isthmus; anteroventral lobes converging anteriorly to midline of proglottid, but not fused. Vitelline follicles extending length of proglottid, 10–49 (n = 27) long by 7–27 (n = 27) wide. Eggs not observed.

Remarks. This species was only known for the Rio Negro, parasitizing *P. orbignyi* [16]. However, the examination of newly collected material from Venezuela revealed that *A. lisae* also infects *P. schroederi* from the mid-Orinoco. Rio Negro and Orinoco are known to share freshwater fauna [56], including these two species of hosts. Interestingly, *A. lisae* has never been found in *P. schroederi*, despite the examination of 31 specimens from the Rio Negro and one specimen from Ilha do Catalão, and has not been observed in *P. orbignyi* after examining 12 specimens from the mid-Orinoco (Marques, unpubl. data). Despite these hosts and the biogeographical distribution, we have no evidence to consider the presence of two populations or distinct lineages based on molecular data.

The redescription of this species provided additional information not observed by Marques et al. [16]. The examination of new material expanded the ranges of morphometric and meristic variables when we compare the original description with the present study as evidenced in bothridial width (322–775 vs. 297–838, respectively), number of poral anterior (5–13 vs. 3–15, respectively) and antipotral testes (21–38 vs. 15–43 respectively). We also provide information on the morphology and patterns of distribution of microtrix as well as cross sections of the ovary for the first time. Also, the redescription provided a better understanding of the bothridial morphology of this species, for which the original account was based on the interpretation and illustration of an immature specimen (see Marques et al. [16]; fig. 2B).

Compared to *A. anacolum*, *A. lisae* can be easily distinguished by the morphology of the bothridia. *Anindobothrium lisae* has only marginal loculi on the bothridia, whereas *A. anacolum* possesses marginal and facial loculi due to the presence of a longitudinal septum and transversal septa on each bothridium. Despite the differences in the bothridial morphology, the proglottids of both species are very similar and the molecular data suggest that both lineages belong to a coherent clade.

***Anindobothrium inexpectatum* n. sp.**
(Figs. 12, 13, 14, 15)

Type-host: *Himantura schmardae* (Werner) (Myliobatiformes: Dasyatidae).
Type-locality: Caribbean Sea, Tobacco Caye, Dangriga, Stann Creek, Belize (16°54'15.2"N, 88°03'38.2"W).
Additional localities: Caribbean Sea, Head Caye, Punta Gorda, Toledo (16°13'N, 88°35'W), north of Southwater Caye, Dangriga, Stann Creek, Belize (16°49'N, 88°04'W); Caribbean Sea, Almirante, Bocas del Toro Province, Panama (09°17'N, 82°20'W and 09°17'N, 82°21'W).
Site of infection: Spiral intestine.
Specimens deposited: #######.
Etymology: From the Latin "*inexpectatus*" meaning unexpected, referring to the surprise of finding another species of the genus in the same host and locality.

Description. [Based on 46 mature specimens: 41 whole mounts, three worms observed with SEM, and two serially-sectioned]: Worms acraspedote, apolytic, 4.3–14.1 mm (n = 39) long, composed of 15–39 (n = 39) proglottids (Fig. 12, Table 6). Scolex with greatest width 458–1,015 (n = 39), composed by four stalked bothridia (Fig. 13A, 14A). Bothridia elliptoid-shaped, 596–1273 (n = 41) long by 222–642 (n = 41) wide, divided by 23–30 (n = 39) transverse septa and one medial longitudinal septum into 47–61 (n = 39) loculi. Medial longitudinal septum extending from posterior margin of apical sucker to posterior margin of bothridium. Apical sucker 40–86 (n = 37) long by 42–83 (n = 36) wide. Short cephalic peduncle; neck varying in length. Proximal surface of apical sucker covered by acicular filitriches (Fig. 14D) and proximal medial bothridia covered by gladiate spinitriches (Fig. 14E). Distal surfaces of bothridia covered by gladiate spinitriches and acicular filitriches (Fig. 14B, C, F, G). Neck covered by capilliform filitriches (Fig. 14H).

Immature proglottids wider than long. Mature proglottids 765–2,058 (n = 39) long by 201–391 (n = 39) wide, 2–6 (n = 39) in number. Terminal proglottids could present sperm-filled vas deferens (Fig. 13B). Gravid proglottids not observed. Testes in anterior ¾ of proglottid, in two irregular columns, extending from near anterior margin of proglottid to anterior margin of ovary, round to oval, 22–57 (n = 38) long by 19–44 (n = 38) wide; 23–44 (n = 37) in number; 3–8 (n = 37) pre-poral, 7–16 (n = 38) post-poral, 12–26 (n = 37) anti-poral (Fig. 15A). Cirrus sac in anterior ¼ of proglottid, round, 78–145 (n = 39) long by 84–183 (n = 39) wide, armed with spinitriches eversible cirrus (Fig. 13C). Genital atrium present. Genital pores 17–27 % (n = 39) of proglottid length from anterior end, irregularly alternating. The vagina runs anterior to the cirrus sac and then turns posteriorly towards the ootype. Ovary near posterior end of proglottid, bilobed in dorso-ventral view, tetra-lobed in cross-section (Fig. 15B), follicular, symmetrical, 228–651 (n = 39) long by 97–235 (n = 39) wide at isthmus; antero-ventral lobes converging anteriorly to midline of proglottid, but not fusing. Vitelline follicles extending throughout length of proglottid, 11–44 (n = 39) long by 11–26 (n = 39) wide. Eggs not observed.

Remarks. *Anindobothrium inexpetatum* n. sp. was found in specimens of *H. schmardae* collected off the coast of Belize and northeastern coast of Panama in the Caribbean Sea. This species appears to have a disjunctive distribution with respect to *A. anacolum*, despite sharing the same host and the fact that both species have been reported from relatively near localities. Nonetheless, we found molecular support to segregate these sister-lineages (see Figure 3C). The congeneric status is also supported by a linear discriminant analysis, indicating that both species could also be separated by morphometric data. It should be noticed that, although we are able to discriminate these two species morphologically using a linear discriminant functions, pairwise comparisons of variables between them show that they overlap to a great extend. For instance, *A. anacolum* and *A. inexpectatum* n. sp. possess overlapping values for the total length (3.1–15.1 vs. 4.3–14.1, respectively), number of proglottids (8–33 vs. 15–39, respectively), and total number of testes (24–50 vs. 23–44, respectively). This might pose a problem to diagnose a specimen of either species, especially without precise locality information. In this particularly scenario, sequence data provide unequivocal assignment, especially for ITS-1, since both species are characterized by having a set of unequivocal and unique synapomorphies (see Fig. 3C). However, this new species can be readily distinguished from *A. lisae* by the morphology of the bothridia using the same characters we distinguished the latter from *A. anacolum* (see above).

***Anindobothrium carrioni* n. sp.**
(Figs. 16, 17, 18, 19)

Type-host: *Himantura pacifica* (Beebe and Tee-Van) (Myliobatiformes: Dasyatidae).
Type-locality: Pacific Ocean, Playa Caleta, Montijo, Veráguas, Panamá (07°29'N, 81°13'W).
Site of infection: Spiral intestine.
Specimens deposited: #######.
Etymology: This species is named in honor of Señor Agustín Carrión, who is a gifted and witty fisherman that guided us to find *Himantura pacifica* in the Gulf of Montijo during our collecting trip to the eastern Pacific cost of Panama.

Description. (Based on 36 mature specimens: 32 whole mounts, two worms observed with SEM, and two serially-sectioned): Worms acraspedote, apolytic, 4.8–13.9 mm (n = 32) long, composed of 20–33 (n = 32) proglottids (Fig. 16, Table 6). Scolex with greatest width 368–763 (n = 32) composed by four stalked bothridia (Fig. 17A, 18A). Bothridia elliptoid-shaped, 531–1,000 (n = 32) long by 162–375 (n = 32) wide, divided by 21–26 (n = 28) transverse septa and one medial longitudinal septum into 43–53 (n = 28) loculi. Medial longitudinal septum extending from posterior margin of apical sucker to posterior margin of bothridium. Apical sucker 38–64 (n = 23) long by 52–86 (n = 23) wide. Short cephalic peduncle; neck varying in length. Proximal surface of apical sucker covered by acicular filitriches (Fig. 18D) and proximal medial bothridia covered by gladiate spinitriches (Fig, 18E). Distal surfaces of bothridia covered by gladiate spinitriches and acicular filitriches (Fig. 18B, C, F, G). Neck covered by capilliform filitriches (Fig. 18H).

Immature proglottids wider than long. Mature proglottids 648–1,752 (n = 30) long by 164–353 (n = 30) wide, 3–6 (n = 32) in number. Terminal proglottids could present sperm-filled vas deferens (Fig 17B). Gravid proglottids not observed. Testes in anterior ¾ of proglottid in two irregular columns, extending from near anterior margin of proglottid to anterior margin of ovary, round to oval, 32–65 (n = 27) long by 26–45 (n = 27) wide; 21–31 (n = 29) in number; 2–5 (n = 29) pre-poral, 6–11 (n = 29) post-poral, 11–19 (n = 29) anti-poral (Fig. 19A). Cirrus sac in anterior ¼ of proglottid, round, 58–135 (n = 26) long by 72–134 (n = 26) wide, armed with spinitriches eversible cirrus (Fig. 17C). Genital atrium present. Genital pores 15–29 % (n = 29) of proglottid length from anterior end, irregularly alternating. The vagina runs anterior to the cirrus sac and then turns posteriorly towards the ootype. Ovary near posterior end of proglottid, bilobed in dorso-ventral view, tetra-lobed in cross-section (Fig. 19B), follicular, symmetrical, 230–570 (n = 28) long by 112–199 (n = 27) wide at isthmus; anteroventral lobes converging anteriorly to midline of proglottid, but not fusing. Vitelline follicles extending length of proglottid, 15–56 (n = 27) long by 12–29 (n = 27) wide. Eggs not observed.

Remarks. *Anindobothrium carioni* n. sp. is the only species of the genus known from the eastern Pacific Ocean. This species is sister to the clade comprised by the remaining marine species of the genus reported from the Caribbean Sea, *A. anacolum* and *A. inexpectatum* n. sp. All marine species of the genus have a very similar morphology, especially with regards to the bothridial architecture that share the presence of longitudinal septa and facial loculi. As stated above, the bothridial morphology can be used to distinguish the marine lineages from the only species known to be restricted to potamotrygonids in freshwater systems of South America, *A. lisae*. However, there is no morphological variable that could be used to distinguish *A. carioni* n. sp. from either *A. anacolum* or *A. inexpectatum* n. sp., since all morphometric attributes overlap among them; with the exception of the use of the linear discriminant function as discussed earlier. Based on the PCA analysis, *A. carioni* n. sp. seems to be phenetically closer to *A. inexpectatum* n. sp., since the centroids of these two species have a greater area of overlap than each of them with respect to the centroid of *A. anacolum* (Fig. 3A).

Although the morphometric data provide no unequivocal diagnostic character to recognize marine species of *Anindobothrium*, all marine species can be diagnosed on a molecular basis (Fig. 2B, C). *Anindobothrium carioni* n. sp. possesses nine base pairs from COI and 16 nuclear positions as unique, unequivocal synapomorphies. *Anindobothrium inexpectatum* n. sp. possesses six base pairs from COI and 11 nuclear positions as unique, unequivocal synapomorphies. Only *A. anacolum* can be solely diagnosed based on six nucleotide positions of COI that comprise unique, unequivocal synapomorphies. Although it is unusual to use those positions as diagnostic characters, there is neither justification to ignore the usefulness of molecular data for this purpose, nor the requirement of morphological attributes as the only source of characters to define taxa.

Discussion

Phylogeny of Rhinebothriidea and the position of *Anindobothrium*

The order Rhinebothriidea was erected by Healy et al. [17] as the result of a phylogenetic analysis based on molecular data for a selected group of the polyphyletic Tetraphyllidea. Healy et al. [17] circumscribed the taxonomic representation of their study upon Euzet's [57,58] concept of the Rhinebothriinae, which was conceptualized to accommodate tetraphyllideans that lack a myzorhynchus in adult forms and which had subdivided and unarmed bothridia. Healy et al. [17] found molecular support for a number of tetraphyllideans that not only had the diagnostic features Euzet [57] proposed to define the Rhinebothriinae, but which were also characterized by possessing stalked bothriidia. Accordingly, the original concept of the order Rhinebothriidea included members of *Anthocephalum* Linton, 1890, *Echeneibothrium* van Beneden, 1850, *Rhabdobothrium* Euzet, 1953, *Rhinebothroides* Mayes, Brooks and Thorson, 1981, *Rhinebothrium* Linton, 1890, *Rhodobothrium* Linton, 1889, *Scalithrium* Ball, Neifar and Euzet, 2003, *Spongiobothrium* Linton, 1889, and the "New Genera 1-4" (*sensu* Healy et al. [17]). These authors also acknowledged 14 putative members of the newly erected order, for which they could not provide molecular data, which included *Anindobothrium*. However, apart from creating the new order, Healy et al. [17]) did not circumscribe any families within it, arguing that future analysis including a broader taxonomic representation should be provided first, before establishing the intra-ordinal arrangements.

The internal relationships and composition of the Rhinebothriidea was revisited recently in two studies. Ruhnke et al. [18] expanded the taxonomic representation of Healy et al. [17] by adding putative members of the order, in addition to most species of *Anthocephalum* recognized to date. Based on their phylogenetic hypothesis, they recognized four families within the Rhinebothriidea: Rhinebothriidae, Echeneibothriidae, Anthocephaliidae and Escherbothriidae, from which the later two families were newly erected. Marques and Caira [20] corroborated Ruhnke et al.'s [18] suspicion that *Pararhinebothroides* Zamparo, Brooks and Barriga, 1999 not only was a member of the Anthocephaliidae, but in fact a member of *Anthocephalum*. In both studies, however, no members of *Anindobothrium* were included.

Our phylogenetic analysis provided clear evidence that *Anindobothrium* is a member of the Rhinebothriidea. However, the phylogenetic position of this genus seems to be unstable, as are most internal nodes within the order. A comparison between the phylogenetic hypotheses proposed by Ruhnke et al. [18] and Marques and Caira [20] is an example of this instability (Fig. 20A, B). Although both studies suggested that the Acanthocephalidae and the Escherbothriidae are sister taxa, they proposed different phylogenetic arrangements for the remaining taxa. Comparing the present results to previous studies, we observe that each study provided different sets of sister-group relationships for the families. All studies had different composition of terminals and some phylogenies were based on different optimality criteria. However, we believe that most of the discrepancies among these studies are due to the low

support of internal nodes. That points out to the limiting resolution power of 18S and 28S rDNA to recover a robust phylogenetic hypothesis for Rhinebothriidea.

Although sister-group relationships within Rhinebothriidea are unstable, all families – including the Anindobothriidae n. fam. – are well supported by the data regardless of optimality criteria (Fig. 1A, B). This support spawns from the large sets of molecular synapomorphies for each clade, albeit not all of them are unique and unambiguous (see Fig. 4). Based on the stability of these clades, our data justifies accommodating *Anindobothrium* in a separate family. The internal relationships among families of the Rhinebothriidea will only be achieved with the inclusion of additional data.

Species discovery within *Anindobothrium*

Prior to this study, only two species were recognized for *Anindobothrium*, *A. anacolum* of *H. schmardae* from Caribbean coastal waters of Colombia and *A. lisae* of *P. orbignyi* from the mid-Rio Negro. Since the marine host *H. schmardae* is the sister-species of *H. pacifica*, we started our study under the assumption that a new species of *Anindobothrium* would be recovered by sampling the eastern Pacific coast of Panama. Our expectation was met, but surprisingly, we also discovered a new species of this genus in the Caribbean. Thus, at present we recognize four species within this genus: *A. lisae*, *A. anacolum*, *A. inexpectatum*, and *A. carioni*.

The discovery of new members of the genus and the re-evaluation of their morphology, especially with respect to the bothridial architecture, required to provide an amended diagnosis of the genus. The diagnosis was modified to accommodate the bothridial morphology of *A. lisae*, which possesses only marginal loculi and an apical sucker, while the marine lineages possess additional longitudinal septa and facial loculi on the bothridia. Due to these obvious morphological differences on the bothridial morphology, *A. lisae* can be easily distinguished from marine members of the genus. However, among marine species, we were unable to provide morphological characters that could be used to differentiate them.

The lack of discrete morphological characters that would serve as diagnostic to recognize marine lineages of *Anindobothrium* imposes a challenge. There is no single or sets of characters that exhibit discontinuous distribution that would serve to this purpose. As such, for these species we can only rely on molecular data and/or discriminant functions to diagnose them. Both are untraditional means of assigning group membership in taxonomy of cestodes. However, linear discriminant functions have already been used successfully by others to recognize parasite lineages [59,60]. The relevance of molecular characters in recognizing species have also been stressed elsewhere [61,62,63]; although, we are unaware of any example in cestode systematics. Be that as it may, within the context of integrated taxonomy, we should approach species discovery by searching congruence among different sources of data without giving preference to one or another method [64,65]. We think this contribution is a step towards this approach taxonomy.

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TABLE 1. Nucleotide sequences for members of *Anindobothrium* generated in the presente study. ####, NCBI/Genbank accession numbers.

Locality [specimens #]	Species [voucher number]	Host (field trip)	18S	28S	Cal	ITS-1	COI
Caribbean/Belize [10]	*Anindobothrium* n. sp. c. [MZUSP 7767]	*Himantura schmardae* (BE-002)	####	####	####	####	####
	Anindobothrium n. sp. c. [MZUSP 7768]	*Himantura schmardae* (BE-002)	####	####	####	####	####
	Anindobothrium n. sp. c. [MZUSP 7769]	*Himantura schmardae* (BE-002)	####	####	####	####	####
	Anindobothrium n. sp. c. [MZUSP 7770]	*Himantura schmardae* (BE-003)	####	####	####	####	####
	Anindobothrium n. sp. c. [MZUSP 7771]	*Himantura schmardae* (BE-005)	####	####	####	####	####
	Anindobothrium n. sp. c. [MZUSP 7772]	*Himantura schmardae* (BE-005)	####	####	####	####	####
	Anindobothrium n. sp. c. [MZUSP 7773]	*Himantura schmardae* (BE-011)	####	####	####	####	####
	Anindobothrium n. sp. c. [MZUSP 7774]	*Himantura schmardae* (BE-009)	####	####	####	####	####
	Anindobothrium n. sp. c. [MZUSP 7775]	*Himantura schmardae* (BE-003)	####	####	####	####	####
	Anindobothrium n. sp. c. [MZUSP 7776]	*Himantura schmardae* (BE-005)	####	####	####	####	####
Caribbean/Panama [1]	*Anindobothrium* n. sp. c. [MZUSP 7777]	*Himantura schmardae* (PN15-54)	####	####	####	####	####

TABLE 1. (Continued).

Caribbean/Trinidad & Tobago [4]	*Anindobothrium anacolum* [MZUSP 7778]	*Himantura schmardae* (TT14-06)	####	####	####	####	####
	Anindobothrium anacolum [MZUSP 7779]	*Himantura schmardae* (TT14-06)	####	####	####	####	####
	Anindobothrium anacolum [MZUSP 7780]	*Himantura schmardae* (TT14-06)	####	####	####	####	####
	Anindonothrium anacolum [MZUSP 7789]	*Himantura schmardae* (TT14-06)	####	####	####	####	####
Eastern Pacific/Panama [4]	*Anindobothrium* n. sp. p. [MZUSP 7785]	*Himantura pacifica* (PN15-14)	####	####	####	####	####
	Anindobothrium n. sp. p. [MZUSP 7786]	*Himantura pacifica* (PN15-14)	####	####	####	####	####
	Anindobothrium n. sp. p. [MZUSP 7787]	*Himantura pacifica* (PN15-14)	####	####	####	####	####
	Anindobothrium n. sp. p. [MZUSP 7788]	*Himantura pacifica* (PN15-25)	####	####	####	####	####
Eastern Pacific/Ecuador [2]	*Anthocephalum hobergi* [MZUSP 7754]	*Urobatis tumbesensis* (EC-14)	KU295561	KU295565	####	####	####
	Anthocephalum hobergi [MZUSP 7756]	*Urobatis tumbesensis* (EC-56)	KU295562	KU295566	####	####	####
Neotropical Freshwater/Delta of Orinoco [2]	*Caulobothrium* sp. [MZUSP 7790]	*Potamotrygon* sp. (VZ13-48)	####	####	####	####	####
	Caulobothrium sp. [MZUSP 7791]	*Potamotrygon* sp. (VZ13-48)	####	####	####	####	####
Neotropical Freshwater/Maracaibo [1]	*Anindobothrium anacolum* [MZUSP 7781]	*Potamotrygon yepezi* (VZ11-001)	####	####	####	####	####

TATBLE 1. (Continued).

Neotropical Freshwater/Rio Negro [3]	*Anindobothrium lisae* [MZUSP 7782]	*Potamotrygon orbignyi* (RN11-028)	####	####	####	####	####
	Anindobothrium lisae [MZUSP 7783]	*Potamotrygon schroederi* (RN11-058)	####	####	####	####	####
	Anindobothrium lisae [MZUSP 7784]	*Potamotrygon orbignyi* (VZ11-029)	####	####	####	####	####
Neotropical Freshwater/Rio Negro [2]	*Rhinebothroides* sp. [MZUSP 7792]	*Potamotrygon wallacei* (RN11-009)	####	####	–	–	–
	Rhinebothroides sp. [MZUSP 7793]	*Potamotrygon wallacei* (RN11-030)	####	####	–	–	–

TABLE 2. Partition model tests for ML phylogenetic analyses including haplotypes of *Anindobothrium*. #taxa, number of taxons analysed; **#branches**, number of branches; **#EPSM**, number of free (estimated) parameters in substitution models; K, total number of free parameter, which indludes tolopolgy, branch lengths, and free parameters in the substitution model(s); **#Char**, number of characters considered in ML analyses (unique patterns); **-lnL**, negative Log-Likelihood scores; **AIC**, Akaike Information Criterion score of partition models; **AICc**, Corrected Akaike Information Criterion score of partition models. Gray rows indicate selected partition model according to AICc.

Model	**#taxa**	**#branches**	**#EPSM**	**K**	**#Char**	**-lnL**	**AIC**	**AICc**
All genes single	24	45	9	79	356	-11012.38731	22182.77463	22227.42680
All genes partition	24	45	32	102	483	-10598.46585	21400.93171	21455.15276
Nuclear single 10	24	45	7	77	220	-8222.02870	16598.05741	16680.47994
Nuclear partition	24	45	25	95	320	-8065.99067	16321.98134	16401.71348

TABLE 3. Correlation Matrix (Pearson) between morphometric variables considered for marine lineages of *Anindobothrium*.

Variable	TL	T#Prog	T#MProg	T#IProg	SCW	BtL	BtW	AsL	AsW	#Loc	ProgL	ProgW	ProgR	GPD	GPP	TesPL	TesPW	#TesPA	#TesPP	TesAL	TesAW	#TesA	T#Tes	CsL	CsW	OL	OW	VL	VW
Total length	1.00	0.63	0.45	0.60	0.52	0.53	0.43	0.35	0.17	0.27	0.57	0.32	0.27	0.54	-0.07	0.17	0.16	0.33	0.22	0.15	0.06	0.20	0.27	0.33	0.34	0.64	0.28	0.22	0.18
Total number of proglottids	0.63	1.00	0.67	**0.97**	0.40	0.48	0.17	0.30	0.34	0.28	-0.08	0.27	-0.23	-0.08	-0.04	-0.14	-0.03	-0.02	-0.05	-0.14	-0.08	-0.07	-0.06	-0.14	-0.04	0.19	0.12	0.13	0.16
Number of mature proglottids	0.45	0.67	1.00	0.48	0.22	0.23	0.09	0.06	0.09	0.17	0.01	0.15	-0.08	0.01	0.01	-0.05	0.05	-0.10	-0.10	-0.07	-0.11	-0.19	-0.16	-0.23	-0.28	0.15	-0.16	0.10	0.06
Number of immature proglottids	0.60	**0.97**	0.48	1.00	0.41	0.49	0.17	0.33	0.38	0.28	-0.10	0.26	-0.24	-0.10	-0.04	-0.15	-0.04	0.00	-0.03	-0.13	-0.06	-0.03	-0.03	-0.10	0.03	0.17	0.19	0.12	0.18
Scolex width	0.52	0.40	0.22	0.41	1.00	0.60	0.64	0.38	0.24	0.46	0.36	0.27	0.13	0.34	-0.02	0.02	0.12	0.25	0.29	0.07	0.14	0.18	0.26	0.09	0.18	0.34	0.07	0.03	0.06
Bothridial length	0.53	0.48	0.23	0.49	0.60	1.00	0.51	0.63	0.43	0.51	0.18	0.24	-0.01	0.17	0.02	-0.23	-0.16	0.10	0.11	-0.17	-0.13	0.06	0.10	0.10	0.20	0.33	0.16	-0.01	0.06
Bothridial width	0.43	0.17	0.09	0.17	0.64	0.51	1.00	0.37	0.17	0.39	0.44	0.31	0.17	0.41	-0.07	0.06	0.03	0.34	0.33	0.15	0.18	0.25	0.33	0.29	0.33	0.41	0.07	0.01	0.07
Apical sucker length	0.35	0.30	0.06	0.33	0.38	0.63	0.37	1.00	0.48	0.34	0.13	0.27	-0.06	0.12	-0.04	-0.27	-0.14	0.08	0.15	-0.16	-0.12	0.05	0.10	0.05	0.26	0.25	0.18	0.07	0.14
Apical sucker width	0.17	0.34	0.09	0.38	0.24	0.43	0.17	0.48	1.00	0.13	-0.17	0.17	-0.25	-0.15	0.11	-0.17	-0.07	-0.21	-0.23	-0.03	-0.02	-0.29	-0.29	-0.16	-0.02	0.08	0.17	0.22	0.30
Number of loculi	0.27	0.28	0.17	0.28	0.46	0.51	0.39	0.34	0.13	1.00	0.12	0.09	0.06	0.11	-0.05	-0.25	-0.16	0.08	0.03	-0.24	-0.10	0.04	0.06	-0.05	0.06	0.16	-0.04	-0.08	0.05
Proglottid length	0.57	-0.08	0.01	-0.10	0.36	0.18	0.44	0.13	-0.17	0.12	1.00	0.08	**0.79**	**0.99**	0.12	0.25	0.11	0.35	0.42	0.30	0.13	0.35	0.43	0.61	0.42	0.78	0.04	0.13	0.12
Proglottid width	0.32	0.27	0.15	0.26	0.27	0.24	0.31	0.27	0.17	0.09	0.08	1.00	-0.53	0.03	-0.24	0.20	0.31	0.21	0.24	0.15	0.15	0.10	0.19	0.08	0.38	0.07	0.43	0.25	0.30
Proglottid length-width ratio	0.27	-0.23	-0.08	-0.24	0.13	-0.01	0.17	-0.06	-0.25	0.06	**0.79**	-0.53	1.00	0.80	0.22	0.09	-0.08	0.15	0.21	0.14	0.01	0.22	0.23	0.46	0.10	0.58	-0.24	-0.04	-0.11
Genital pore distance	0.54	-0.08	0.01	-0.10	0.34	0.17	0.41	0.12	-0.15	0.11	**0.99**	0.03	**0.80**	1.00	0.25	0.21	0.07	0.30	0.40	0.27	0.10	0.32	0.39	0.59	0.37	0.80	0.01	0.12	0.11
Genital pore position	-0.07	-0.04	0.01	-0.04	-0.02	0.02	-0.07	-0.04	0.11	-0.05	0.12	-0.24	0.22	0.25	1.00	-0.19	-0.24	-0.26	-0.08	-0.13	-0.19	-0.17	-0.18	-0.04	-0.22	0.27	-0.19	-0.03	-0.03
Poral testes length	0.17	-0.14	-0.05	-0.15	0.02	-0.23	0.06	-0.27	-0.17	-0.25	0.25	0.20	0.09	0.21	-0.19	1.00	0.66	0.25	0.11	0.64	0.56	0.08	0.14	0.21	0.27	0.03	0.18	0.29	0.15
Poral testes width	0.16	-0.03	0.05	-0.04	0.12	-0.16	0.03	-0.14	-0.07	-0.16	0.11	0.31	-0.08	0.07	-0.24	0.66	1.00	0.18	0.10	0.51	0.64	-0.01	0.07	0.14	0.19	-0.08	0.29	0.23	0.15
Number of pre-poral testes	0.33	-0.02	-0.10	0.00	0.25	0.10	0.34	0.08	-0.21	0.08	0.35	0.21	0.15	0.30	-0.26	0.25	0.18	1.00	0.55	0.14	0.18	0.62	**0.76**	0.31	0.48	0.20	0.17	-0.07	-0.09
Number of post-poral testes	0.22	-0.05	-0.10	-0.03	0.29	0.11	0.33	0.15	-0.23	0.03	0.42	0.24	0.21	0.40	-0.08	0.11	0.10	0.55	1.00	0.10	0.12	0.66	**0.85**	0.32	0.46	0.23	-0.01	-0.05	-0.19
Anti-poral testes length	0.15	-0.14	-0.07	-0.13	0.07	-0.17	0.15	-0.16	-0.03	-0.24	0.30	0.15	0.14	0.27	-0.13	0.64	0.51	0.14	0.10	1.00	**0.71**	0.03	0.08	0.32	0.24	0.08	0.22	0.30	0.19
Anti-poral testes width	0.06	-0.08	-0.11	-0.06	0.14	-0.13	0.18	-0.12	-0.02	-0.10	0.13	0.15	0.01	0.10	-0.19	0.56	0.64	0.18	0.12	**0.71**	1.00	0.02	0.10	0.17	0.20	-0.07	0.29	0.32	0.22
Number of anti-poral testes	0.20	-0.07	-0.19	-0.03	0.18	0.06	0.25	0.05	-0.29	0.04	0.35	0.10	0.22	0.32	-0.17	0.08	-0.01	0.62	0.66	0.03	0.02	1.00	**0.93**	0.33	0.40	0.20	0.04	-0.18	-0.17
Total number of testes	0.27	-0.06	-0.16	-0.03	0.26	0.10	0.33	0.10	-0.29	0.06	0.43	0.19	0.23	0.39	-0.18	0.14	0.07	**0.76**	**0.85**	0.08	0.10	**0.93**	1.00	0.36	0.49	0.24	0.06	-0.13	-0.19
Cirrus sac length	0.33	-0.14	-0.23	-0.10	0.09	0.10	0.29	0.05	-0.16	-0.05	0.61	0.08	0.46	0.59	-0.04	0.21	0.14	0.31	0.32	0.32	0.17	0.33	0.36	1.00	0.57	0.44	0.31	0.10	0.08
Cirrus sac width	0.34	-0.04	-0.28	0.03	0.18	0.20	0.33	0.26	-0.02	0.06	0.42	0.38	0.10	0.37	-0.22	0.27	0.19	0.48	0.46	0.24	0.20	0.40	0.49	0.57	1.00	0.33	0.47	0.09	0.14
Ovary length	0.64	0.19	0.15	0.17	0.34	0.33	0.41	0.25	0.08	0.16	**0.78**	0.07	0.58	**0.80**	0.27	0.03	-0.08	0.20	0.23	0.08	-0.07	0.20	0.24	0.44	0.33	1.00	0.11	0.18	0.27
Ovary width	0.28	0.12	-0.16	0.19	0.07	0.16	0.07	0.18	0.17	-0.04	0.04	0.43	-0.24	0.01	-0.19	0.18	0.29	0.17	-0.01	0.22	0.29	0.04	0.06	0.31	0.47	0.11	1.00	0.28	0.34
Vitelline follicles length	0.22	0.13	0.10	0.12	0.03	-0.01	0.01	0.07	0.22	-0.08	0.13	0.25	-0.04	0.12	-0.03	0.29	0.23	-0.07	-0.05	0.30	0.32	-0.18	-0.13	0.10	0.09	0.18	0.28	1.00	0.64
Vitelline follicles width	0.18	0.16	0.06	0.18	0.06	0.06	0.07	0.14	0.30	0.05	0.12	0.30	-0.11	0.11	-0.03	0.15	0.15	-0.09	-0.19	0.19	0.22	-0.17	-0.19	0.08	0.14	0.27	0.34	0.64	1.00

TABLE 4. Loadings values for each variable in Principal Component Analysis (Fig. 3A).

VARIABLE	PCA1	PCA2
Total length	**-0.373**	
Total number of proglottids	-0.257	-0.262
Number of mature proglottids	-0.136	-0.247
Scolex width	**-0.327**	
Bothridia length	**-0.338**	-0.223
Bothridia width	-0.312	
Apical sucker length	-0.280	-0.169
Apical sucker width	-0.174	-0.234
Number of loculi	-0.210	-0.219
Proglottid width	-0.235	0.109
Proglottid length-width ratio		0.133
Genital Pore Position		-0.150
Poral testes length		**0.388**
Poral testes width		0.312
Total number of testes	-0.140	0.242
Cirrus sac length	-0.160	**0.350**
Cirrus sac width	-0.226	**0.340**
Ovary length	-0.275	
Ovary width	-0.177	0.228
Vitelline follicles length	-0.122	0.136
Vitelline follicles width	-0.154	

TABLE 5. Loadings values for each variable in Linear Discriminat Analysis (Fig. 3B).

Variable	LD1	LD2
Bothridia length	0.004	-0.004
Bothridia width	-0.007	-0.004
Cirrus sac length	-0.009	-0.018
Cirrus sac width	-0.013	-0.038
Genital pore position	0.045	0.232
Numver of loculi	0.162	-0.341
Number of mature proglottids	0.088	**-0.666**
Total number of proglottids	0.193	0.178
Total number of testes	**-0.232**	-0.158
Ovary length	0.015	-0.003
Ovary width	-0.004	0.054
Proglottid length-width ratio	**-0.902**	-0.275
Proglottid width	-0.010	-0.048
Scolex width	0.005	-0.013
Apical sucjer length	0.060	-0.025
Apical sucker width	0.068	0.151
Poral testes length	-0.001	0.083
Poral testes width	-0.021	0.101
Total length	-0.136	0.065
Vitelline follicles length	0.140	**0.442**
Vitelline follicles width	-0.109	-0.082

TABLE 6. Morphometric and meristic characters for species of *Anindobothrium*. Data presented as ranges followed by number os specimens measured between parantheses.

Character	***A. anacolum***	***A. lisae***	***A. inexpectatum* n. sp.**	***A. carioni* n. sp.**
Total Length (mm)	3.1–15.1 (63)	2.5–11.7 (25)	4.3–14.1 (39)	4.8–13.9 (32)
Number of proglottids	8–33 (61)	7–24 (26)	15–39 (39)	20–33 (32)
Scolex width	366–1,041 (45)	386–1,197 (31)	458–1,015 (39)	368–763 (32)
Botridium length	356–889 (62)	225–643 (17)	596–1,273 (41)	531–1,000 (32)
Botridium width	128–707 (62)	297–838 (12)	222–642 (41)	162–375 (32)
Apical sucker length	27–70 (51)	20–79 (8)	40–86 (37)	38–64 (23)
Apical sucker width	25–74 (49)	30–79 (9)	42–83 (36)	52–86 (23)
Number of loculi	39–55 (50)	40–58 (10)	47–61 (39)	43–53 (28)
Number of mature proglottids	1–7 (61)	1–4 (26)	2–6 (39)	3–6 (32)
Mature/Terminal proglottid length	686–2,484 (63)	722–2,395 (28)	765–2,058 (39)	648–1,752 (30)
Mature/Terminal proglottid width	176–425 (63)	257–643 (28)	201–391 (39)	164–353 (30)
Proglottid length-width ratio	3–10 (63)	2–6 (28)	2–10 (39)	3–7 (30)
Genital pore position (%)	15–32 (63)	18–44 (27)	17–27 (39)	15–29 (29)
Total number of testes	24–50 (59)	30–72 (27)	23–44 (37)	21–31 (29)
Number of pre-poral testes	2–8 (59)	3–15 (27)	3–8 (37)	2–5 (29)
Number of post-poral testes	7–20 (59)	10–21 (27)	7–16 (37)	6–11 (29)
Number of anti-poral testes	13–26 (59)	15–43 (27)	12–26 (37)	11–19 (29)
Testes length	32–96 (59)	44–101 (27)	22–57 (38)	32–65 (27)
Testes width	23–79 (59)	19–61 (27)	19–44 (38)	26–45 (27)

TABLE 6. (Continued).

Cirrus sac length	36–163 (63)	45–130 (27)	78–145 (39)	58–135 (26)
Cirrus sac width	61–180 (63)	65–212 (27)	84–183 (39)	72–134 (26)
Ovary length	137–613 (63)	55–159 (26)	228–651 (39)	230–570 (28)
Ovary width	67–267 (63)	138–385 (26)	97–235 (39)	112–199 (27)
Vitelline follicles length	12–49 (53)	10–49 (27)	14–44 (39)	15–56 (27)
Vitelline follicles width	8–35 (53)	7–27 (27)	11–26 (39)	12–29 (27)

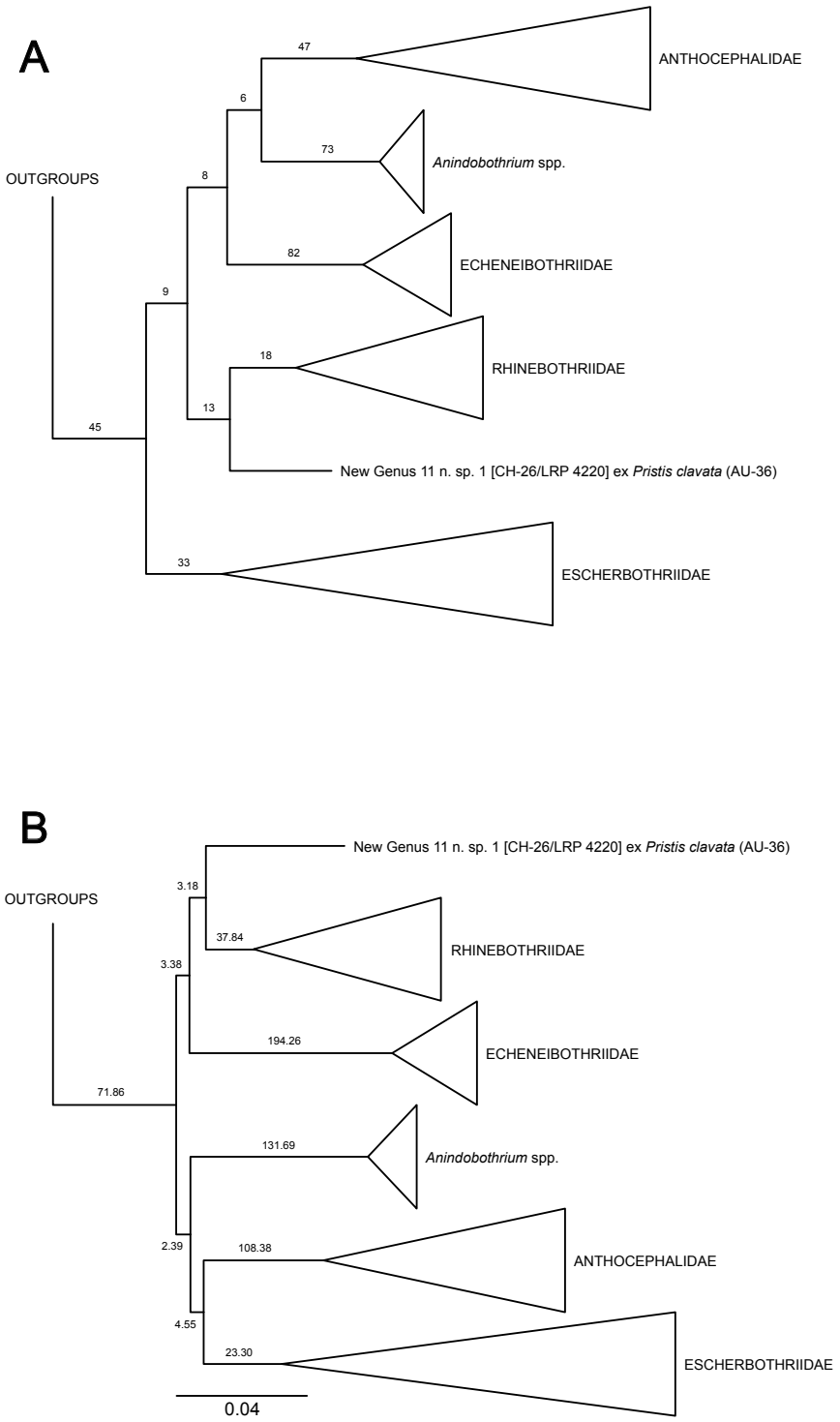

FIGURE 1. Summary of sister-group relationships within Rhinebothriidea based on the simultaneous analysis of 18S and 28S rDNA regions. A. Topology inferred under parsimony as the optimality criteria with 6346 steps in length and Goodman-Bremer support values for selected clades. **B.** Topology inferred under Maximum Likelihood as the optimality criteria (-lnL 28523.9385) and Likelihood Length Difference support values for selected clades.

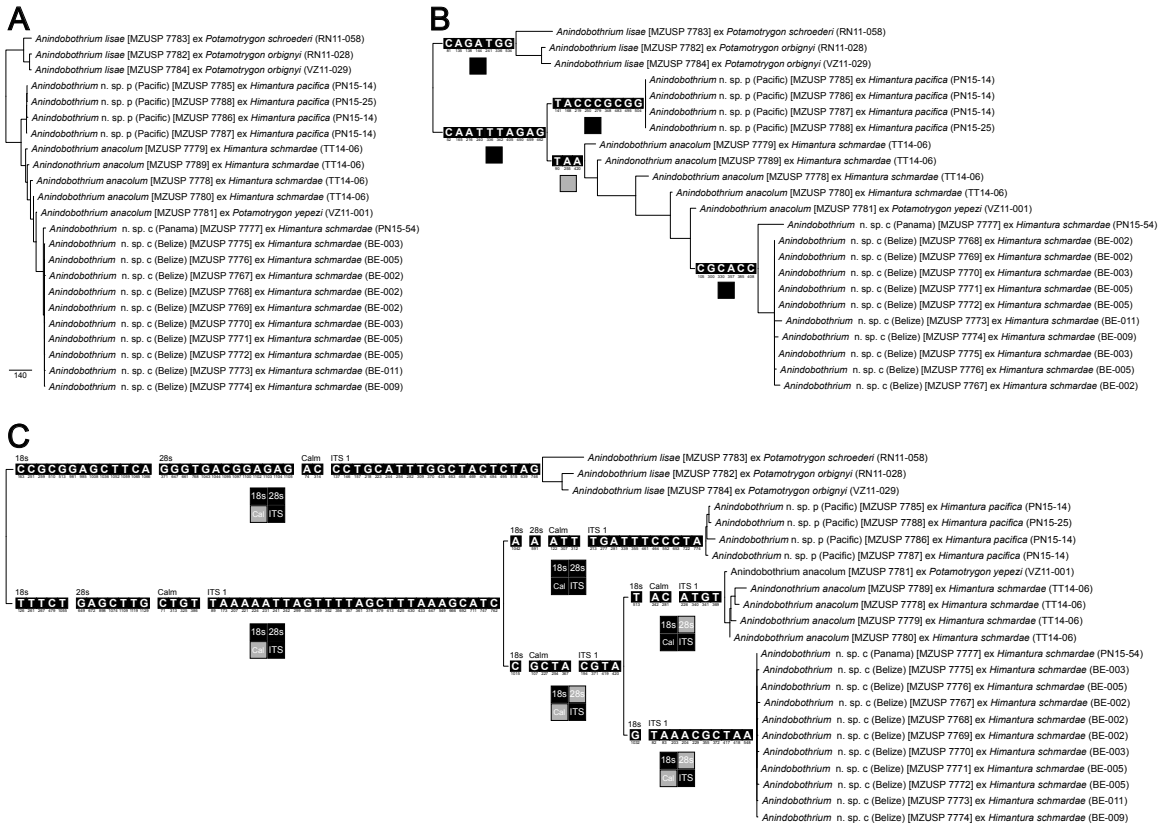

FIGURE 2. Phylogenetic relationships among haplotypes of *Anindobothrium* based on parsimony analysis of nucleotide sequences. A. Phylogeny based on the simultaneous analysis of 18S, 28S, COI, and ITS-1 for members of *Anindobothrium* by direct optimization of nucleotide data (tree length = 1,245). **B**. Phylogeny based on the parsimony analysis of COI (tree length = 371). **C**. Phylogeny based on the simultaneous analysis of nuclear regions (18S, 28S and ITS-1) by direct optimization of nucleotide data (tree length = 863). Base pairs on branches represent unambiguous unique synapomorphies for respective clades. Squares under branches represent presence (black) or absence (gray) of selected clades in the ML analysis.

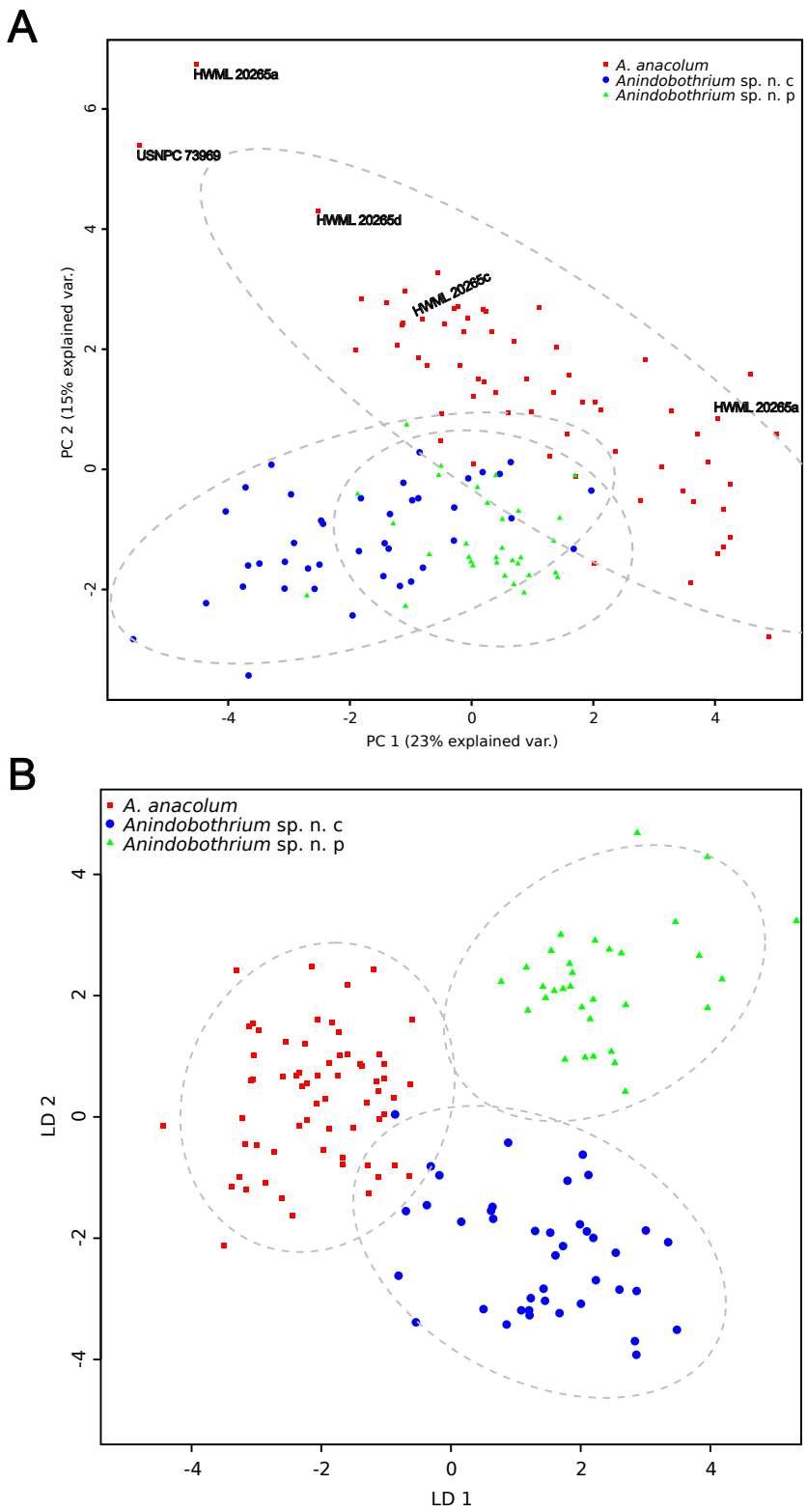

FIGURE 3. Multivariate statistical analysis of morphological data from marine lineages of *Anindobothrium*. A. Principal Component Analysis (PCA). **B**. Linear Discriminant Analysis (LDA).

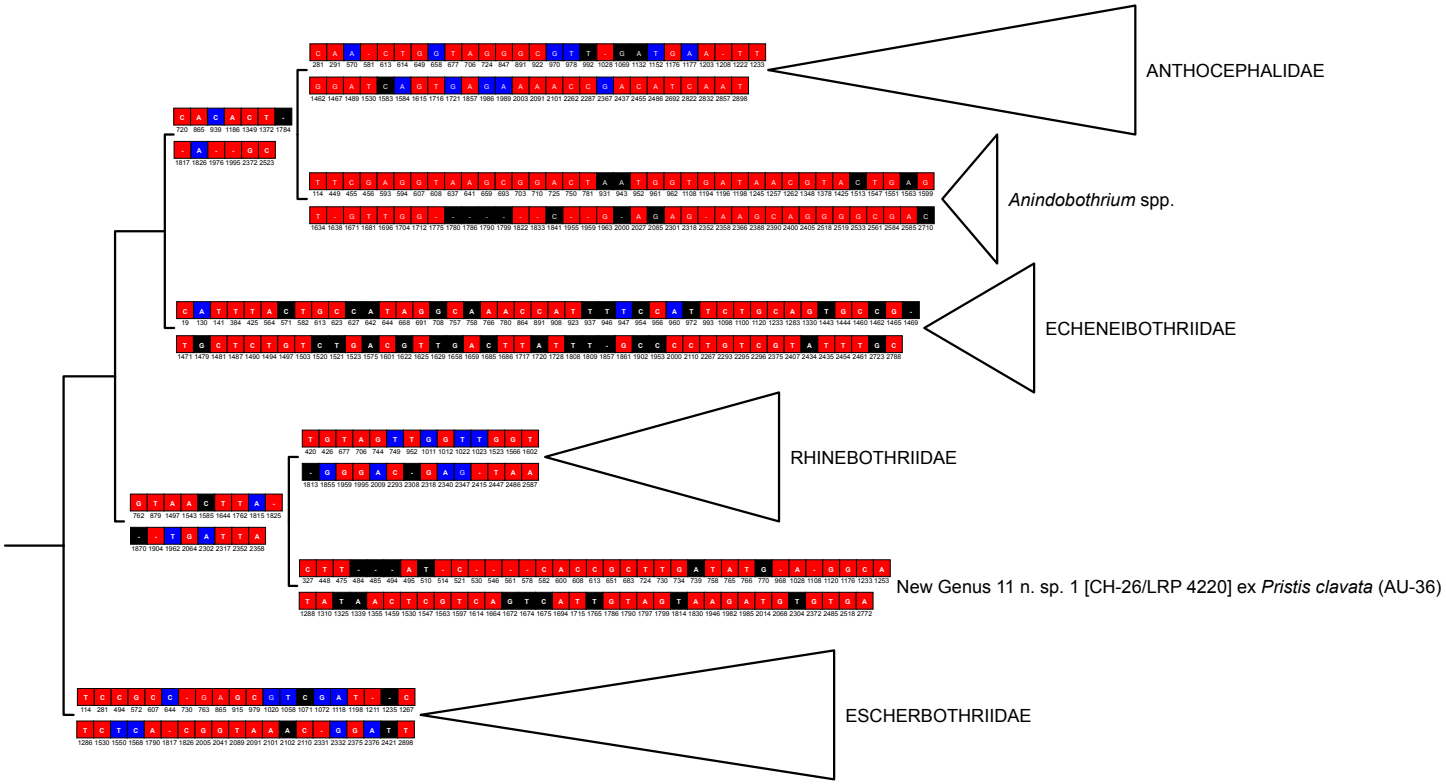

FIGURE 4. Summary of sister-group relationships based on the simultaneous analysis of 18S and 28S rDNA regions using parsimony as the optimality criteria for rhinebothriideans. Division between sets of synapomorphies indicates transformations from 18S and 28S rDNA, respectively. For each base pair, black cell represent unambiguous unique synapomorphies, red cells represent unique homoplastic synapomorphies, and blue cells represent non-unique homoplastic synapomorphies (see 45). Numbers below each cell represent alignment position for respective gene regions.

FIGURE 5. Whole worm. Light micrograph of *Anindobothrium anacolum* (Brooks, 1977) Marques, Brooks & Lasso, 2001 collected from the type locality.

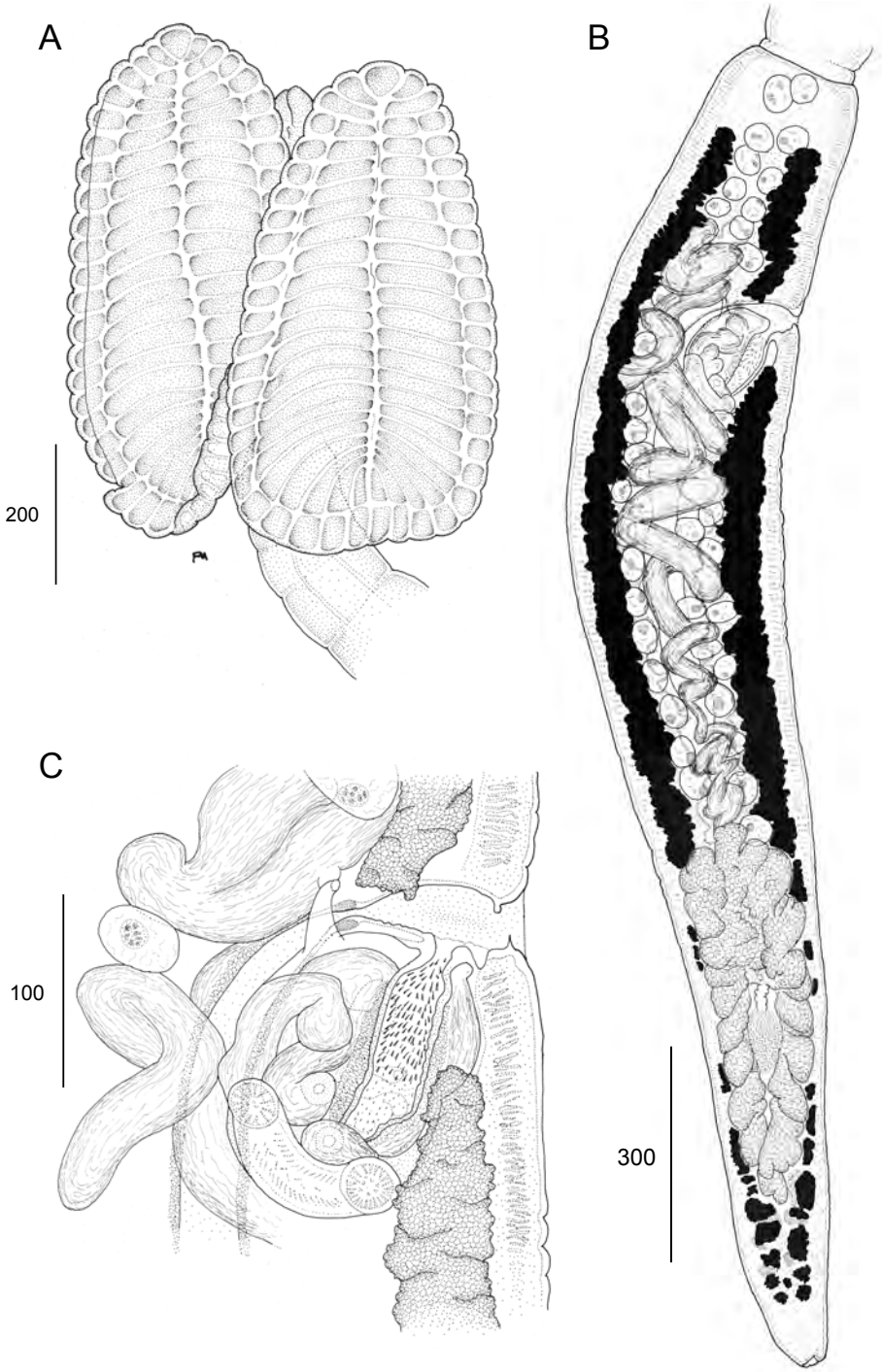

FIGURE 6. Line drawings of *Anindobothrium anacolum*. A. Scolex. **B.** Terminal proglottid. **C.** Cirrus sac.

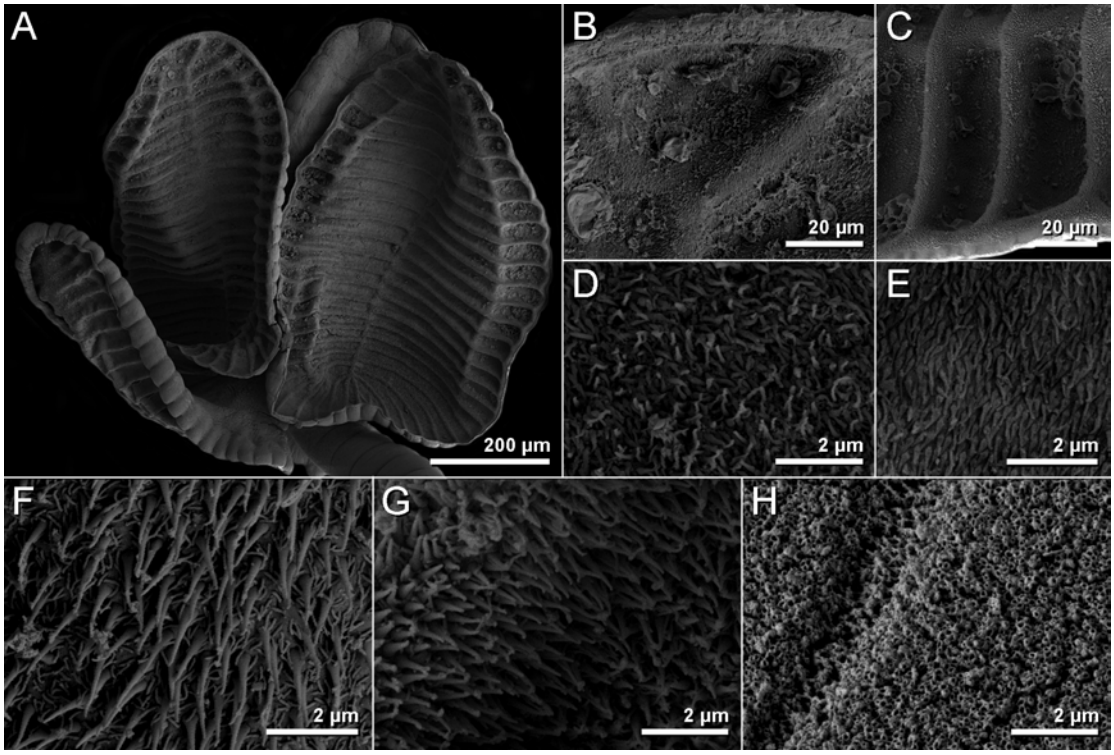

FIGURE 7. Scanning electron micrographs of *Anindobothrium anacolum*. A. scolex. **B.** distal surface of anterior loculus. **C.** distal surface of medial loculi. **D.** proximal surface of anterior loculus. **E.** proximal bothridial surface near centre of bothridium. **F.** distal anterior surface of longitunal septum. **G.** distal posterior surface of longtidinal septum. **H.** cephalic peduncle.

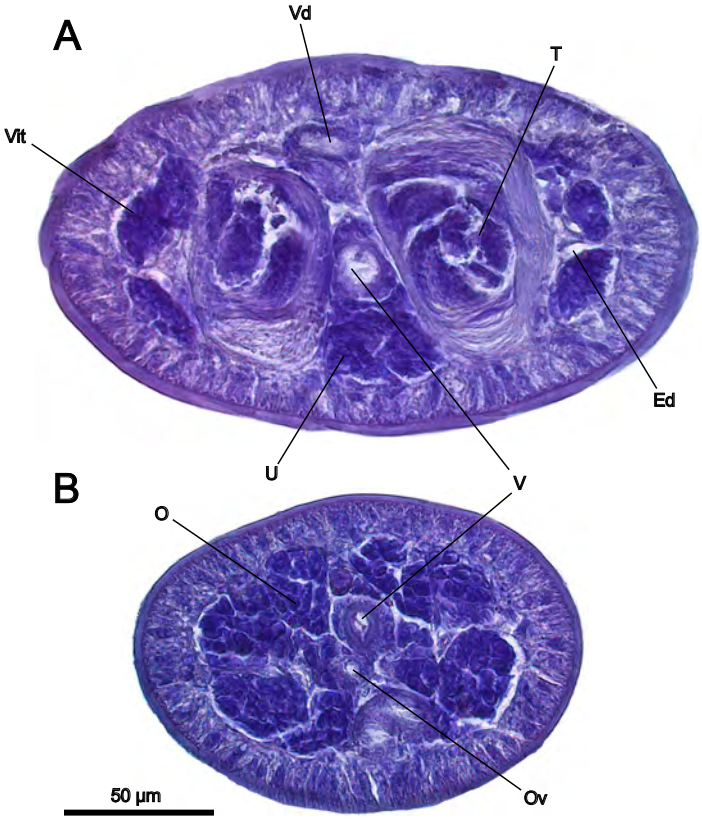

FIGURE 8. Micrographs of transversal histological sections of *Anindobothrium anacolum*. A. section at level of testes; **B**. section at level of ovary. *Abbreviations*: **Ed.** excretory duct; **O**. ovary; **Ov.** Oviduct; **T**. testes; **U**. uterus; **V.** vagina; **Vit.** vitelline follicles.

FIGURE 9. Whole worm. Light micrograph of *Anindobothrium lisae* (Brooks, 1977) Marques, Brooks & Lasso, 2001 collected from the type locality.

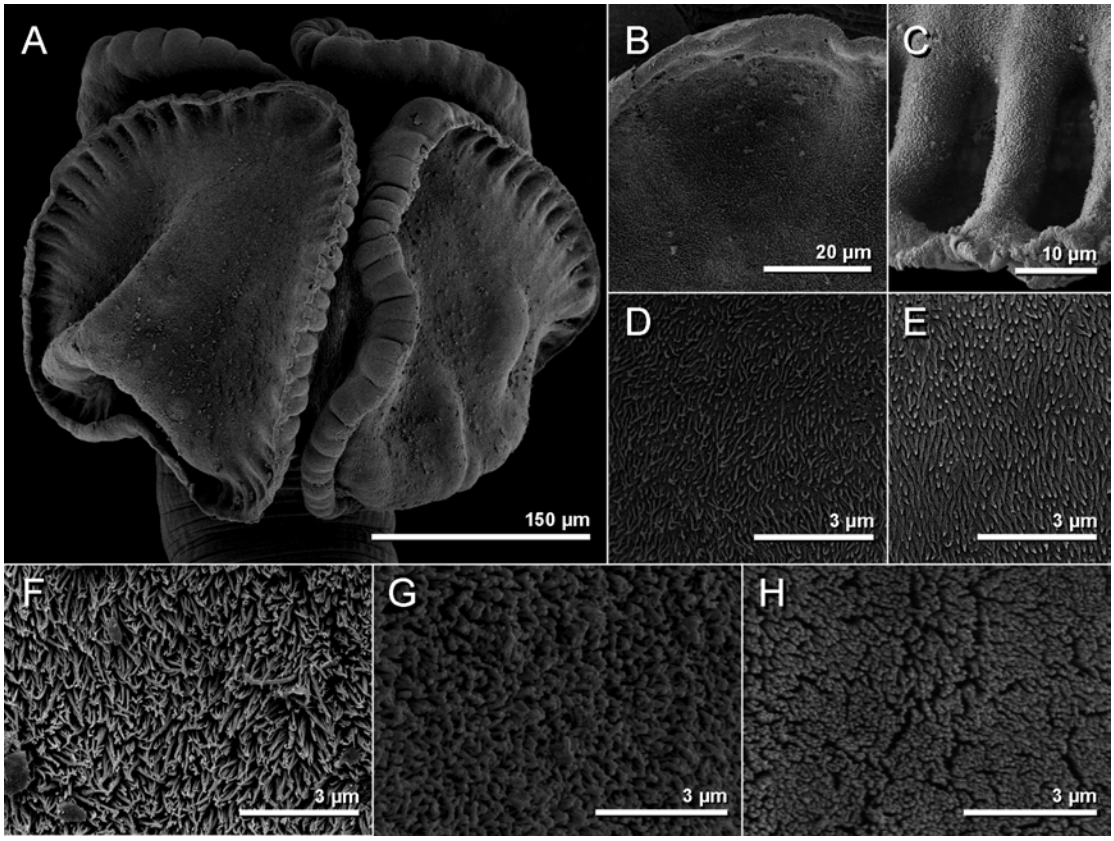

FIGURE 10. Scanning electron micrographs of *Anindobothrium lisae*. A. scolex. **B.** distal surface of anterior loculus. **C.** distal surface of medial loculi. **D.** proximal surface of anterior loculus. **E.** proximal bothridial surface near centre of bothridium. **F.** distal anterior bothridia surface. **G.** distal bothridia posterior surface. **H.** cephalic peduncle.

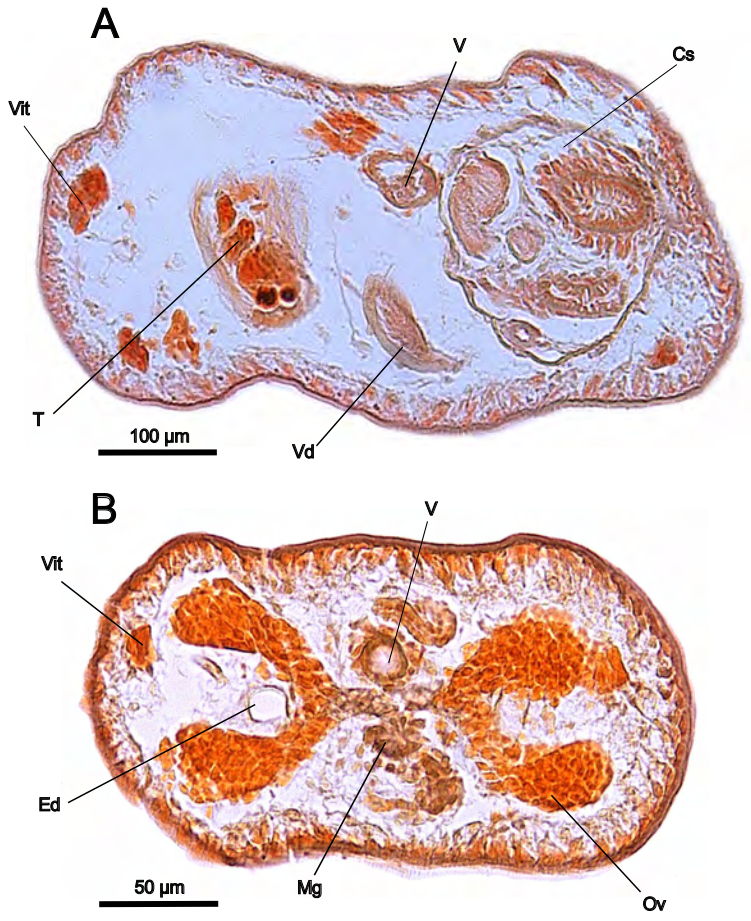

FIGURE 11. Micrographs of transversal histological sections of *Anindobothrium lisae*. A. section at level of testes; **B**. section at level of ovary. *Abbreviations*: **Cs.** cirrus sac; **Ed.** excretory duct; **Mg.** Mehlis' gland; **O**. ovary; **Ov.** oviduct; **T**. testes; **V.** vagina; **Vd.** deferens vas; **Vit.** vitelline follicles.

FIGURE 12. Whole worm. Light micrograph of *Anindobothrium inexpectatum* n. sp. collected from the type locality.

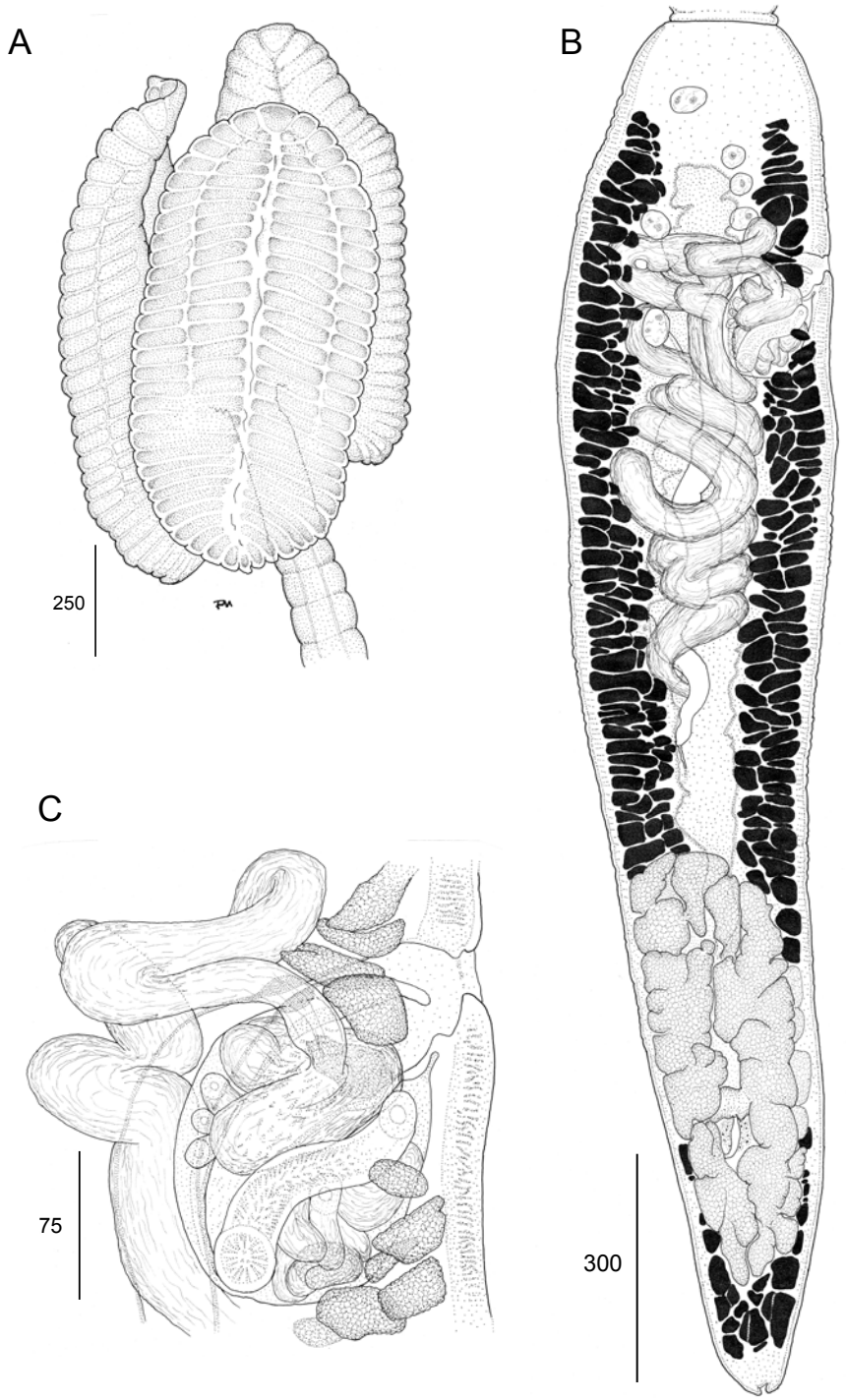

FIGURE 13. Line drawings of *Anindobothrium inexpectatum* n. sp. A. scolex. **B.** terminal proglottid. **C.** cirrus sac.

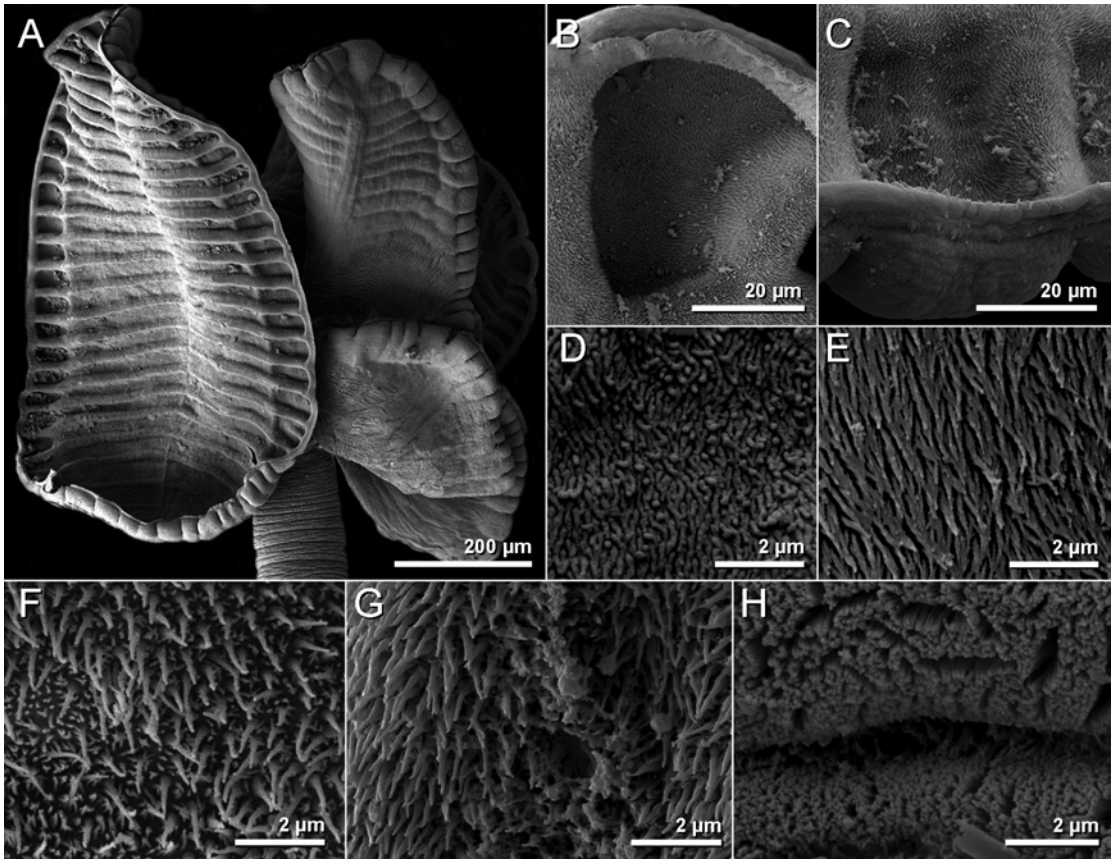

FIGURE 14. Scanning electron micrographs of *Anindobothrium inexpectatum* n. sp. A. scolex. **B.** distal surface of anterior loculus. **C.** distal surface of medial loculi. **D.** proximal surface of anterior loculus. **E.** proximal bothridial surface near centre of bothridium. **F.** distal anterior surface of longitunal septum. **G.** distal posterior surface of longtidinal septum. **H.** cephalic peduncle.

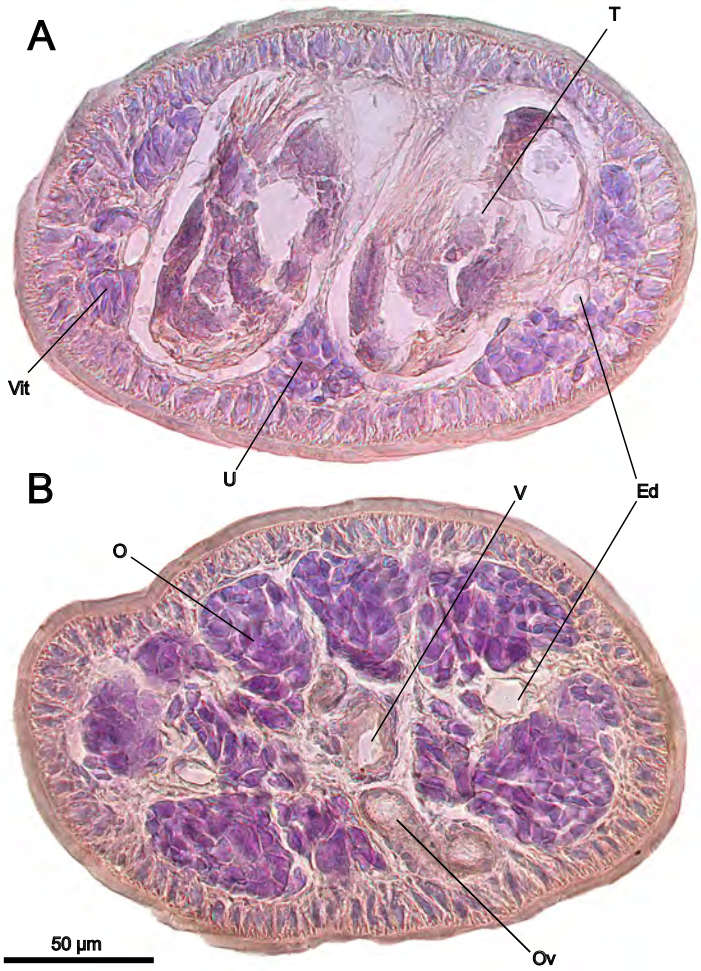

FIGURE 15. Micrographs of transversal histological sections of *Anindobothrium inexpectatum* n. sp. A. section at level of testes; **B**. section at level of ovary. *Abbreviations*: **Ed.** excretory duct; **O**. ovary; **Ov.** oviduct; **T**. testes; **U**. uterus; **V.** vagina; **Vit.** vitelline follicles.

FIGURE 16. Whole worm. Light micrograph of *Anindobothrium carioni* n. sp. collected from the type locality.

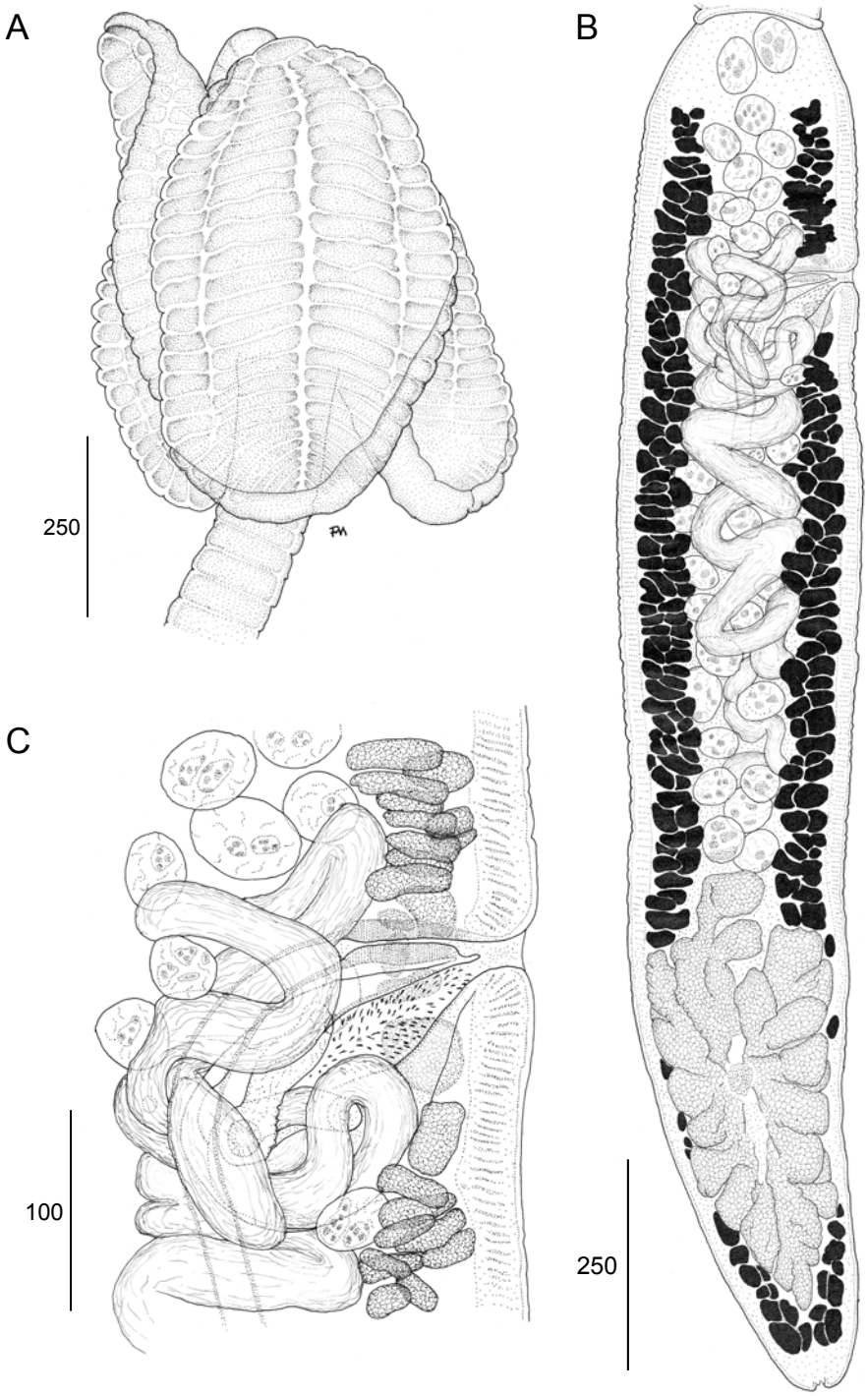

FIGURE 17. Line drawings of *Anindobothrium carioni* n. sp. A. scolex. **B.** terminal proglottid. **C.** cirrus sac.

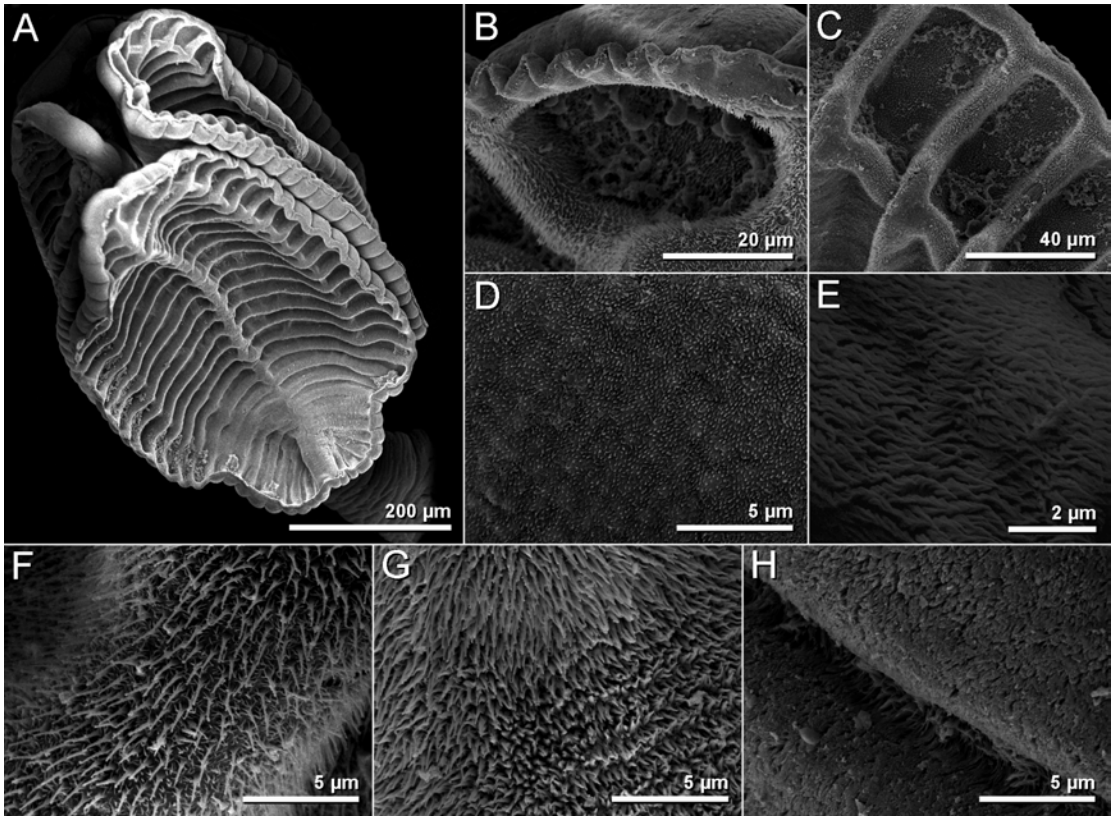

FIGURE 18. Scanning electron micrographs of *Anindobothrium carioni* n. sp. A. scolex. **B.** distal surface of anterior loculus. **C.** distal surface of medial loculi. **D.** proximal surface of anterior loculus. **E.** proximal bothridial surface near centre of bothridium. **F.** distal anterior surface of longitunal septum. **G.** distal posterior surface of longtidinal septum. **H.** cephalic peduncle.

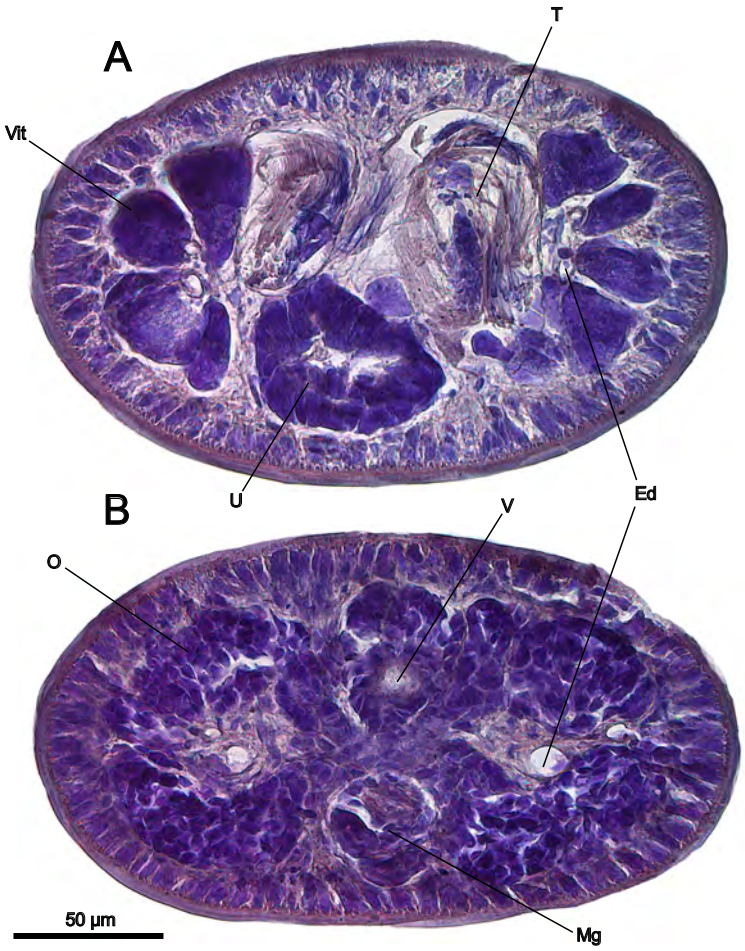

FIGURE 19. Micrographs of transversal histological sections of *Anindobothrium carioni* n. sp. A. section at level of testes; **B**. section at level of ovary. *Abbreviations*: **Ed.** excretory duct; **Mg.** Mehlis' gland; **O**. ovary; **T**. testes; **U**. uterus; **V.** vagina; **Vit.** vitelline follicles.

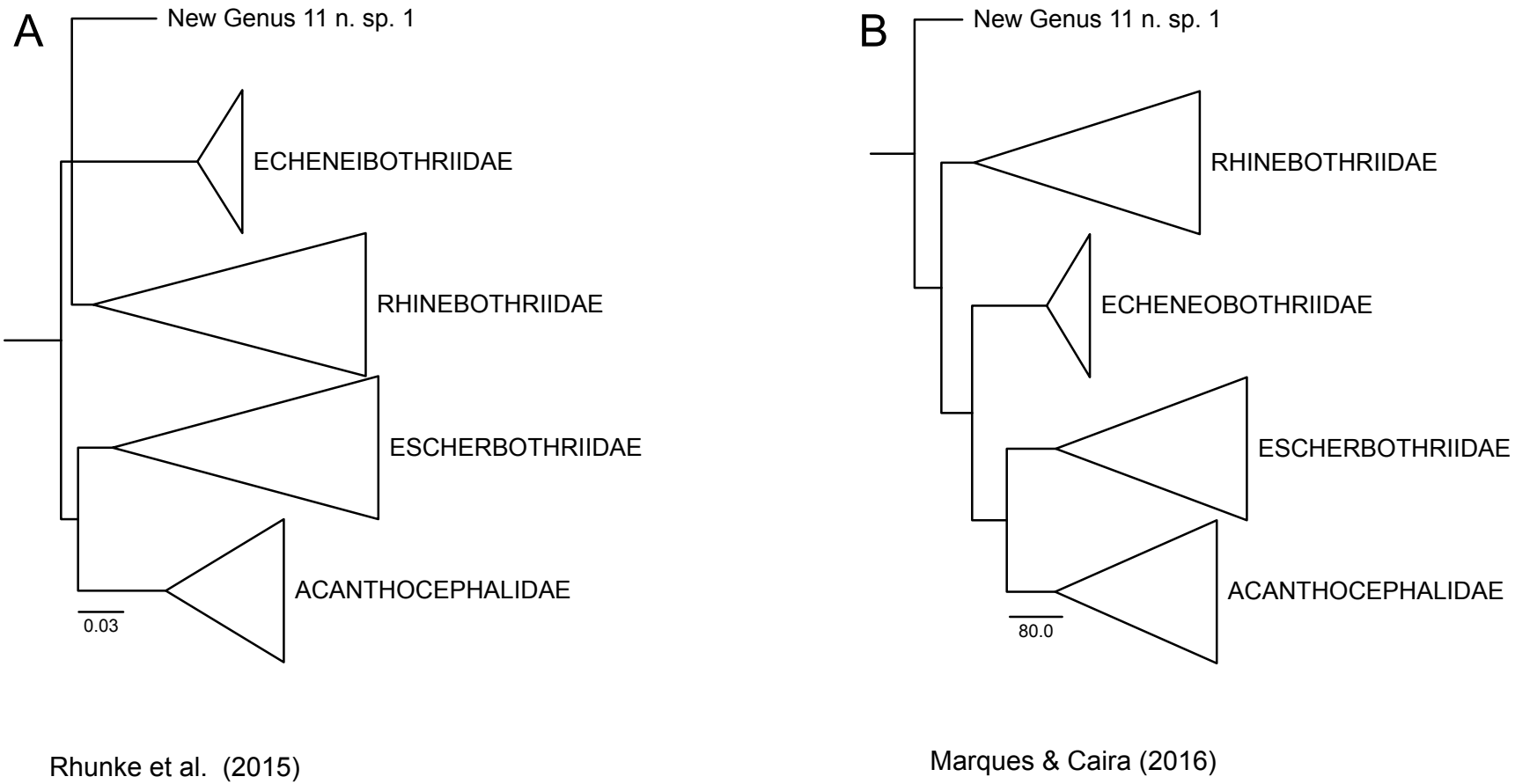

FIGURE 20. Previous phylogenetic hypotheses for members of the Rhinebothriidea. **A**. Modified from Ruhnke *et al.* (2015). **B**. Modified from Marques and Caira (2016).

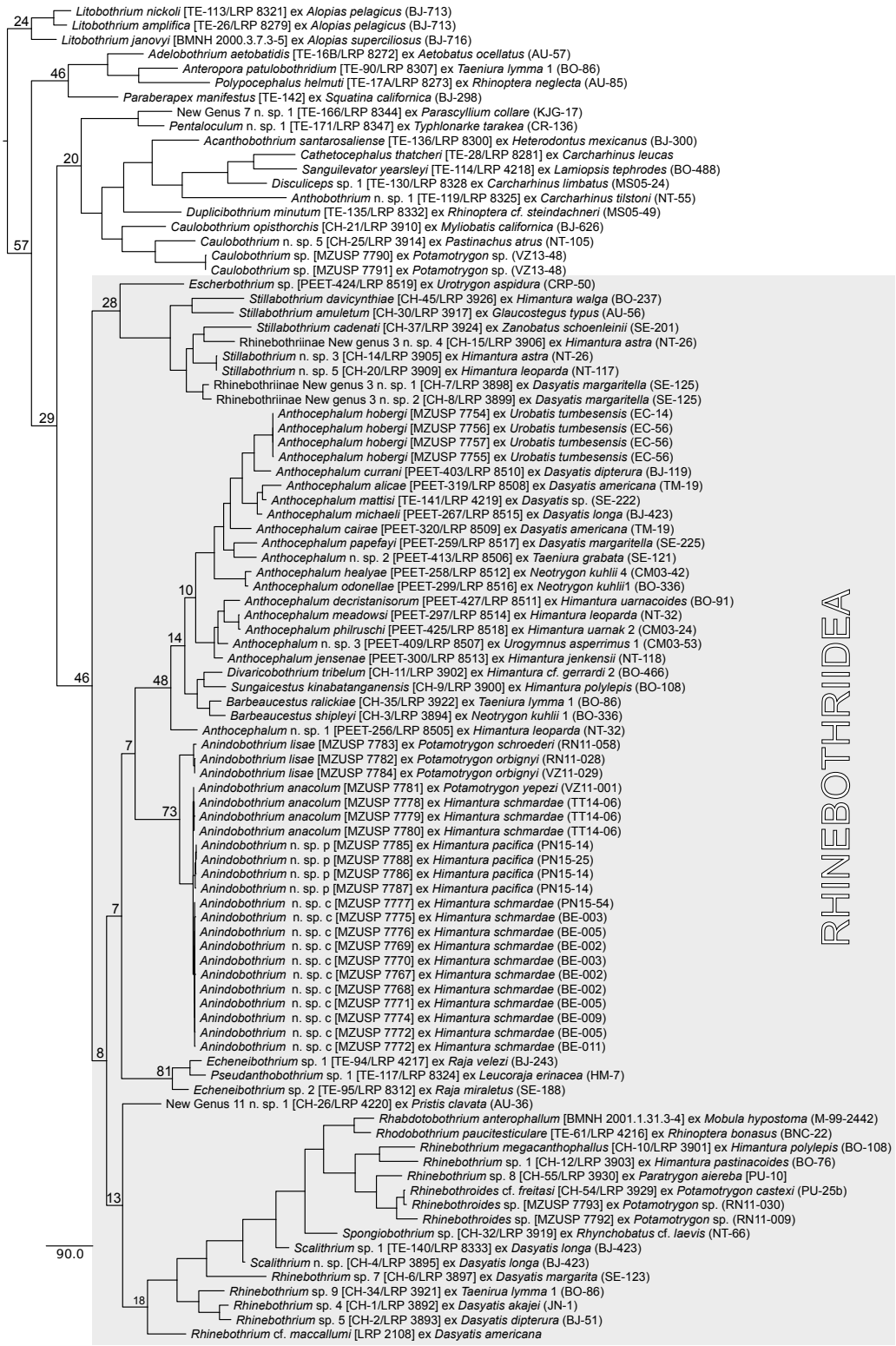

FIGURE S1. Sister-group relationships based on the simultaneous analysis of 18S and 28S rDNA regions using parsimony as the optimality criteria. Contents between brackets represent molecular codes and/or accession number for vouchers, contents between parentheses refer to host accession code in http://tapewormdb.uconn.edu/index.php/hosts/specimen_search/elasmobranch. Tree length = 26346.

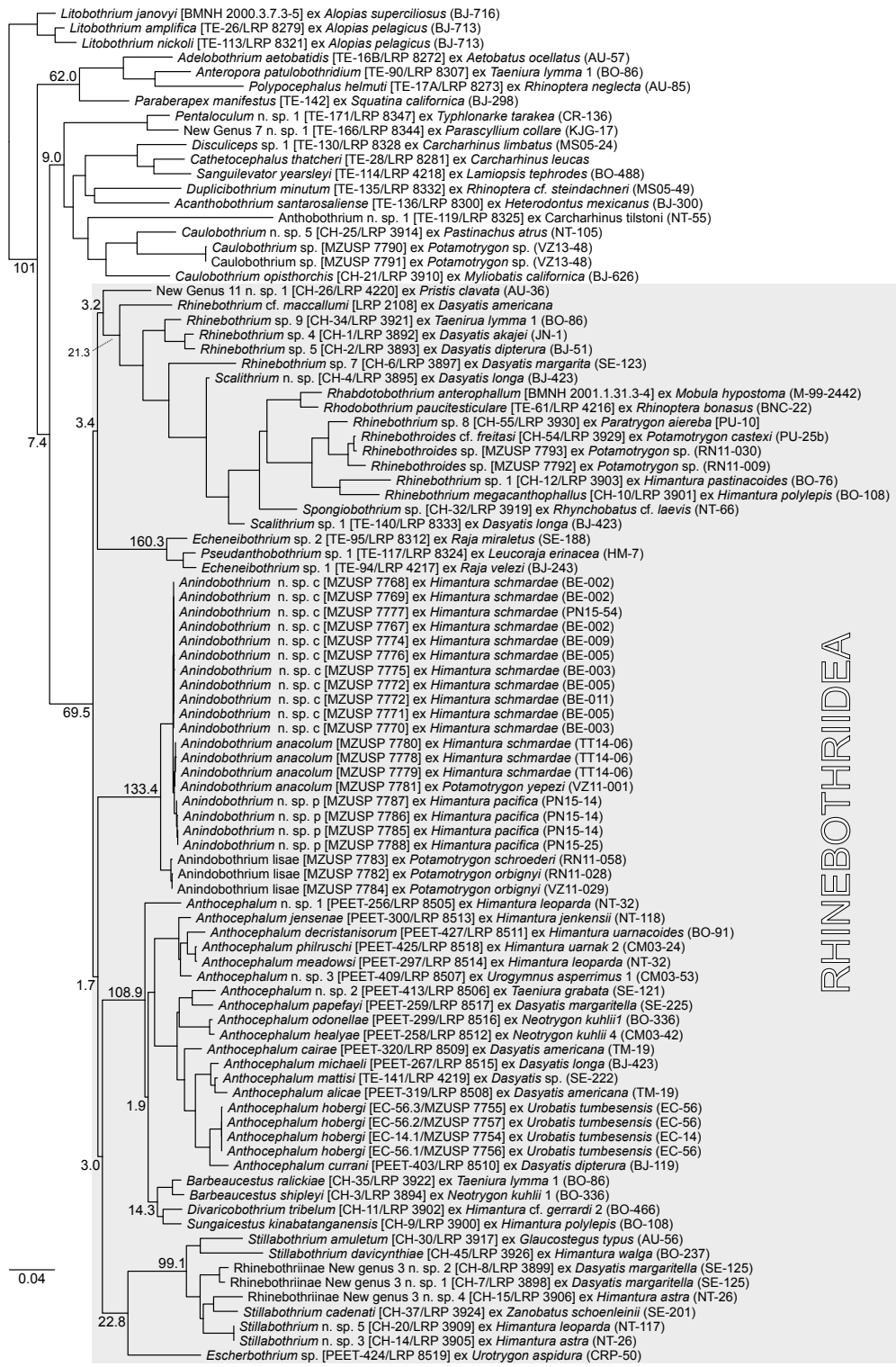

FIGURE S2. Sister-group relationships based on the simultaneous analysis of 18S and 28S rDNA regions using Maximum Likelihood as the optimality criteria. Numbers above branches represent Likelihood Length Difference support values. Contents between brackets represent molecular codes and/or accession number for vouchers, contents between parentheses refer to host accession code in http://tapewormdb.uconn.edu/index.php/hosts/specimen_search/elasmobranch. -lnL = 28523.9385.

Capítulo 3

Species diversity of *Acanthobothrium* Blanchard, 1848 (Eucestoda: Onchoproteocephalidea) from amphi-American species of *Himantura* (Myliobatiformes: Dasyatidae)

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Abstract

Historical associations within host-parasite systems can potentially reveal co-evolutionary events. In this case, it is possible that shared lineages of parasites of marine and freshwater batoids could provide useful information about the phylogenetic relationships and biogeographical history of their hosts. Within this framework, the present study surveyed species of *Acanthobothrium* Blanchard, 1848 from amphi-American species of *Himantura* Müller & Henle, the alleged sister-group of batoids of the family Potamotrygonidae - a unique group of stingrays restricted to the Neotropical freshwater systems of South America. Toward our goal to reveal the historical associations within this system, our results also increased the diversity of the genus to a total of 194 species, revealing eight new species of *Acanthobothrium*. Five novel taxa were found parasitizing *Himantura schmardae* (Werner) from the western Atlantic Ocean while three were infecting *H. pacifica* (Beebe & Tee-Van) from the eastern Pacific Ocean. Moreover, a redescription of *Acanthobothrium himanturi* Brooks, 1977 is provided, including two additional locality records. For the fist time, it was possible to provide the phylogenetic position of the marine lineages of *Acanthobothrium*, which revealed that some marine taxa are sister-clade of lineages infecting potamotrygonid hosts. This advanced phylogenetic framework, furthermore, demonstrated that events of co-divergence shaped the historical associations among amphi-American species of *Himantura*, potamotrygonids as well as their respective lineages of *Acanthobothrium*. Overall, our results support the most recent hypothesis about the origin and diversification of the Potamotrygonidae, from a marine ancestor that originated in waters which now includes tropical eastern Pacific Ocean and the Caribbean Sea.

Key-words: phylogenetic analysis, historical association, co-evolution, eastern Pacific Ocean, western Atlantic Ocean, Neotropical freshwater system, Potamotrygonidae.

Introduction

The widespread distribution and richness of the genus *Acanthobothrium* Blanchard, 1848 makes it one of the most intriguing genus within the Onchoproteocephalidea and could therefore represent a good indicator for co-evolutionary studies (Marques *et al.*, 1997; Fyler, 2009). Currently, there are 186 species of *Acanthobothrium* reported worldwide infecting several families of elasmobranch hosts (*e.g.*, Dasyatidae, Urolophidae, Narcinidae, Rajidae, Potamotrygonidae, Triakidae, among others [Caira *et al.*, 2012]). Within the Dasyatidae, amphi-American species of *Himantura*, *H. pacifica* (Beebe & Tee-Van) and *H. schmardae* (Werner) are distributed throughout the tropical eastern Pacific and western Atlantic coasts of the Americas, respectively. There is robust evidence suggesting that amphi-American species of *Himantura* represent a different lineage of dasyatids with respect of other members of the genus distributed throughout the Indo-West Pacific Ocean, hence the genus is paraphyletic (Aschliman, 2011; Naylor *et al.*, 2012). Also, morphological and molecular data support the hypothesis that Neotropical species of *Himantura* form a clade sister to freshwater stingrays of the family Potamotrygonidae and are presently distributed throughout the putative area of derivation of these freshwater stingrays (Lovejoy, 1996; Lovejoy *et al.*, 1998; Marques, 2000; Aschliman, 2011; Naylor *et al.*, 2012).

In studies of historical associations within host-parasite systems, in which parasites are associated with their hosts through a long period of time, it is possible to predict that sister-groups of hosts are likely to house sister-groups of parasites. These shared lineages could provide information about the phylogenetic relationships and biogeographical history of their hosts (Page, 1994; Caira and Jensen, 2001; Paterson and Banks, 2001). Within this framework, studying the parasite fauna of the alleged sister-group of potamotrygonids could clarify the processes that are responsible for their diversification and geographical distribution (Brooks *et al.*, 1981b; Klassen, 1992; Brooks and McLennan, 1993; Page and Charleston, 1998).

Considerable efforts have been made to reveal the species diversity of *Acanthobothrium* from freshwater stingrays in South America (Rego and Dias, 1976; Mayes *et al*., 1978; Brooks *et al*., 1981a; Ivanov, 2005; Reyda, 2008, Cardoso Jr., 2010; Machado, 2012) resulting in the recognition of six species and two potential new species (Cardoso Jr., 2010). The diversity of *Acanthobothrium* from amphi-American species of *Himantura* currently includes two species, both reported from *H. schmardae* off the coast of Colombia, namely: *A. himanturi* Brooks, 1977 and *A. tasajerasi* Brooks, 1977. The impressive species diversity of *Acanthobothrium* worldwide and overwhelming evidence that the closure of the Panamanian isthmus generated sets of sister species (Marques *et al.*, 1995; Craig *et al.*, 2004) suggests that an extensive survey of the cestode parasites of amphi-American species of *Himantura* potentially would reveal new lineages of *Acanthobothrium*.

The diversity of *Acanthobothrium* and the relationships among its lineages are important pieces in the biogeographical puzzle. The use of parasitological data to understand the evolution of this system, however, requires a refined documentation of

parasites and hosts as well as hypotheses for their phylogenetic relationships. In fact, parasites provided the initial set of evidence that led Brooks *et al.* (1981b) to propose a biogeographical hypothesis for the origin and diversification of this unique group of restricted freshwater stingrays (*i.e.*, the Potamotrygonidae). According to these authors (1981b), potamotrygonids derived from a Pacific dwelling urolophid (today considered urotrygonids of the genus *Urobatis*; Naylor *et al.*, 2012) long time before the closure of the Panamanian isthmus. This hypothesis has been challenged ever since, either by the inadequacies associated with their data and analyses (Straney, 1982; Lovejoy, 1997; Caira, 1990; Caira, 1994), or by our recent knowledge about the phylogeny of batoids (Aschliman, 2011; Naylor *et al.*, 2012).

Here we present our results of a recent survey of parasites of amphi-American species of *Himantura*. Our results reveal many new lineages of *Acanthobothrium* associated with the putative sister-group of potamotrygonids. We also identify the marine lineages of this genus that act as sister-taxa to those found in potamotrygonids and demonstrate that events of co-divergency shaped the historical associations among amphi-American species of *Himantura*, potamotrygonids, and some lineages of *Acanthobothrium*. Moreover, we discuss the implications of our findings to Brooks *et al.*'s (1981b) hypothesis for the diversification of potamotrygonids.

Materials and methods

A total of 33 spiral intestines of amphi-American species of *Himantura* were examined in the present study. Of those, five specimens of *H. schmardae* were collected off the coast of Belize in May 2012 (Head Caye, Punta Gorda, Toledo, 16°13'20.8"N, 88°35'38.3"W; north of Southwater Caye and Tobacco Caye, Dangriga, Stann Creek, 16°49'43.1"N, 88°04'48.1"W and 16°54'15.2"N, 88°03'38.2"W) and 11 from the Caribbean coast of Panama in January 2015 (Almirante, Bocas del Toro, 9°17'39.1N, 82°20'42.0"W and 9°17'20.3"N, 82°21'18.9"W), and 11 specimens of *H. pacifica* from the eastern Pacific cost of Panama in January 2015 (Playa Caleta, Montijo, Veráguas, 07°29'37.9"N, 81°13'21.9"W). All collecting activities followed the guidelines of two collecting permits issued to Dr. Janine Caira by the Ministry of Forest, fisheries and sustainable development (Belize Fisheries Department – Proc. No 000016–12, issued in 2012) and F.P.L.M. by the Autoridad Nacional del Ambiente (ANAM – Proc. no SE/A101-14, issued in 2014) (respectively). After collection, stingrays were euthanized and their spiral intestines removed, opened with a mid-ventral incision, washed with distilled water and fixed in a 4 % seawater-buffered formalin solution (for morphological studies) or pure ethanol (stored in a freezer for molecular analysis). After a few days both wash and spiral valve fixed with formalin were transferred to 70 % ethanol for long-term storage and subsequent examination in the laboratory.

Morphological data

Specimens of *Acanthobothrium* selected for light microscopy were prepared as whole mounts, hydrated in a regressive alcoholic series, stained with Delafield`s hematoxylin (9:1 solution), destained in a 1 % acid (HCl) ethanol solution, followed by 1 % basic (NaOH) ethanol solution, dehydrated in a graded ethanol series, cleared in methyl salicylate and mounted in Canada balsam on microscope slides. Morphometric data and photographic documentation were obtained using an Olympus SC30 camera and analysis 5.0 software (Olympus Soft Images Solutions) attached to an Olympus BX51 microscope. Hook measurements were taken following Euzet (1959). For both the lateral and medial hooks the measurements taken consist of hook handle length (AC, A'C') as the distance from the anterior extremity of the hook base to the anteriormost point of the curve connecting the axial and abaxial prongs; inner or axial prong length (CD, C'D') as the distance from the anteriormost point of the curve connecting the axial and abaxial prongs to the posteriormost extremity of the axial prong; outer or abaxial prong (CB, C'B') as the distance from the anteriormost point of the curve connecting the axial and abaxial prongs to the posterior extremity of the abaxial prong; and total hook length (AD, A'D') as the distance from the anterior extremity of the hook handle to the posterior extremity of the axial prong.

Fiji/ImageJ (Schindelin *et al.*, 2012) and WormBox (Vellutini and Marques, 2014) were used to process the images and compute the data (respectively). Only complete specimens with mature proglottids (*i.e.*, genital pore open) or further developed proglottids (*e.g.*, with atrophied testes or vas deferens filled) were considered in this study. All measurements were taken from terminal proglottids, unless in cases where terminal proglottids presented atrophied testes. In these cases, testes data are presented from subterminal mature proglottids in which testes remained sufficiently intact to be measured. All measurements are in micrometers unless otherwise stated, and are presented as ranges and number of specimens from which the variable was taken in parentheses. Repeated measurements for the number and dimensions of testes and for the dimensions of vitelline follicles were averaged for individuals.

Morphological comparisons of valid species of *Acanthobothrium*

Specimens selected for histological sectioning were embedded in paraffin and sectioned at 7 µm intervals using a LEICA RM 2025 retracting rotary microtome. Sections were mounted on glass slides and dried first on a slide warmer for five minutes and later in an oven for 30 min. Cross sections of mature proglottids were stained with Mayer's hematoxylin and counterstained with eosin, dehydrated in a graded ethanol series, cleared in xylene, and mounted in Entellan (Merck). The scolex of each worm sectioned was prepared as a whole mount and kept as a voucher (as described above).

Scoleces of two or more specimens of each species were prepared and examined with SEM. Each scolex was cut from the strobila, which was prepared as a whole mount, as described above, and kept as a voucher. The scoleces were cleaned with brushes to remove the host tissue, hydrated in a graded ethanol series, transferred to 1 % osmium tetroxide overnight, dehydrated in a graded ethanol series, and placed in hexamethyldisilazane (HMDS). They were allowed to air-dry overnight and were subsequently mounted on carbon tape on an aluminum stub, sputter–coated with gold/palladium and examined with a FEI Quanta 600 FEG scanning electron microscope. Microthrix terminology follows Chervy (2009).

Newly recognized lineages of *Acanthobothrium* were morphologically compared to those located in the tropical eastern Pacific and western Atlantic coasts of the Americas. The decision of limiting the scope of comparisons was based on the great number of species assigned to the genus (186) and on the observation that species from similar geographic regions are most likely to be conspecific (see Goldstein, 1967; Williams, 1969; Ivanov and Campbell, 1998; Ghoshroy and Caira, 2001). This is a practice that has also been applied in previous studies (*e.g.*, Ivanov and Campbell, 1998; Ghoshroy and Caira, 2001) and we think the same could be undertaken here.

Museum abbreviations used are as follows: **USNPC**, United States National Parasite Collection, Beltsville, Maryland, U.S.A. (material now allocated in **USNM**, National Museum of Natural History, Smithsonian Institution, Washington, D.C, U.S.A.); **HWML**, Harold W. Manter Laboratory, University of Nebraska, Lincoln, Nebraska, U.S.A; **MZUSP**, Museu de Zoologia da Universidade de São Paulo, São Paulo, SP, Brazil; **MIUP**, Museu de Invertebrados G. B. Fairchild, Estafeta Universitaria, Universidad de Panamá, Panama City, Panama.

Molecular data acquisition

Specimens submitted to molecular phylogenetic analyses had their scolex and posterior portion of the strobila prepared as whole mounts as described above, and these hologenophores (sensu Pleijel *et al.*, 2008) were deposited in the MZUSP. The middle portion of the strobila of each specimen was removed and allowed to air dry for about 5 min at room temperature. Total genomic DNA was extracted using the Agencourt DNAdvance - Nucleic Acid Isolation Kit (Beckman Coulter) following the manufacturer's instructions. Genomic DNA was quantified using a micro-volume spectrophotometer, NanoDrop 2000 (Thermo Scientific). Polymerase Chain Reaction (PCR) was used to amplify partial sequences of the nuclear regions 28S rDNA (D1-D3) and the mitochondrial region of 16S rDNA. These regions were selected based on existing dataset (see Caira *et al.*, in prep), which allow us to verify the phylogenetic position of putative lineages found in amphi-American species of *Himantura* with respect to many other lineages of *Acanthobothrium* from different hosts and biogeographical regions (see below). Doublestranded amplifications were performed in a 25 µl volume containing 1 µl of DNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 200 lM dNTPs, 1.0–3.0 mM MgCl2, 0.4 lM of each primer, and 1 U of Taq DNA

polymerase recombinant (Fermentas, Thermo Scientific). General PCR conditions included initial denaturation for 5 min at 95° C, 35 cycles of denaturation for 30 sec at 95° C, annealing for 30 sec at specific temperatures (see below), extension for 1 min to 1 min and 10 sec at 72° C, and a final extension for 7 min at 72° C. The amplification and sequencing were performed with the following primer sets: 28S rDNA (D1-D3) with LSU-5F 5' – TAGGTCGACCCGCTGAAYTTAAGCA – 3' and LSU-1500R 5' – GCTATCCTGAGGGAAACTTCG – 3' at 58° C; with and Cyclo-16S-F 5' – TGCCTTTTGCATCATGCT – 3' and Cyclo-16SR 5' – AATAGATAAGAACCGACCTGG – 3' at 55° C. PCR products were purified using an Agencourt AMPuret XP DNA Purification and Cleanup kit (Beckman Coulter). Products were subsequently cycle-sequenced directly from both forward and reverse directions using ABI Big-Dye Sequence Terminator version 3.1, cleaned with ethanol precipitation, and sequenced on an ABI Prism Genetic Analyzer (3131XL) automated sequencer (Applied Biosystems/ThermoFisher).

Contiguous sequences were assembled using the package Consed/PhredPhrap (Ewing and Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998, 2001). Sequences were aligned using MAFFT (Katoh *et al.*, 2002) and visualized and edited in BioEdit (version 7.1.3.0; Hall, 1999) to remove leading and trailing regions that varied in length.

Phylogenetic analyses

Nucleotide sequences of 16S rDNA and 28S rDNA (D1-D3) were implemented in a phylogenetic analysis by direct optimization (**DO**; Wheeler, 1996) using POY (version 5.1.1; Varón *et al.*, 2010) under parsimony as the optimality criterion. Initial tree searches included 10 iterations of one independent search for 1 h using the command search (*i.e.*, search(max_time:0:01:00)) assuming equal weights for character transformations. This search was conducted in a 10 X 2.83 GHz Intel® Core™2 Quad Processor Q9550 computer cluster. After compiling candidate trees by DO, we submitted unique topologies to tree refinement by tree-fusing algorithm (Goloboff, 1999) and re-diagnosis by iterative pass alignment (**DO/IP+Fuse**; Wheeler, 2003a). Finally, the results of POY were verified by performing a phylogenetic analysis of the implied alignment (sensu Wheeler, 2003b) generated by the previous step in TNT (Goloboff *et al.*, 2008) using its New Technology searches (Goloboff, 1999; Nixon, 1999) with the following parameters: rep 1000 hold 100 fuse 20 ratchet 20. We evaluated nodal support of selected nodes by using the Goodman-Bremer values (GBS, Goodman *et al.*, 1982; Bremer, 1988, 1994; see Grant and Kluge, 2008a). To obtain this metric, we considered the shortest tree found by TNT based on the implied alignment above and executed a modified version of the script BREMER.RUN distributed with TNT. This script considered 1,000 replicates with 10 repetitions of ratchet and drift (Goloboff, 1999; Nixon, 1999) in constrained searches and the remaining default parameters. Trees with branch lengths were obtained in PAUP* (Version 4.0a147, Swofford, 2003) using the implied alignment from POY.

Results

Phylogenetic analyses

Genetic representation: In total, we obtained sequences of 16S and 28S for 24 specimens of *Acanthobothrium* from amphi-American species of *Himantura* (Table 1). With the exception of *Acanthobothrium* n. sp. 2, all putative species initially recognized on the basis of morphological data were represented. These 24 terminals were added to the dataset of Caira *et al.* (in prep.). The dataset considers 115 terminals of *Acanthobothrium* from all major bodies of water, including 18 representatives from potamotrygonids of Neotropical freshwater systems and 10 onchoproteocephalids used as out-group taxa following Caira *et al.* (2014). Hence, the entire dataset considered nucleotide sequences for 149 terminals for which unaligned sequences of 16S varied from 276 to 561 base pairs (**bp**) in length, while 28S sequences ranged from 712 to 1,239 bp. The aligned sequenced of 16S and 28S from MAFTT presented 600 and 1,375 positions, respectively.

Phylogenetic inference: POY found 48 unique trees ranging from 5,779 to 6,007 steps in length after 10 iterations of direct optimization. After submitting these trees to IP+Fuse, POY found five trees with 5,757 steps in length for which the implied alignment had 2,284 positions. The phylogenetic analysis of this implied alignment in TNT resulted in three topologies 5,754 steps long, which differ from each other in branches with near-zero length (see Figure 1).

Most of the lineages we recognized morphologically nested as monophyletic groups in our phylogenetic analysis (Fig. 1). For instance, *Acanthobothrium* n. sp. 6 from *Himantura pacifica* nested as sister of a clade of species of *Acanthobothrium* mainly found in Rajiformes, but also some dasyatids. *Acanthobothrium* n. sp. 8, also recovered from *H. pacifica*, is sister to a clade comprised by member of the genus found in Atlantic species of *Dasyatis* and *H. schmardae*. Within this sister clade, *Acanthobothrium* n. sp. 4 resulted as monophyletic and was recognized as *A. humanturi* Brooks, 1977, whereas *Acanthobothrium* n. sp. 5 seems to be paraphyletic with respect to an undescribed species found in *Dasyatis guttata* (Bloch & Schneider) off the coast of Belize. This clade also includes another undescribed species of *Acanthobothrium* not recognized morphologically in the samples from Belize. The same paraphyletic pattern was recovered for *Acanthobothrium* n. sp. 7 from *Himantura pacifica*. Members of this putative species nested with two other putative species from *Urotrygon aspidura* (Jordan & Gilbert) and *Zapteryx xyster* Jordan & Evermann from the eastern Pacific coast of Costa Rica. *Acanthobothrium* n. sp. 1 resulted as sister of a clade comprised by three terminals from the eastern Pacific parasitizing *Dasyatis longa* (Garman), *Gymnura crebripunctata* (Peters) and *G. marmorata* (Cooper). *Acanthobothrium* n. sp. 9 from *H. schmardae* nested as the sister of an undescribed species of the genus parasite of the Neotropical freshwater

stingrays *Potamotrygon schroederi* Fernández-Yépez. This pattern of host association also replicated for *Acanthobothroides thorsoni* Brooks, 1977, which nested as sister of the remaining species of the genus endemic to potamotrygonids (see Discussion). Based on these results, the taxonomic actions we propose for the species we recognize are as follows.

***Acanthobothrium* n. sp. 1**
(Figs. 2, 3)

Type-host: *Himantura schmardae* (Werner) (Myliobatiformes: Dasyatidae).
Type-locality: Caribbean Sea, Tobacco Caye, Dangriga, Stann Creek, Belize (16°54'N, 88°03'W).
Additional locality: Caribbean Sea, Head Caye, Punta Gorda, Toledo, Belize (16°13'N, 88°35'W); Caribbean Sea, North of Southwater Caye, Dangriga, Stann Creek, Belize (16°49'N, 88°04'W); Caribbean Sea, Almirante, Bocas Del Toro, Panama (09°17'N, 82°20'W).
Site of infection: Spiral intestine.
Specimens deposited: #######.
Etymology: to be defined.

Description. (Based on 36 specimens: 33 whole mounts, two worms observed with SEM, and one used for cross sections). Worms acraspedote (Fig. 2A, Table 2), apolytic, 1.6–4.4 mm (n = 33) long, composed of 6–16 (n = 33) proglottids. Scolex composed by a proper and cephalic peduncle (Fig. 2B, 3A). Scolex proper with four bothridia, 440–586 (n = 31) long, by 255–334 (n = 28) wide (maximum width of scolex at level of anterior loculus), each with three loculi: anterior loculus 210–328 (n = 16) long, middle loculus 60–103 long (n = 16), and posterior loculus 60–101 (n = 16) long; loculus length ratio (A:M:P) 1:0.3–0.4:0.2–0.4 (n = 33). Anterior region of scolex in form of muscular pad 79–127 (n = 24) long by 115–170 (n = 25) wide, bearing an apical sucker 13–27 (n = 26) long by 20–45 (n = 27) wide, and one pair of hooks below posterior margin and triangular in shape (Fig. 2B, 3B). Velum absent. Hooks bipronged, hollow, with inconspicuous tubercle on proximal surface of axial prong; internal channels of axial and abaxial prongs continuous and smooth; axial and abaxial prongs equal in length; lateral and medial hooks approximately equal in size (Fig. 2C). Lateral hook measurements: AC 48–85 (n = 31), CD 100–148 (n = 31), CB 111–155 (n = 31), AD 147–197 (n = 31). Medial hook measurements: AC’ 42–63 (n = 31), CD’ 106–155 (n = 30), CB’ 107–149 (n = 29) and AD’ 151–199 (n = 31). Medial and lateral hook base with approximately same width. Thin layer of tissue covering each prong of both hooks. Lateral bothridia of anterior loculus covered with gladiate spinitriches and papilliform filitriches (Fig 3C). Distal surface of anterior loculus covered with papilliform filitriches (Fig. 3D). Cephalic peduncle covered with gladiate spinitriches (Fig. 3E).

Immature proglottids wider than long, 4–14 (n = 33) in number. Mature proglottids longer than wide, 592–1,390 (n = 30) long by 180–344 (n = 30) wide, 1–4 (n = 33) in number; mature proglottid length-width ratio 2.4–4.7 (n = 26) (Fig. 2D, E). Absence of gravid proglottids. Testes oval, 17–50 (n = 30) long by 12–33 (n = 30) wide, arranged in two irregular columns, extending from ovarian isthmus to anterior margin of proglottid, 21–42 (n = 30) in total number, 5–11 (n = 31) pre-poral, 4–11 (n = 31) post-poral and 10–24 (n = 31) anti-poral. Genital pores irregularly alternating,

30–43 % (n = 31) from anterior end of proglottid. Cirrus sac pyriform, with posterior region tilted anteriorly, 67–207 (n = 31) long by 66–150 (n = 31) wide, containing spined eversible cirrus. Vagina thick-walled, sinuous, extending from ootype along medial line of proglottid to anterior margin of cirrus sac to common genital atrium. Vaginal sphincter absent. Ovary located near posterior end of proglottid, essentially symmetrical, reaching or almost reaching cirrus sac, inverted A-shaped in frontal view, and tetra-lobed in cross section, lobulated, 215–500 (n = 31) long by 69–120 (n = 29) wide at isthmus. Ovarian isthmus located at approximate midpoint of ovary. Mehlis' gland posterior to ovarian isthmus. Vitellarium follicular, consisting of two lateral bands each consisting of small follicles, 8–27 (n = 30) long by 7–22 (n = 30) wide; bands extending from near anterior margin of proglottid to posterior margin of ovary, interrupted by vagina and cirrus sac. Uterus thick-walled, sacciform, extending from isthmus to near anterior margin of proglottid.

Remarks. *Acanthobothrium* n. sp. 1 has a unique combination of features that distinguishes it from the other 20 species of *Acanthobothrium* from the western Atlantic Ocean. Among these species *Acanthobothrium* n. sp. 1 closely resembles 11 congeners in total length, number of proglottids and number of testes (Table 3). Within these species, two of them (*A. fogeli* Goldstein, 1964 and *A. brevissime* Linton 1908) can be distinguished from *Acanthobothrium* n. sp. 1 by possessing axial and abaxial prongs from medial hooks unequal in length, instead of equal, respectively. *Acanthobothrium* n. sp. 1 can be distinguished from *A. westi* Vardo–Zalik & Campbell, 2011 and *A. ulmeri* Vardo-Zalik & Campbell, 2011 by the presence of testes between the ovarian lobes, which are not present in the latter two species. Furthermore, *A. westi* possesses a shorter ovary (*i.e.*, 152–176 vs. 215–500 long, respectively) and *A. ulmeri* has shorter lateral hooks in comparison to *Acanthobothrium* n. sp. 1 (*i.e.*, AD 80–103 vs. 147–197, respectively). Two additional species can be distinguished from *Acanthobothrium* n. sp. 1 by the position of the genital pore, namely *A. schalli* Vardo-Zalik & Campbell, 2011 (*i.e.*, 49–66 % vs. 30–43 % from anterior end, respectively) and *A. lintoni* (posterior vs. anterior, respectively). *Acanthobothrium tasajerasi* Brooks, 1977 differs from the new species by possessing a prominent genital atrium instead of an inconspicuous one and by having a smaller scolex length (*i.e.*, 309–384 vs. 440–586, respectively). *Acanthobothrium zapterycum* Núñez, 1971 can be distinguished from *Acanthobothrium* n. sp. 1 by the total length of hooks (*i.e.*, AD 84–123 vs. 147–197, respectively). In addition, *A. zapterycum* also possesses a narrower terminal proglottid (*i.e.*, 70–100 vs. 180–334, respectively). *Acanthobothrium himanturi* Brooks, 1977 differs from the new species by possessing angulated hooks with prominent tubercles on the proximal part of the axial prong instead of a slightly angulated hook shape with an inconspicuous tubercle (respectively). *Acanthobothrium lentiginosum* Vardo-Zalik & Campbell, 2011 can be distinguished from the new species by possessing smaller mature proglottids length (*i.e.*, 350–570 vs. 592–1,390, respectively) and smaller lateral hooks (*i.e.*, AC 35–40 vs. 48–85 and AD 90–135 vs. 147–197, respectively). *Acanthobothrium lineatum* Campbell, 1969 is the species that most closely resembles *Acanthobothrium* n. sp. 1, but it differs by possessing axial and abaxial prongs of medial hooks that could be equal or unequal in length (vs. always equal in length, respectively); thinner hooks (vs. robust, respectively) and a narrower ovary (*i.e.*, 20–50 vs. 69–120, respectively).

***Acanthobothrium* n. sp. 2**
(Figs. 4, 5)

Type-host: *Himantura schmardae* (Werner) (Myliobatiformes: Dasyatidae).
Type-locality: Caribbean Sea, Tobacco Caye, Dangriga, Stann Creek, Belize (16°54'N, 88°03'W).
Additional locality: Caribbean Sea, Head Caye, Punta Gorda, Toledo, Belize (16°13'N, 88°35'W); Caribbean Sea, North of Southwater Caye, Dangriga, Stann Creek, Belize (16°49'N, 88°04'W).
Site of infection: Spiral intestine.
Specimens deposited: #######.
Etymology: to be defined.

Description. (Based on 31 specimens: 28 whole mounts, two worms observed with SEM, and one used for cross sections). Worms acraspedote (Fig. 4A, Table 2), apolytic, 2.8–7.5 mm (n = 28) long, composed of 9–23 (n = 28) proglottids. Scolex composed by a proper and cephalic peduncle (Fig. 4B, 5A). Scolex proper with four bothridia, 294–400 (n = 25) long, by 222–325 (n = 25) wide (maximum width of scolex at level of anterior loculus), each with three loculi: anterior loculus 125–195 long (n = 24), middle loculus 29–65 long (n = 25), and posterior loculus 35–70 long (n = 25); loculus length ratio (A:M:P) 1:0.2–0.4:0.2–0.4 (n = 25). Muscular striations on anterior loculus present. Anterior margin of scolex with a region in form of muscular pad 40–100 (n = 21) long by 82–165 (n = 21) wide, bearing an apical sucker 12–35 (n = 16) long by 12–40 (n = 16) wide, and one pair of hooks below posterior margin and triangular in shape (Fig. 5B). Velum absent. Hooks bipronged, hollow, with prominent tubercle on proximal surface of axial prong; internal channels of axial and abaxial prongs continuous, smooth; axial prongs longer than abaxial prongs in medial hooks; lateral and medial hooks differing in angulation because of the tubercle (Fig. 4C). Lateral hook measurements: AC 32–49 (n = 23), CD 72–97 (n = 23), CB 57–82 (n = 23), AD 101–129 (n = 23). Medial hook measurements: AC’ 28–48 (n = 24), CD’ 88–110 (n = 24), CB’ 63–94 (n = 24) and AD’ 117–150 (n = 24). Medial and lateral hook base with approximately the same width. Thin layer of tissue covering anteriorly each prong of both hooks. Lateral bothridia of anterior loculus covered with gladiate spinitriches and capilliform filitriches (Fig. 4D). Distal surface of anterior loculus covered with papilliform filitriches (Fig. 4C). Cephalic peduncle surface covered with gladiate spinitriches interspersed with acicular filitriches (Fig. 4E).

Immature proglottids wider than long, 8–20 (n = 28) in number. Mature proglottids longer than wide, 691–1,162 (n = 25) long by 133–270 (n = 27) wide, 1–4 (n = 28) in number; mature proglottid length to width ratio 2.7–5.6 (n = 24) (Fig. 4D, E). Absence of gravid proglottids. Testes round to oval, 20–57 (n = 28) long by 17–40 (n = 28) wide, arranged in two regular columns, extending from ovarian isthmus to anterior margin of proglottid, 23–33 (n = 27) in total number, 3–9 (n = 28) pre-poral, 4–10 (n = 28) post-poral and 11–18 (n = 27) anti-poral. Genital pores irregularly alternating, 22–35 % (n = 21) from anterior end of proglottid. Cirrus sac pyriform, 45–160 (n = 28) long by 80–187 (n = 24) wide, containing spined eversible cirrus. Vagina thick-walled, sinuous, extending from ootype along medial line of proglottid to anterior margin of cirrus sac to common genital atrium. Vaginal sphincter absent. Ovary located near posterior end of proglottid, essentially symmetrical or slightly

asymmetrical in posteriormost proglottids, reaching or almost reaching the cirrus sac, inverted A-shaped in frontal view, and tetra-lobed in cross section, lobulated, 180–680 (n = 27) long by 27–115 (n = 27) wide at isthmus. Ovarian isthmus located at approximate midpoint of ovary. Mehlis' gland posterior to ovarian isthmus. Vitellarium follicular, consisting of two lateral bands each consisting of small follicles, 6–25 (n = 28) long by 4–25 (n = 28) wide; bands extending from near anterior margin of proglottid to posterior margin of ovary, interrupted by vagina and cirrus sac. Uterus thick-walled, sacciform, extending from isthmus to near anterior margin of proglottid.

Remarks. *Acanthobothrium* n. sp. 2 closely resembles 8 out of 21 species reported from the western Atlantic Ocean based on the total length, number of proglottids and number of testes (Table 3). From these congeners, a total of four species, namely *A. lintoni*, *A. schalli*, *A. ulmeri* and *Acanthobothrium* n. sp. 1, can be distinguished from *Acanthobothrium* n. sp. 2 by possessing axial and abaxial prongs from medial hooks that are equal in length instead of unequal as it in new species. *Acanthobothrium* n. sp. 2 can be distinguished from *A. tasajerasi* on the basis of genital pore position (*i.e.*, 25–35 % vs. 37–43 % from anterior end of proglottid, respectively). *Acanthobothrium paulum* Linton, 1890 differs from *Acanthobothrium* n. sp. 2 by a different position of the genital pore (*i.e.*, in the mid-posterior part of the segment vs. anterior, respectively). *Acathobothrium lineatum* can be distinguished from *Acanthobothrium* n. sp. 2 by possessing larger lateral hooks length (*i.e.*, CB 92–186 vs. 57–82, respectively) and by having an inconspicuous tubercle on the proximal part of the axial prong of the medial hooks and not a prominent tubercle as in the new species. *Acanthobothrium brevissime* most closely resembles *Acanthobothrium* n. sp. 2 in total length, number of proglottids and number of testes (Table 3), but differs from the latter by possessing different genital pore position which is located at the middle of proglottid or a slightly anterior vs. anterior (25–35% from anterior end, respectively). *Acanthobothrium* n. sp. 2 further differs from *A. brevissime* by the presence of axial hooks with a prominent tubercle on the proximal part instead of an inconspicuous tubercle (respectively) and by lappets that do not protrude out of the apical sucker (vs. lappets extending out, respectively).

***Acanthobothrium* n. sp. 3**
(Figs. 6, 7)

Type-host: *Himantura schmardae* (Werner) (Myliobatiformes: Dasyatidae).
Type-locality: Caribbean Sea, Tobacco Caye, Dangriga, Stann Creek, Belize (16°54'N, 88°03'W).
Additional locality: Caribbean Sea, Head Caye, Punta Gorda, Toledo, Belize (16°13'N, 88°35'W); Caribbean Sea, North of Southwater Caye, Dangriga, Stann Creek, Belize (16°49'N, 88°04'W); Caribbean Sea, Almirante, Bocas Del Toro, Panama (09°17'N, 82°20'W).
Site of infection: Spiral intestine.
Specimens deposited: #######.
Etymology: to be defined

Description. (Based on 43 specimens: 40 whole mounts, two worms observed with SEM, and one used for cross sections). Worms acraspedote (Fig. 6A, Table 2), apolytic, 5.7–11.7 mm (n = 40) long, composed of 37–64 (n = 39) proglottids. Scolex composed by a proper and cephalic peduncle (Fig. 6B, 7A). Scolex proper with four

bothridia, 391–540 (n = 39) long by 198–502 (n = 37) wide (maximum width of scolex at level of anterior loculus), each with three loculi: anterior loculus 157–234 long (n = 39), middle loculus 50–90 long (n = 37), and posterior loculus 50–111 long (n = 38); loculus length ratio (A:M:P) 1:0.3–0.5:0.2–0.6 (n = 37). Anterior region of scolex in form of muscular pad 97–165 (n = 31) long by 125–205 (n = 31) wide, bearing an apical sucker 26–55 (n = 34) long by 30–60 (n = 34) wide, and one pair of hooks below posterior margin and triangular in shape (Fig. 7B). Velum present, extending from anterior to posterior loculus. Hooks bipronged, hollow, with prominent tubercle on proximal surface of axial prong; internal channels of axial and abaxial prongs continuous, smooth; axial prongs longer than abaxial prongs; lateral and medial hooks approximately equal in size (Fig. 6C). Lateral hook measurements: AC 52–72 (n = 32), CD 66–115 (n = 29), CB 51–91 (n = 26), AD 115–173 (n = 29). Medial hook measurements: AC' 51–79 (n = 34), CD' 75–116 (n = 32), CB' 46–87 (n = 25) and AD' 129–179 (n = 32). Medial and lateral hook base with approximately same width. Thin layer of tissue covering entire prong of both hooks. Lateral bothridia of anterior loculus covered with acicular filitriches and gladiate spinitriches (Fig. 7D). Distal surface of anterior loculus covered with papilliform filitriches (Fig. 7C). Cephalic peduncle covered with gladiate spinithriches (Fig 7E).

Immature proglottids wider than long, 30–60 (n = 39) in number. Mature proglottids longer than wide, 592–1,355 (n = 35) long by 167–388 (n = 37) wide, 1–9 (n = 39) in number; mature proglottid length to width ratio 2.2–5.1 (n = 33) (Fig. 6D, E). Absence of gravid proglottids. Testes round to oval, 27–51 (n = 37) long by 19–41 (n = 37) wide, arranged in two irregular columns, extending from ovarian isthmus to anterior margin of proglottid, 28–74 (n = 33) in total number, 10–26 (n = 32) pre-poral, 3–8 (n = 35) post-poral and 20–39 (n = 32) anti-poral. Genital pores irregularly alternating, 38–56 % (n = 38) from anterior end of proglottid. Cirrus sac pyriform, with posterior region tilted slightly anteriorly, 44–137 (n = 35) long by 73–198 (n = 34) wide, containing spined eversible cirrus. Vagina thick-walled, sinuous, extending from ootype along medial line of proglottid to anterior margin of cirrus sac to common genital atrium. Vaginal sphincter absent. Ovary located near posterior end of proglottid, essentially symmetrical, inverted A-shaped in frontal view, and tetra-lobed in cross section, reaching or almost reaching cirrus sac, lobulated, 244–490 (n = 34) long by 98–190 (n = 31) wide at isthmus. Ovarian isthmus located at approximate midpoint of ovary. Mehlis' gland posterior to ovarian isthmus. Vitellarium follicular, consisting of two lateral bands each consisting of small follicles, 11–40 (n = 33) long by 5–23 (n = 33) wide; bands extending from near anterior margin of proglottid to posterior margin of ovary, interrupted by vagina and cirrus sac. Uterus thick-walled, sacciform, extending from isthmus to near anterior margin of proglottid.

Remarks. *Acanthobothrium* n. sp. 3 closely resembles three species of *Acanthobothrium* from the western Atlantic Ocean by its total length and the number of proglottids and testes (Table 3). *Acanthobothrium. floridensis* Goldstein, 1964 differs from the new species by a longer scolex loculi (*i.e.*, anterior: 251–459 vs. 157–234 long, middle: 153–255 vs. 50–90 long, and posterior: 153–240 vs. 50–111 long, respectively), a wider apical sucker (*i.e.*, 72–102 vs. 30–60, respectively), larger diameter of testes (*i.e.*, 100 vs. 21–63, respectively) and by possessing an ovary that reaches half the distance to the cirrus sac than reaching the entire distance towards the cirrus sac (respectively). Goldstein (1964) considered *A. paulum* as the synonym of *A. woodsholei* Baer 1948, but Campbell (1969) considered *A. paulum* as valid species and *A. woodsholei* should be the synonym of *A. paulum*. Given the dubious taxonomic status of these nominal species, we will compare *Acanthobothrium* n. sp. 3

with both. *Acanthobothrium paulum* can be distinguished from *Acanthobothrium* n. sp. 3 by possessing axial and abaxial prongs equal in length instead of unequal and by a different size of the ovary (*i.e.*, 676 long by 70 wide vs. 244–490 long by 98–190 wide, respectively). *Acanthobothrium woodsholei* most closely resembles *Acanthobothrium* n. sp. 3 but differs from it by the presence of a larger apical sucker (*i.e.*, 104 vs. 30–60, respectively) and a longer cirrus sac (*i.e.*, 252 vs. 44–137, respectively).

***Acanthobothrium himanturi* Brooks, 1977**
(Figs. 8, 9)

Type-host: *Himantura schmardae* (Werner) (Myliobatiformes: Dasyatidae).
Type-locality: Caribbean Sea, 15 km west of La Cienaga, Magdalena, Colombia (11°01'N, 74°15'W).
Additional locality: Caribbean Sea, Head Caye, Punta Gorda, Toledo, Belize (16°13'N, 88°35'W); Caribbean Sea, North of Southwater Caye, Dangriga, Stann Creek, Belize (16°49'N, 88°04'W); and Caribbean Sea, Tobacco Caye, Dangriga, Stann Creek, Belize (16°54'N, 88°03'W); Caribbean Sea, Almirante, Bocas Del Toro, Panama (09°17'N, 82°20'W).
Site of infection: Spiral intestine.
Type-material: Holotype (USNPC 73963) and 4 paratypes (HWML 20260).
Additional specimens deposited: #######.

Redescription. [Based on the type series comprised of holotype (USNPC 73963) and four paratypes (HWML 20260), and 52 additional mature specimens which included: 49 whole mounts, two worms observed with SEM, and one used for cross sections]. Worms acraspedote (Fig. 8A, Table 2), apolytic, 3.4–12.4 mm (n = 49) long, composed of 14–32 (n = 47) proglottids. Scolex composed by a proper and cephalic peduncle (Fig. 8B, 9A). Scolex proper with four bothridia, 341–630 (n = 43) long by 226–480 (n = 42) wide (maximum width of scolex at level of anterior loculus), each with three loculi: anterior loculus 148–295 long (n = 44), middle loculus 52–120 long (n = 43), and posterior loculus 61–149 long (n = 44); loculus length ratio (A:M:P) 1:0.2–0.6:0.3–0.6 (n = 42). Anterior region of scolex in form of muscular pad 47–145 (n = 35) long by 110–220 (n = 37) wide, bearing an apical sucker 16–45 (n = 37) long by 19–60 (n = 38) wide, and one pair of hooks below posterior margin and triangular in shape (Fig. 9B). Velum present, extending from anterior to posterior loculus. Hooks bipronged, hollow, with prominent tubercle on proximal surface of axial prong; internal channels of axial and abaxial prongs continuous, smooth; axial prongs approximately same length than abaxial prongs; lateral and medial hooks approximately equal in size (Fig. 8C). Lateral hook measurements: AC 38–89 (n = 38), CD 70–129 (n = 35), CB 63–115 (n = 37), AD 97–181 (n = 37). Medial hook measurements: AC’ 32–78 (n = 38), CD’ 80–126 (n = 37), CB’ 52–106 (n = 38) and AD’ 111–188 (n = 37). Medial and lateral hook base with approximately same width. Thin layer of tissue covering completely each prong of both hooks. Lateral bothridia of anterior loculus covered with capilliform and papilliform filitriches and gladiate spinitriches (Fig. 9D). Distal surface of anterior loculus covered with papilliform filitriches (Fig. 9C). Cephalic peduncle covered with gladiate spinithriches (Fig. 9E).

Immature proglottids wider than long, 12–28 (n = 47) in number. Mature proglottids longer than wide, 805–1,870 (n = 46) long by 172–375 (n = 47) wide, 2–8 (n = 49) in number; mature proglottid length to width ratio 2.9–6 (n = 44) (Fig. 8D,

E). Absence of gravid proglottids. Testes round to oval, 22–72 (n = 49) long by 22–50 (n = 48) wide, arranged in two irregular columns, extending from ovarian isthmus to anterior margin of proglottid, 37–67 (n = 43) in total number, 9–18 (n = 45) pre-poral, 5–14 (n = 46) post-poral and 20–37 (n = 44) anti-poral. Genital pores irregularly alternating, 32–47 % (n = 46) from anterior end of proglottid. Cirrus sac round, with posterior region tilted slightly posteriorly in most posterior proglottids, 72–190 (n = 47) long by 75–200 (n = 48) wide, containing spined eversible cirrus. Vagina thick-walled, sinuous, extending from ootype along medial line of proglottid to anterior margin of cirrus sac to common genital atrium. Vaginal sphincter absent. Ovary located near posterior end of proglottid, essentially symmetrical, inverted A-shaped in frontal view, and tetra-lobed in cross section, reaching or almost reaching the cirrus sac, lobulated, 371–960 (n = 46) long by 90–200 (n = 47) wide at isthmus. Ovarian isthmus located at approximate midpoint of ovary. Mehlis' gland posterior to ovarian isthmus. Vitellarium follicular, consisting of two lateral bands each consisting of small follicles, 7–34 (n = 46) long by 6–26 (n = 46) wide; bands extending from near anterior margin of proglottid to posterior margin of ovary, interrupted by vagina and cirrus sac. Uterus thick-walled, sacciform, extending from isthmus to near anterior margin of proglottid.

Remarks. *Acanthobothrium himanturi* was originally described by Brooks (1977) to accommodate a new species found in *H. schmardae* from Colombia. Until now, this species has only been reported from the type locality. For the first time it was possible to provide a new locality record for this species outside Colombia from off the coast of Belize. We also provide data on the morphology of the microtriches and nucleotide sequences for this species for the first time. The newly collected material allowed us to extend the range for most morphometric attributes [*e.g.*, total length: 3.4–12.4 mm in the present redescription (R) vs. 3.8–9.3 from the original description (O) (respectively); number of proglottids 14–32 (R) vs. 17–26 (O); position of the genital pore 32–47 % (R) vs. 40–48 % (O) from anterior end of proglottid, respectively, among others]. The photomicrographs provided herein are also more informative then the illustrations provided in original description, including the characterization of the whole worm.

***Acanthobothrium* n. sp. 5**
(Figs. 10, 11)

Type-host: *Himantura schmardae* (Werner) (Myliobatiformes: Dasyatidae).
Type-locality: Caribbean Sea, Almirante, Bocas Del Toro, Panama (09°17'N, 82°20'W).
Additional locality: Maracas bay, Maracas, San Juan-Laventille, Trinidad & Tobago (10°45'N, 61°26'W) and Caribbean Sea, Almirante, Bocas Del Toro, Panama (09°17'N, 82°21'W).
Site of infection: Spiral intestine.
Specimens deposited: ######.
Etymology: to be defined.

Description. (Based on 52 specimens: 49 whole mounts, two worms observed with SEM, and one used for cross sections). Worms acraspedote (Fig. 10A, Table 2), apolytic, 1.4–4.6 mm (n = 49) long, composed of 6–11 (n = 49) proglottids. Scolex composed by a proper and cephalic peduncle (Fig. 10B, 11A). Scolex proper with four bothridia, 128–315 (n = 42) long by 102–254 (n = 42) wide (maximum width of

scolex at level of anterior loculus), each with three loculi: anterior loculus 58–155 long (n = 42), middle loculus 24–69 long (n = 42), and posterior loculus 26–79 long (n = 42); loculus length ratio (A:M:P) 1:0.2–0.5:0.3–0.7 (n = 42). Anterior region of scolex in form of muscular pad 32–61 (n = 12) long by 74–100 (n = 12) wide, bearing an apical sucker 9–19 (n = 7) long by 11–24 (n = 7) wide, and one pair of hooks below posterior margin and triangular in shape (Fig. 11B). Velum present extending from end of anterior loculus to posterior loculus. Hooks bipronged, hollow, with prominent tubercle on proximal surface of axial prong; internal channels of axial and abaxial prongs continuous; axial prongs longer than abaxial on medial hooks; lateral and medial hooks approximately equal in size (Fig. 10C). Lateral hook measurements: AC 27–41 (n = 29), CD 47–73 (n = 28), CB 43–65 (n = 28), AD 79–102 (n = 28). Medial hook measurements: AC' 21–38 (n = 29), CD' 51–79 (n = 29), CB' 40–70 (n = 29) and AD' 75–111 (n = 29). Medial and lateral hook base with approximately same width. Thin layer of tissue covering anteriorly each prong of both hooks. Lateral bothridia of anterior loculus covered with gladiate spinitriches, capilliform and acicular filitriches (Fig. 11D). Distal surface of anterior loculus covered with papilliform filitriches (Fig. 11C). Cephalic peduncle covered with gladiate spinitriches (Fig. 11E).

Immature proglottids wider than long, 5–9 (n = 49) in number. Mature proglottids longer than wide, 733–1,414 (n = 28) long by 99–195 (n = 48) wide, 1–3 (n = 39) in number; mature proglottid length to width ratio 4.8–10.2 (n = 45) (Fig. 10D, E). Absence of gravid proglottids. Testes round to oval, 18–47 (n = 35) long by 15–36 (n = 35) wide, arranged in two regular columns, extending from ovarian isthmus to anterior margin of proglottid, 28–44 (n = 36) in total number, 8–12 (n = 37) pre-poral, 6–10 (n = 37) post-poral and 13–25 (n = 37) anti-poral. Genital pores irregularly alternating, 26–44 % (n = 47) from anterior end of proglottid. Cirrus sac elongated, tilted posteriorly, 57–159 (n = 47) long by 51–128 (n = 47) wide, containing eversible cirrus; cirrus armed with spinitriches. Vagina thick-walled, sinuous, extending from ootype along medial line of proglottid to anterior margin of cirrus sac to common enlarged genital atrium. Vaginal sphincter absent. Ovary located near posterior end of proglottid, essentially symmetrical, inverted A-shaped in frontal view, and tetra-lobed in cross section, reaching or almost reaching cirrus sac, lobulated, 310–738 (n = 46) long by 32–85 (n = 46) wide at isthmus. Ovarian isthmus located at approximate midpoint of ovary. Mehlis' gland posterior to ovarian isthmus. Vitellarium follicular, consisting of two lateral bands each consisting of small follicles, 8–19 (n = 18) long by 6–15 (n = 18) wide; bands extending from near anterior margin of proglottid to posterior margin of ovary, interrupted by vagina and cirrus sac. Uterus thick-walled, sacciform, extending from isthmus to near anterior margin of proglottid.

Remarks. *Acanthobothrium* n. sp. 5 has a unique combination of features that distinguishes it from all species of *Acanthobothrium* reported from the western Atlantic Ocean. From the 23 species reported from this locality, eight of them closely resemble *Acanthobothrium* n. sp. 5 in their total length, number of proglottids and number of testes (Table 3). *Acanthobothrium lintoni* and *Acanthobothrium* n. sp. 1 differ from this new species by axial and abaxial prongs from medial hooks equal in length instead of unequal, respectively. Two additional species, *A. schalli* and *A. lineatum*, can be distinguished from *Acanthobothrium* n. sp. 5 by possessing a longer scolex (*i.e.*, 396–520, 380–600 vs. 128–315, respectively) and longer medial hooks (*i.e.*, AD 130–175, 118–216 vs. 75–111, respectively). *Acanthobothrium brevissime* and *A. tasajerasi* differ from *Acanthobothrium* n. sp. 5 by possessing 2–5 post-poral

testes instead of 6–10 in terminal proglottids, respectively. *Acanthobothrium lengitinosum* differs from the new species by longer lateral hooks (*i.e.*, CD 80–100 vs. 43–65, respectively), shorter proglottids (*i.e.*, 350–570 vs. 733–1,414, respectively), and a lower number of pre-poral testes (*i.e.*, 5–7 vs. 8–12, respectively). *Acanthobothrium* n. sp. 2 can be distinguished from *Acanthobothrium* n. sp. 5 by having longer medial hooks (*i.e.*, CD 88–110 vs. 51–79; AD 117–150 vs. 75–111, respectively) and a smaller cephalic peduncle in comparison to the longer cephalic peduncle typical of *Acanthobothrium* n. sp. 5.

***Acanthobothrium* n. sp. 6**
(Figs. 12, 13)

Type-host: *Himantura pacifica* (Beebe & Tee-Van) (Myliobatiformes: Dasyatidae).
Type-locality: eastern Pacific Ocean, Playa Caleta, Montijo, Veráguas, Panama (07°29'N, 81°13'W).
Site of infection: Spiral intestine.
Specimens deposited: ######.
Etymology: to be defined.

Description. (Based on 21 specimens: 18 whole mounts, two worms observed with SEM, and one used for cross sections). Worms acraspedote (Fig. 12A, Table 4), apolytic, 4.2–9 mm (n = 18) long, composed of 60–97 (n = 18) proglottids. Scolex composed by a proper and cephalic peduncle (Fig. 12B, 13A). Scolex proper with four bothridia, 369–498 (n = 17) long, by 376–511 (n = 17) wide (maximum width of scolex at level of anterior loculus), each with three loculi: anterior loculus 137–231 (n = 15) long, middle loculus 45–76 (n = 15) long, and posterior loculus 53–82 (n = 15) long; loculus length ratio (A:M:P) 1:0.2–0.5:0.2–0.5 (n = 15). Anterior region of scolex in form of muscular pad 81–143 (n = 14) long by 147–188 (n = 14) wide, bearing an apical sucker 26–44 (n = 11) long by 30–61 (n = 11) wide, and one pair of hooks below posterior margin and triangular in shape (Fig. 13B). Velum present, extending from middle to posterior loculus. Hooks bipronged, hollow, with prominent tubercle on proximal surface of axial prong; internal channels of axial and abaxial prongs continuous, smooth; axial prongs longer than abaxial prongs; lateral and medial hooks approximately equal in size (Fig. 12C). Lateral hook measurements: AC 58–85 (n = 17), CD 104–122 (n = 17), CB 63–83 (n = 17), AD 150–186 (n = 17). Medial hook measurements: AC’ 62–85 (n = 17), CD’ 101–122 (n = 17), CB’ 63–86 (n = 17) and AD’ 159–188 (n = 17). Medial and lateral hook base with approximately same width. Absence of tissue layer covering each prong of both hooks. Lateral bothridia of anterior loculus covered with acicular filitriches and gladiate spinitriches (Fig. 13D). Distal surface of anterior loculus covered with papilliform filitriches (Fig. 13C). Cephalic peduncle covered with gladiate spinitriches (Fig. 13E).

Immature proglottids wider than long, 59–92 (n = 18) in number. Mature proglottids longer than wide, 349–649 (n = 18) long by 153–298 (n = 18) wide, 1–6 (n = 18) in number; mature proglottid length to width ratio 1.6–3.3 (n = 18) (Fig. 12D, E). Absence of gravid proglottids. Testes round to oval, 30–51 (n = 14) long by 18–35 (n = 14) wide, arranged in two irregular columns, extending from or almost from ovarian isthmus to anterior region of proglottid, 24–36 (n = 14) in total number, 6–11 (n = 15) pre-poral, 3–6 (n = 15) post-poral and 12–19 (n = 14) anti-poral. Genital pores irregularly alternating, 29–40 % (n = 18) from anterior end of proglottid. Cirrus sac pyriform, with posterior region tilted anteriorly, 36–59 (n = 13) long by 75–114 (n

= 13) wide, containing eversible cirrus armed with spinitriches. Vagina thick-walled, sinuous, extending from ootype along medial line of proglottid to anterior margin of cirrus sac to common genital atrium. Vaginal sphincter absent. Ovary located near posterior end of proglottid, essentially symmetrical, inverted A-shaped in frontal view, and tetra-lobed in cross section, not reaching cirrus sac, lobulated, 150–330 (n = 13) long by 62–120 (n = 13) wide at isthmus. Ovarian isthmus located at approximate midpoint of ovary. Mehlis' gland posterior to ovarian isthmus. Vitellarium follicular, consisting of two lateral bands each consisting of small follicles, 8–18 (n = 11) long by 5–12 (n = 11) wide; bands extending from near anterior margin of proglottid to posterior margin of ovary, interrupted by vagina and cirrus sac. Uterus thick-walled, sacciform, extending from isthmus to near anterior margin of proglottid.

Remarks. *Acanthobothrium* n. sp. 6 has a unique combination of characters, which distinguishes it from all the other 39 species of *Acanthobothrium* that have been described from the eastern Pacific Ocean. These attributes include the total length varying from 4.2 to 9.0 mm, composed by 60 to 97 proglottids, 24 to 36 testes per proglottid, and the possession of axial prongs of both medial and lateral hooks longer than of the abaxial prongs. Of the 39 valid species reported from the eastern Pacific Ocean, only *A. atahualpai* Marques, Brooks & Barriga, 1997 closely resembles *Acanthobothrium* n. sp. 6 in its total length, number of proglottids and number of testes (Table 4). However, *A. atahualpai* can be differentiated from the new species by a longer anterior loculus (*i.e.*, 272–310 vs. 137–231, respectively), a wider muscular pad (*i.e.*, 189–227 vs. 147–188, respectively), larger lateral hooks dimensions (*i.e.*, AC 32–35 vs. 58–85; CD 144–147 vs. 104–122; CB 154–166 vs. 63–83; AD 193–195 vs. 150–186, respectively), larger terminal proglottids (*i.e.*, 918–1,168 long by 355–426 wide vs. 349–689 long by 153–298 wide, respectively), and posteriorly positioned genital pores vs. anteriorly positioned (*i.e.*, 53–58 % vs. 29–40 %, respectively).

***Acanthobothrium* n. sp. 7**
(Figs. 14, 15)

Type-host: *Himantura pacifica* (Beebe & Tee-Van) (Myliobatiformes: Dasyatidae).
Type-locality: eastern Pacific Ocean, Playa Caleta, Montijo, Veráguas, Panama (07°29'N, 81°13'W).
Site of infection: Spiral intestine.
Specimens deposited: ######.
Etymology: to be defined.

Description. (Based on 40 specimens: 37 whole mounts, two worms observed with SEM, and one used for cross sections). Worms acraspedote (Fig. 14A, Table 4), apolytic, 1.7–3.0 mm (n = 37) long, composed of 6–11 (n = 37) proglottids. Scolex composed by a proper and cephalic peduncle (Fig. 14B, 15A). Scolex proper with four bothridia, 347–456 (n = 36) long by 239–321 (n = 36) wide (maximum width of scolex at level of anterior loculus), each with three loculi: anterior loculus 165–238 long (n = 36), middle loculus 41–85 long (n = 36), and posterior loculus 54–101 long (n = 36); loculus length ratio (A:M:P) 1:0.2–0.4:0.2–0.5 (n = 36). Anterior region of scolex in form of muscular pad 62–97 (n = 20) long by 109–158 (n = 20) wide, bearing an apical sucker 16–29 (n = 22) long by 18–42 (n = 22) wide, and one pair of hooks below posterior margin and triangular in shape (Fig. 15B). Velum absent. Hooks bipronged, hollow, with insconspicuous tubercle on proximal surface of axial

prong; internal channels of axial and abaxial prongs continuous, smooth; axial prongs as same length as abaxial prongs; lateral and medial hooks approximately equal in size (Fig. 14C). Lateral hook measurements: AC 40–57 (n = 36), CD 80–120 (n = 36), CB 71–125 (n = 36), AD 106–170 (n = 36). Medial hook measurements: AC' 37–57 (n = 36), CD' 83–129 (n = 36), CB' 71–121 (n = 36) and AD' 113–169 (n = 36). Medial and lateral hook base with approximately same width. Thin layer of tissue covering anteriorly each prong of both hooks. Lateral bothridia of anterior loculus covered with gladiate spinitriches and acicular filitriches (Fig. 15D). Distal surface of anterior loculus covered with papilliform filitriches (Fig. 15C). Cephalic peduncle covered with gladiate spinitriches (Fig. 15E).

Immature proglottids wider than long, 5–9 (n = 37) in number. Mature proglottids longer than wide, 551–948 (n = 34) long by 160–299 (n = 34) wide, 1–2 (n = 37) in number; mature proglottid length to width ratio 2.3–5 (n = 34) (Fig. 14D). Absence of gravid proglottids. Testes round to oval, 34–67 (n = 27) long by 26–40 (n = 27) wide, arranged in two regular columns, extending from ovarian isthmus to anterior region of proglottid, 23–42 (n = 22) in total number, 8–16 (n = 23) pre-poral, 3–7 (n = 22) post-poral and 12–23 (n = 25) anti-poral. Genital pores irregularly alternating, 36–53 % (n = 34) from anterior end of proglottid. Cirrus sac pyriform, 47–81 (n = 33) long by 59–120 (n = 33) wide, containing eversible cirrus armed with spinitriches. Vagina thick-walled, sinuous, extending from ootype along medial line of proglottid to anterior margin of cirrus sac to common genital atrium. Vaginal sphincter absent. Ovary located near posterior end of proglottid, symmetrical or asymmetrical, inverted A-shaped in frontal view, and tetra-lobed in cross section, reaching or almost reaching cirrus sac, lobulated, 192–400 (n = 34) long by 59–123 (n = 34) wide at isthmus. Ovarian isthmus located at approximate midpoint of ovary. Mehlis' gland posterior to ovarian isthmus. Vitellarium follicular, consisting of two lateral bands each consisting of small follicles, 8–24 (n = 34) long by 6–18 (n = 34) wide; bands extending from near anterior margin of proglottid to posterior margin of ovary, interrupted by vagina and cirrus sac. Uterus thick-walled, sacciform, extending from isthmus to near anterior margin of proglottid.

Remarks. *Acanthobothrium* n. sp. 7 closely resembles *A. campbelli* Marques, Brooks & Monks, 1995, *A. coquimbensis* Carvajal & Jeges, 1980, *A. dasi* Ghoshroy & Caira, 2001, and *A. vargasi* Marques, Brooks & Monks, 1995, out of 40 valid species of *Acanthobothrium* reported from the eastern Pacific Ocean. The resemblance among these species is due to the total length, number of proglottids and number of testes (Table 5). *Acanthobothrium campbelli* can be distinguished from *Acanthobothrium* n. sp. 7 by possessing a shorter scolex (*i.e.* 211–255 vs. 347–456, respectively) and a narrower ovary (*i.e.*, 9–22 vs. 59–123, respectively). *Acanthobothrium coquimbensis* differs from the new species by a longer cirrus (*i.e.*, 200–400 vs. 47–81, respectively) and by possessing mature proglottids with different size along the strobili (*i.e.*, same proportion of length and width vs. longer than wide, respectively). *Acanthobothrium dasi* can be distinguished from *Acanthobothrium* n. sp. 7 by unequally long axial and abaxial prongs unequal in length and angulated instead of equal and slightly curved (respectively), a longer scolex (*i.e.*, 525–1,075 vs. 347–456, respectively) and longer cirrus sac (*i.e.*, 100–153 vs. 47–81, respectively). *Acanthobothrium vargasi* most closely resembles *Acanthobothrium* n. sp. 7, but differs from the new species by possessing a wider scolex (*i.e.*, 378 vs. 239–321, respectively).

***Acanthobothrium* n. sp. 8**
(Figs. 16, 17)

Type-host: *Himantura pacifica* (Beebe & Tee-Van) (Myliobatiformes: Dasyatidae).
Type-locality: eastern Pacific Ocean, Playa Caleta, Montijo, Veráguas, Panama (07°29'N, 81°13'W).
Site of infection: Spiral intestine.
Specimens deposited: ######.
Etymology: to be defined.

Description. (Based on 29 specimens: 26 whole mounts, two worms observed with SEM, and one used for cross sections). Worms acraspedote (Fig. 16A, Table 4), apolytic, 3.7–6.3 mm (n = 26) long, composed of 13–23 (n = 26) proglottids. Scolex composed by a proper and cephalic peduncle (Fig. 16B, 17A). Scolex proper with four bothridia, 392–512 (n = 23) long by 266–346 (n = 22) wide (maximum width of scolex at level of anterior loculus), each with three loculi: anterior loculus 170–235 long (n = 21), middle loculus 60–105 long (n = 21), and posterior loculus 68–135 long (n = 21); loculus length ratio (A:M:P) 1:0.3–0.5:0.3–0.7 (n = 21). Anterior region of scolex in form of muscular pad 74–119 (n = 11) long by 128–164 (n = 11) wide, bearing an apical sucker 16–25 (n = 8) long by 24–39 (n = 8) wide, and one pair of hooks below posterior margin and triangular in shape (Fig. 17B). Velum absent. Hooks bipronged, hollow, with prominent tubercle on proximal surface of axial prong; internal channels of axial and abaxial prongs continuous, smooth; axial prongs approximately with same length of abaxial prongs; lateral and medial hooks approximately equal in size (Fig. 16C). Lateral hook measurements: AC 44–74 (n = 26), CD 83–109 (n = 26), CB 73–97 (n = 26), AD 115–153 (n = 26). Medial hook measurements: AC’ 39–66 (n = 26), CD’ 86–114 (n = 26), CB’ 67–107 (n = 26) and AD’ 125–152 (n = 26). Medial and lateral hook base with approximately same width. Thin layer of tissue covering anteriorly each prong of both hooks. Lateral bothridia of anterior loculus covered by gladiate spinitriches, capilliform and acicular filitriches (Fig. 17D). Distal surface of anterior loculus covered with papilliform filitriches (Fig. 17C). Cephalic peduncle covered with gladiate spinitriches (Fig. 17E).

Immature proglottids wider than long, 10–20 (n = 26) in number. Mature proglottids longer than wide, 840–1,414 (n = 23) long by 203–299 (n = 26) wide, 2–4 (n = 26) in number; mature proglottid length to width ratio 3.1–6.7 (n = 23) (Fig. 16D, E). Absence of gravid proglottids. Testes round to oval, 27–69 (n = 27) long by 23–43 (n = 27) wide, arranged in two irregular columns, extending from ovarian isthmus to anterior region of proglottid, 43–63 (n = 26) in total number, 13–23 (n = 27) pre-poral, 4–9 (n = 27) post-poral and 22–34 (n = 26) anti-poral. Genital pores irregularly alternating, 44–55 % (n = 23) from anterior end of proglottid. Cirrus sac pyriform, with posterior region tilted anteriorly, 66–107 (n = 27) long by 93–131 (n = 27) wide, containing eversible cirrus armed with spinitriches. Vagina thick-walled, sinuous, extending from ootype along medial line of proglottid to anterior margin of cirrus sac to common genital atrium. Vaginal sphincter absent. Ovary located near posterior end of proglottid, slightly asymmetrical, inverted A-shaped in dorsal or ventral view, and tetra-lobed in cross section, reaching the cirrus sac, lobulated, 294–764 (n = 24) long by 75–134 (n = 27) wide at isthmus. Ovarian isthmus located at approximate midpoint of ovary. Mehlis’ gland posterior to ovarian isthmus. Vitellarium follicular, consisting of two lateral bands each consisting of small follicles, 10–24 (n = 22) long by 9–18 (n = 22) wide; bands extending from near anterior margin of proglottid to almost

posterior margin of ovary, interrupted by vagina and cirrus sac. Uterus thick-walled, sacciform, extending from isthmus to near anterior margin of proglottid.

Remarks. *Acanthobothrium* n. sp. 8 can be distinguished from all the 41 valid species of *Acanthobothrium* reported from the Pacific Ocean by a unique set of morphological attributes. Three of them closely resemble *Acanthobothrium* n. sp. 8 in their total lengths, numbers of proglottids, and numbers of testes (Table 5). *Acanthobothrium bullardi* Ghoshroy & Caira, 2001 and *Acanthobothrium costarricense* Marques, Brooks & Monks, 1995 can be distinguished from the new species by axial and abaxial prongs from medial hooks unequal in length instead of equal (respectively). In addition, *A. bullardi* possessess a longer scolex (*i.e.*, 633–943 vs. 392–512, respectively) and *A. costarricense* possessess a shorter outer prong of the lateral hooks (*i.e.*, CB 54–66 vs. 73–97, respectively) and narrower ovary (*i.e.*, 26–51 vs. 75–134, respectively). *Acanthobothrium cimari* Marques, Brooks & Monks, 1995 is the species that most closely resembles *Acanthobothrium* n. sp. 8, but it differs from the new species by a shorter anterior loculi length (*i.e.*, 117–164 vs. 170–235, respectively) and a shorter cirrus sac (*i.e.*, 145–180 vs. 66–107, respectively).

***Acanthobothrium* n. sp. 9**
(Figs. 18, 19)

Type-host: *Himantura schmardae* (Werner).
Type-locality: Caribbean Sea, Almirante, Bocas Del Toro, Panama (09°17'N, 82°20'W)
Additional locality: Caribbean Sea, Almirante, Bocas Del Toro, Panama (09°17'N, 82°21'W).
Site of infection: Spiral intestine.
Specimens deposited: #######.
Etymology: to be defined.

Description. (Based on 36 specimens: 33 whole mounts, two worms observed with SEM, and one used for cross sections). Worms acraspedote (Fig. 18A, Table 2), apolytic, 1.9–4.7 mm (n = 33) long, composed of 4–10 (n = 33) proglottids. Scolex composed by a proper and cephalic peduncle (Fig. 18B, 19A). Scolex proper with four bothridia, 197–319 (n = 32) long, by 128–221 (n = 31) wide (maximum width of scolex at level of anterior loculus), each with three loculi: anterior loculus 97–139 long (n = 30), middle loculus 23–54 long (n = 30), and posterior loculus 26–54 long (n = 30); loculus length ratio (A:M:P) 1:0.2–0.5:0.2–0.5 (n = 30). Anterior region of scolex in form of muscular pad 36–62 (n = 19) long by 45–96 (n = 19) wide, bearing an apical sucker 9–16 (n = 11) long by 8–16 (n = 11) wide, and one pair of hooks below posterior margin and triangular in shape (Fig. 19B). Velum present, extending from anterior to posterior loculus. Hooks bipronged, hollow, with inconspicuous tubercle on proximal surface of axial prong; internal channels of axial and abaxial prongs continuous, smooth; axial prongs with the same length of abaxial prongs; lateral and medial hooks approximately equal in size (Fig. 18C). Lateral hook measurements: AC 23–40 (n = 28), CD 48–74 (n = 27), CB 45–65 (n = 27), AD 75–101 (n = 27). Medial hook measurements: AC’ 27–39 (n = 27), CD’ 52–75 (n = 27), CB’ 44–75 (n = 27) and CD’ 76–104 (n = 27). Medial and lateral hook base with approximately same width. Thin layer of tissue covering each prong of both hooks. Lateral bothridia of anterior loculus covered with capilliform, acicular filitriches and

gladiate spinitriches (Fig. 19D). Distal surface of anterior loculus covered with papilliform filitriches (Fig. 19C). Cephalic peduncle covered with gladiate spinitriches (Fig. 19E).

Immature proglottids wider than long, 3–8 (n = 33) in number. Mature proglottids longer than wide, 686–1,192 (n = 28) long by 109–203 (n = 29) wide, 1–2 (n = 33) in number; mature proglottid length to width ratio 3.7–8.1 (n = 28) (Fig. 18D, E). Absence of gravid proglottids. Testes round to oval, 23–48 (n = 16) long by 13–32 (n = 16) wide, arranged in two regular columns, extending from or almost from ovarian isthmus to anterior region of proglottid, 24–37 (n = 23) in total number, 7–12 (n = 24) pre-poral, 3–6 (n = 23) post-poral and 12–19 (n = 24) anti-poral. Genital pores irregularly alternating, 30–48 % (n = 29) from anterior end of proglottid Cirrus sac pyriform tilted posteriorly, 72–165 (n = 26) long by 53–125 (n = 25) wide, containing eversible cirrus armed with spinitriches. Vagina thick-walled, sinuous, extending from ootype along medial line of proglottid to anterior margin of cirrus sac to common genital atrium. Vaginal sphincter absent. Ovary located near posterior end of proglottid, essentially asymmetrical, inverted A-shaped in frontal view, and tetra-lobed in cross section, reaching or almost reaching the cirrus sac, lobulated, 247–665 (n = 25) long by 37–82 (n = 27) wide at isthmus. Ovarian isthmus located at approximate ⅔ of ovary end. Mehlis' gland posterior to ovarian isthmus. Vitellarium follicular, consisting of 2 lateral bands each consisting of small follicles, 8–22 (n = 15) long by 4–19 (n = 15) wide; bands extending from near anterior margin of proglottid to posterior margin of ovary, interrupted by vagina and cirrus sac. Uterus thick-walled, sacciform, extending from isthmus to near anterior margin of proglottid.

Remarks. *Acanthobothrium* n. sp. 9 closely resembles ten out of 24 valid species of *Acanthobothrium* reported from the western Atlantic Ocean. The resemblance among these species is based on a similar total length and numbers of proglottids and testes (Table 3). Out of these 10 species, four possess a longer scolex: *A. lineatum* (380–600), *A. schalli* (396–520), *A. ulmeri* (357–464) and *Acanthobothrium* n. sp. 1 (440–586), whereas the scolex of *Acanthobothrium* n. sp. 9 is less than 320 long. *Acanthobothrium lintoni* and *A. westi* differ from *Acanthobothrium* n. sp. 9 by having a shorter ovary (*i.e.*, 19–51 and 152–176 vs. 247–665, respectively). *Acanthobothrium lengitinusum* can be distinguished from the new species by possessing shorter proglottids (*i.e.*, 350–570 vs. 686–1,192, respectively), wider ovary (*i.e.*, 104–152 vs. 37–82, respectively) and ovarian lobes not reaching to the level of the cirrus sac (vs. reaching to the cirrus sac, respectively). *Acanthobothrium* n. sp. 2 differs from *Acanthobothrium* n. sp. 9 by having unequal medial hooks (vs. of equal length, respectively) and longer medial prongs (*i.e.*, CD 88–110 vs. 52–75, AD 117–150 vs. 74–104, respectively). *Acanthobothrium brevissime* differs from the new species by having lappets on the apical sucker that extend out of the same (vs. lappets that do not protrude out of the apical sucker, respectively), a larger apical sucker diameter (*i.e.*, 8–16 vs. 25–48, respectively), and angulated medial hooks (vs. not angulated, respectively). *Acanthobothrium* n. sp. 5 most closely resembles *Acanthobothrium* n. sp. 9 in most features, but differs from the latter by possessing axial and abaxial prongs unequal in length for medial hooks (vs. axial and abaxial prongs equal in length, respectively) and a prominent tubercle on axial prongs (vs. inconspicuous tubercles, respectively).

Discussion

Historical associations studies can potentially help to understand the processes that are responsible for the diversification and geographical distribution of hosts and parasites alike (Brooks *et al.*, 1981b; Page and Charleston, 1998; Marques and Caira, 2016; among others). Theoretically, hosts impose patterns of diversification on their associated lineages, especially when events of co-divergence are prevalent in the historical association (Page and Charleston, 1998). The current evidence from morphological and molecular data supports the hypothesis that a clade composed by amphi-American species of *Himantura* is the sister-group to Neotropical freshwater stingrays (Lovejoy, 1996; Lovejoy *et al.*, 1998; Aschliman, 2011). For this reason, a survey of parasites of members of amphi-American *Himantura* generates the expectation of finding lineages closely associated with potamotrygonids, which might shed some light into the evolutionary history of potamotrygonids, their marine allies, and their parasites.

Previously, the knowledge of species of *Acanthobothrium* infecting amphi-American species of *Himantura* was solely based on two species from *H. schmardae* (*i.e.*, *A. himanturi* and *A. tasajerasi*). *Acanthobothrium* is one of the most diverse genus within onchoproteocephalideans. As such, the two species reported from *H. schmardae* indicates that the diversity of *Acanthobothrium* for this host is underestimated. The same can be stated for its sister species, *H. pacifica*, for which no species of *Acanthobothrium* has been reported thus far. However, only two hosts individuals of this species have been examined for parasites until now (Marques *et al.*, 1996; Hoberg *et al.*, 1998). In addition, the recurrent documentation of geminate species from both sides of the Panamanian isthmus, not only for cestodes (Craig *et al.*, 2004) but also of other metazoan lineages (Donaldson and Wilson, 1999; Marko and Moran 2009), predicted the existence of undiscovered species of *Acanthobothrium* in *H. pacifica*. Therefore, a more extensive search for members of *Acanthobothrium* parasitizing these hosts was long overdue.

Confirming the predictions above, the present study reported eight new species of *Acanthobothrium* hosted by amphi-American species of *Himantura*. Among the new species described, five were recovered from *H. schmardae* (*i.e.*, *Acanthobothrium* n. sp. 1, *Acanthobothrium* n. sp. 2, *Acanthobothrium* n. sp. 3, *Acanthobothrium* n. sp. 5 and *Acanthobothrium* n. sp. 9) while three infected *H. pacifica* (*i.e.*, *Acanthobothrium* n. sp. 6, *Acanthobothrium* n. sp. 7 and *Acanthobothrium* n. sp. 8). With the addition of *A. himanturi*, reported by Brooks (1977) in *H. schmardae* from Colombia, our study increases the number of species of *Acanthobothrium* associated with these hosts to nine and the overall diversity of the genus to a total of 194 species.

It is somewhat puzzling that the richness of *Acanthobothrium* found in *H. schmardae* (*i.e.*, six species) is two-fold larger when compared to its sister *H. pacifica* (*i.e.*, three species). We think that the explanation for that resides on the

biogeographical representation of the samples available to this study. Trevisan and Marques (in prep. – Cap. 1) questioned how cestode richness seems to be biogeographically structured for species of *Rhinebothrium* parasitizing amphi-American species of *Himantura*. For *Rhinebothrium*, they argued, richness is not concentrated at the individual-host level, as it would be expected for systems with a higher α-richness, but tends to increase as different host populations are sampled. As such, total richness is contingent on the biogeographical representation, since cestode communities seem to be dominated by β-richness. This rational might explain the apparent low diversity of *Acanthobothrium* in *H. pacifica*, for which the samples examined in the present study came from a single locality. For its sister, *H. schmardae*, at least three different localities have been sampled to date. If in fact, the richness of *Acanthobothrium* is biogeographically structured, we would predict that additional samples of *H. pacifica* from different localities throughout its distribution will reveal new lineages of parasites for this genus.

The phylogenetic position of the lineages parasitizing amphi-American species of *Himantura* reveals yet some hidden components for the diversity of *Acanthobothrium*. The molecular data recognized an independent lineage, represented by two terminals, namely *Acanthobothrium* sp. [BE-3.13, 14] in *H. schmardae* from Belize (BE-3), which was not recovered among the specimens available for morphological studies. *Acanthobothrium* n. sp. 5 from *H. schmardae*, although the specimens we examined form a morphological coherent group, resulted paraphyletic. The paraphyletic status of this new species is due to the position of one specimen of *Acanthobothrium* sp. from Belize [BE-12.42] in *Dasyatis guttata* (BE-12), which nested between two haplotypes of *Acanthobothrium* n. sp. 5 [TT14-06.5 and PN15-56.4] from Trinidad and Tobago and Panama in *H. schmardae* (TT14-06 and PN15-56, respectively, see Fig. 1). Thus the present concept of *Acanthobothrium* n. sp. 5 might hide cryptic species, which can potentially be recognized with the inclusion of additional nucleotide sequence data and re-evaluation of morphological data, including material from *D. gutatta*, which is beyond the scope of this study. The same paraphyletic pattern is found for *Acanthobothrium* n. sp. 7 for which the monophyly of two haplotypes [PN15-14.4 and 25.2] from *H. schmardae* from Panama was undermined by the position of two undescribed species of *Acanthobothrium* from the eastern Pacific coast of Costa Rica infecting *U. aspidura* (CRP-51) and *Zapteryx xyster* (CRP-71). As for *Acanthobothrium* n. sp. 5, additional sequence and morphological data for all representatives of this clade should refine the concept of this species. The molecular phylogeny clearly indicates that *Acanthobothroides* Brooks, 1997 is a member of *Acanthobothrium*. *Acanthobothroides* was erected to accommodate a new species, *Acanthobothroides thorsoni* Brooks, 1977 from *H. schmardae* from Colombia. Later, Marques *et al.* (1996) included *Acanthobothroides pacificus* Marques, Brooks and Ureña, 1996 from *H. pacifica* collected in Costa Rica. No molecular data is available for *A. pacificus* at this point, but we predict that there cestodes sister species giving their respect hosts.

The phylogeny of *Acanthobothrium* adds relevant information to the historical associations of potamotrygonids, their marine sister-group, and associated parasites.

Brooks *et al.* (1981b) were the first to address the evolution of the historical associations of this system using parasitological data. The general premise of Brooks *et al.*'s (1981b) study was, that it would be possible to ascertain the relationships of the hosts based on the phylogeny of their parasites. They assumed that the phyletic status of their parasites (*e.g.*, mono/para/polyphyletic) would indicate the phyletic status of potamotrygonids. They proceeded, based on morphological data from parasites of potamotrygonids and a selected group of marine cestodes, by concluding that potamotrygonids form a monophyletic group, due to the monophyly of their parasite lineages. Their premise, however, had the underlined assumption that the ancestor of potamotrygonids carried monophyletic lineages of cestodes. Regardless of the overwhelming evidence for the monophyly of potamotrygonids (de Carvalho *et al.*, 2004; Aschliman, 2011; Naylor *et al.*, 2012), which was not known at the time of Brooks et al. (1981b), the phylogenetic pattern recovered for *Acanthobothrium* challenges their initial assumption. It is evident that the lineages of *Acanthobothrium* infecting amphi-American species of *Himantura* are not monophyletic, as it is the case for the lineages of lineages found in potamotrygonids (Fig. 1). The most relevant result of this study, however, is the fact that the two clades of freshwater species of *Acanthobothrium* resemble a sister-group to lineages of amphi-American species of *Himantura*.

The congruence between our recent understanding of phylogenetic relationships of both hosts and parasites refute the initial hypothesis put forward by Brooks *et al.* (1981b) that the genus *Urobatis* (then considered as members of *Urolophus*) is the sister-group of potamotrygonids. Hoberg *et al.* (1998) were the first to provide a representative phylogenetic study for nematodes of the genus *Echinocephalus* Molin, 1858 in which species found in amphi-American species of *Himantura* nested as sister-taxa to those found in potamotrygonids. Most recently, Marques and Caira (2016) demonstrated that *Pararhinebothroides hobergi* (Zamparo, Brooks & Barriga, 1999) described by Zamparo *et al.* (1999) from *Urobatis tumbesensis* from Ecuador and hypothesized sister of *Rhinebothroides* – an endemic genus of rhinebothriidean found in potamotrygonids, is a member of *Anthocephalum* and phylogenetically distant from *Rhinebothroides*. The same congruent pattern was found by Trevisan *et al.* (in prep. - Cap. 2) for species of *Anindobothrium* of amphi-American species of *Himantura* that form a sister clade to a single species found in potamotrygonids, *A. lisae* Marques, Brooks and Lasso, 2001. The present results for *Acathobothrium* also suggest events of codivergence between freshwater and marine lineages (see Figure 1: FW1 and FW2). All these studies illustrate that as taxonomic sampling increases and robust methodological approaches are applied, components severely criticized with respect to Brooks *et al.*'s (1981b) seminal study (Straney, 1982; Caira, 1990; Caira, 1994; Lovejoy, 1997), the congruence between host and parasite phylogenies become visible. Only through this strategy, will it be possible to understand the historical associations among potamotrygonids, marine batoids and their parasites.

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TABLE 1. Sequences of 16S and 28S for 24 specimens of *Acanthobothrium* from amphi-American species of *Himantura* generated in the present study. Mol. Code, molecular code (which will be replaced by NCBI accession numbers); MUZUSP, museum voucher numbers for hologenophores.

Mol. code	Field code	MUZUSP	Species	Host	Locality
b033	PN15-56.4	7794	*Acanthobothrium* n. sp. 5	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b034	PN15-56.5	7795	*Acanthobothrium* n. sp. 9	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b035	PN15-56.6	7796	*Acanthobothrium* n. sp. 9	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b039	PN15-09.5	7797	*Acanthobothrium* n. sp. 8	*H. pacifica*	Coast of Playa Caleta, Montijo, Veraguas, Panama
b040	PN15-09.6	7798	*Acanthobothrium* n. sp. 8	*H. pacifica*	Coast of Playa Caleta, Montijo, Veraguas, Panama
b044	PN15-14.4	7799	Acanthobothrium n. sp. 7	*H. pacifica*	Coast of Playa Caleta, Montijo, Veraguas, Panama
b045	PN15-25.2	7800	*Acanthobothrium* n. sp. 7	*H. pacifica*	Coast of Playa Caleta, Montijo, Veraguas, Panama
b047	PN15-51.1	7801	*Acanthobothrium* n. sp. 9	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b048	PN15-52.5	7802	*Acanthobothrium* n. sp. 4	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b049	PN15-52.6	7803	*Acanthobothrium* n. sp. 4	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b050	PN15-52.7	7804	*Acanthobothrium* n. sp. 4	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b052	PN15-53.4	7805	*Acanthobothrium* n. sp. 4	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama

TABLE 1. (Continued).

b053	PN15-53.5	7806	*Acanthobothrium* n. sp. 4	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b054	PN15-53.6	7807	*Acanthobothrium* n. sp. 4	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b055	PN15-54.4	7808	*Acanthobothrium* n. sp. 3	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b056	BE12-09.16	7809	*Acanthobothrium* n. sp. 3	*H. schmardae*	Coast of Tobacco Caye, Dangriga, Stann Creek, Belize
b057	BE12-09.15	7810	*Acanthobothrium* n. sp. 3	*H. schmardae*	Coast of Tobacco Caye, Dangriga, Stann Creek, Belize
b058	TT14-06.5	7811	*Acanthobothrium* n. sp. 5	*H. schmardae*	Caribbean Sea, Maracas, San Juan-Laventille, Trinidad and Tobago
b076	PN15-09.9	7812	*Acanthobothrium* n. sp. 6	*H. pacifica*	Coast of Playa Caleta, Montijo, Veraguas, Panama
b077	PN15-09.10	7813	*Acanthobothrium* n. sp. 6	*H. pacifica*	Coast of Playa Caleta, Montijo, Veraguas, Panama
b078	PN15-09.11	7814	*Acanthobothrium* n. sp. 6	*H. pacifica*	Coast of Playa Caleta, Montijo, Veraguas, Panama
b079	PN15-53.7	7815	*Acanthobothrium* n. sp. 1	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b080	PN15-53.8	7816	*Acanthobothrium* n. sp. 1	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama
b081	PN15-53.9	7817	*Acanthobothrium* n. sp. 1	*H. schmardae*	Caribbean Sea, Almirante, Bocas del Toro, Panama

TABLE 2. Morphometric data of species of *Acanthobothrium* from *Himantura schmardae* described on this study. Measurements are given as the range, followed by number of worms in parentheses. All measurements are in micrometers, unless otherwise indicated.

Character	**n. sp 1**	**n. sp. 2**	**n. sp. 3**	***A. himanturi* Brooks, 1977**	**n. sp. 5**	**n. sp. 9**
Total Length (mm)	1.6–4.4 (n = 33)	2.8–7.5 (n = 28)	5.7–11.7 (n = 40)	3.4–12.4 (n = 49)	1.4–4.6 (n = 49)	1.9–4.7 (n = 33)
Number of proglottids	6–16 (n = 33)	9–23 (n = 28)	37–64 (n = 39)	14–32 (n = 47)	6–11 (n = 49)	4–10 (n = 33)
Scolex length	440–586 (n = 31)	294–400 (n = 25)	391–540 (n = 39)	341–630 (n = 43)	128–315 (n = 42)	197–319 (n = 32)
Scolex width	255–334 (n = 28)	222–325 (n = 25)	198–502 (n = 37)	226–480 (n = 42)	102–254 (n = 42)	128–221 (n = 31)
Muscular pad length	79–127 (n = 24)	40–100 (n = 21)	97–165 (n = 31)	47–145 (n = 35)	32–61 (n = 12)	36–62 (n = 19)
Muscular pad width	115–170 (n = 25)	82–165 (n = 21)	125–205 (n = 31)	110–220 (n = 37)	74–100 (n = 12)	45–96 (n = 19)
Apical sucker length	13–27 (n = 26)	12–35 (n = 16)	26–55 (n = 34)	16–45 (n = 37)	9–19 (n = 7)	9–16 (n = 11)
Apical sucker width	20–45 (n = 27)	12–40 (n = 16)	30–60 (n = 34)	19–60 (n = 38)	11–24 (n = 7)	8–16 (n = 11)
Anterior loculus length	210–328 (n = 33)	125–195 (n = 24)	157–234 (n = 39)	148–295 (n = 44)	58–155 (n = 42)	97–139 (n = 30)
Middle loculus length	60–103 (n = 33)	29–65 (n = 25)	50–90 (n = 37)	52–120 (n = 43)	24–69 (n = 42)	23–54 (n = 30)
Posterior loculus length	60–101 (n = 32)	35–70 (n = 25)	50–111 (n = 38)	61–149 (n = 44)	26–79 (n = 42)	26–54 (n = 30)
Loculus ratio (A:M:P)	1:0.3–0.4:0.2–0.4 (n = 33)	1:0.2–0.4:0.2–0.4 (n = 24)	1:0.3–0.5:0.2–0.6 (n = 39)	1:0.2–0.6:0.3–0.6 (n = 42)	01:0.2–0.5:0.3–0.7 (n = 42)	01:0.2–0.5:0.2–0.5 (n = 30)
Hooks measurements						
Lateral AC	48–85 (n = 31)	32–49 (n = 23)	52–72 (n = 32)	38–89 (n = 38)	27–41 (n = 29)	23–40 (n = 28)
Lateral CD	100–148 (n = 31)	72–97 (n = 23)	66–115 (n = 29)	70–129 (n = 35)	47–73 (n = 28)	48–74 (n = 27)
Lateral CB	111 –155 (n = 30)	57–82 (n = 23)	51–91 (n = 26)	63–115 (n = 37)	43–65 (n = 28)	45–65 (n = 27)
Lateral AD	147–197 (n = 31)	101–129 (n = 23)	115–173 (n = 29)	97–181 (n = 37)	79–102 (n = 28)	75–101 (n = 27)
Medial AC'	42–63 (n = 31)	28–48 (n = 24)	51–79 (n = 34)	32–78 (n = 38)	21–38 (n = 29)	27–39 (n = 27)
Medial CD'	106–155 (n = 30)	88–110 (n = 24)	75–116 (n = 32)	80–126 (n = 37)	51–79 (n = 29)	52–75 (n = 27)
Medial CB'	107–149 (n = 29)	63–94 (n = 24)	46–87 (n = 25)	52–106 (n = 38)	40–70 (n = 29)	44–75 (n = 27)
Medial AD'	151–199 (n = 31)	117–150 (n = 24)	129–179 (n = 32)	111–188 (n = 37)	75–111 (n = 29)	76–104 (n = 27)
Number of imature proglottids	4–14 (n = 33)	8–20 (n = 28)	30–60 (n = 39)	12–28 (n = 47)	5–9 (n = 49)	3–8 (n = 33)

TABLE 2. (Continued).

Number of mature proglottids	1–4 (n = 33)	1–4 (n = 28)	1–9 (n = 39)	2–8 (n = 49)	1–3 (n = 39)	1–2 (n = 33)
Mature/Terminal proglottid length	592–1,390 (n = 30)	658–1,162 (n = 25)	592–1,355 (n = 35)	805–1,870 (n = 46)	733–1,414 (n = 28)	686–1192 (n = 28)
Mature/Terminal proglottid width	180–344 (n = 30)	133–270 (n = 27)	167–388 (n = 37)	172–375 (n = 47)	99–195 (n = 48)	109–203 (n = 29)
Proglottid length–width ratio	2.4–4.7 (n = 26)	2.7–5.6 (n = 24)	2.2–5.1 (n = 33)	2.9–6 (n = 44)	4.8–10.2 (n = 45)	3.7–8.1 (n = 28)
Genital pore position (%)	30–43 (n = 31)	25–35 (n = 21)	38–56 (n = 38)	32–47 (n = 46)	26–44 (n = 47)	30–48 (n = 29)
Total number of testes	21–42 (n = 30)	23–33 (n = 27)	28–74 (n = 33)	37–67 (n = 43)	28–44 (n = 36)	24–37 (n = 23)
Number of pre-poral testes	5–11 (n = 31)	3–9 (n = 28)	10–26 (n = 32)	9–18 (n = 45)	8–12 (n = 37)	7–12 (n = 24)
Number of post-poral testes	4–11 (n = 31)	4–10 (n = 28)	3–8 (n = 35)	5–14 (n = 46)	6–10 (n = 37)	3–6 (n = 23)
Number of anti-poral testes	10–24 (n = 31)	11–18 (n = 27)	20–39 (n = 32)	20–37 (n = 44)	13–25 (n = 37)	12–19 (n = 24)
Testes length	17–50 (n = 30)	20–57 (n = 28)	27–51 (n = 37)	22–72 (n = 49)	18–47 (n = 35)	23–48 (n = 16)
Testes width	12–33 (n = 30)	17–40 (n = 28)	19–41 (n = 37)	22–50 (n = 48)	15–36 (n = 35)	13–32 (n = 16)
Cirrus sac length	67–207 (n = 31)	45–160 (n = 28)	44–137 (n = 35)	72–190 (n =47)	57–159 (n = 47)	72–165 (n = 26)
Cirrus sac width	66–150 (n = 31)	80–187 (n = 24)	73–198 (n = 34)	75–200 (n = 48)	51–128 (n = 47)	53–125 (n = 25)
Ovary length	215–500 (n = 31)	180–680 (n = 27)	244–490 (n = 34)	371–960 (n = 46)	310–738 (n = 46)	247–665 (n = 25)
Ovary width	69–120 (n = 29)	27–115 (n = 27)	98–190 (n = 31)	90–200 (n = 47)	32–85 (n = 46)	37–82 (n = 27)
Vitelaria length	8–27 (n = 30)	6–25 (n = 28)	11–40 (n = 33)	6–34 (n =46)	8–19 (n = 18)	8–21 (n = 15)
Vitelaria width	7–22 (n = 30)	4–25 (n = 28)	5–23 (n = 33)	6–26 (n =46)	6–15 (n = 18)	4–19 (n = 15)

TABLE 3. Morphometric data for species of *Acanthobothrium* reported from western Atlantic Ocean. All measurements are in micrometers, unless otherwise indicated. TL, total length; # prog, number of proglottids; # loculi, number of loculi; # testes, number of testes. Marine ecoregions provided according to Spalding *et al.* (2007).

Species	Authority	TL	# prog	# testes	Type Host	Locality	Marine Ecoregions*
A. brevissime	(Linton, 1908) Campbell, 1969	1–4.2	7–29	19–40	*Dasyatis say*	Gulf of Mexico, Mexico and Cheasepeak Bay, U.S.A.	Warm Temperate Northwest Atlantic/Northern Gulf of Mexico and Cold Temperate Northwest Atlantic/Virginian
A. floridensis	Goldstein, 1964	2.9–22	38–90	37–82	*Raja eglanteria*	Gulf of Mexico, U.S.A.	Warm Temperate Northwest Atlantic/Northern Gulf of Mexico
A. fogeli	Goldstein, 1964	1.9–4.8	12–32	36–54	*Gymnura micrura*	Gulf of Mexico, U.S.A.	Warm Temperate Northwest Atlantic/Northern Gulf of Mexico
A. himanturi	Brooks, 1977	3.8–9.3	17–26	6–12	*Himantura schmardae*	La Cienaga, Magdalena, Caribbean Sea, Colombia	Tropical Northwestern Atlantic/Southwestern Caribbean
A. lineatum	Campbell, 1969	1.8–6.1	6–19	28–45	*Dasyatis americana*	Cheasepeak Bay, Virginia, U.S.A.	Cold Temperate Northwest Atlantic/Virginian
A. lintoni	Goldstein, Henson & Schlicht, 1968	2.5–22.6	5–60	30–46	*Narcine brasiliensis*	Gulf of Mexico, U.S.A.	Warm Temperate Northwest Atlantic/Northern Gulf of Mexico
A. lengitinosum	Vardo-Zalik & Campbell, 2011	2–3.1	5–7	22–29	*Rhinobatos lengitinosus*	Gulf of Mexico, U.S.A.	Warm Temperate Northwest Atlantic/Northern Gulf of Mexico
A. paulum	Linton, 1890	3–16	22–50	27–59	*Dasyatis centroura*	Woodshole, Massachussets and Cheasepeak Bay, Virginia, U.S.A.	Cold Temperate Northwest Atlantic/Gulf of Maine,Bay of Fundy and Virginian
A. schalli	Vardo-Zalik & Campbell, 2011	3–7	10–23	25–29	*Mustelus canis canis*	Gulf of Mexico, U.S.A.	Warm Temperate Northwest Atlantic/Northern Gulf of Mexico
A. tasajerasi	Brooks, 1977	2.5–5.5	11–18	19–33	*Himantura schmardae*	La Cienaga, Magdalena, Caribbean Sea, Colombia	Tropical Northwestern Atlantic/Southwestern Caribbean
A. ulmeri	Vardo-Zalik & Campbell, 2011	0.7–3.4	3–11	18–26	*Raja texana*	Gulf of Mexico, U.S.A.	Warm Temperate Northwest Atlantic/Northern Gulf of Mexico
A. westi	Vardo-Zalik & Campbell, 2011	1.5–2.3	5–11	20–24	*Raja texana*	Gulf of Mexico, U.S.A.	Warm Temperate Northwest Atlantic/Northern Gulf of Mexico

TABLE 3. (Continued).

A. woodsholei	Baer, 1948	12–15	NA	50–55	*Dasyatis centroura*	Woodshole, Massachussets, U.S.A.	Cold Temperate Northwest Atlantic/Gulf of Maine,Bay of Fundy
Acanthobothrium sp. n. 1	present study	1.6–4.4	6–16	21–42	*Himantura schmardae*	BE, PNa	Tropical Northwestern Atlantic/Western Caribbean and Southwestern Caribbean
Acanthobothrium sp. n. 2	present study	2.8–7.5	9–23	23–33	*Himantura schmardae*	BE	Tropical Northwestern Atlantic/Western Caribbean
Acanthobothrium sp. n. 3	present study	5.7–11.7	37–64	28–74	*Himantura schmardae*	BE, PNa	Tropical Northwestern Atlantic/Western Caribbean and Southwestern Caribbean
Acanthobothrium sp. n. 5	present study	1.4–4.6	6–11	28–44	*Himantura schmardae*	PNa	Tropical Northwestern Atlantic/Southwestern Caribbean
Acanthobothrium sp. n. 9	present study	1.9–4.7	4–10	24–37	*Himantura schmardae*	PNa	Tropical Northwestern Atlantic/Southwestern Caribbean

*BE = North of Southwater Caye and Tobacco Caye, Dangriga, Stann Creek, Belize.
**PNa = Almirante, Bocas del Toro, Panama.

TABLE 4. Morphometric data for species of *Acanthobothium* from *Himantura pacifica* described on this study. Measurements are given as the range, followed by number of worms in parentheses. All measurements are in micrometers, unless otherwise indicated.

Character	**n. sp. 6**	**n. sp. 7**	**n. sp. 8**
Total Length (mm)	4.2–9 (n = 18)	1.7–3.0 (n = 37)	3.7–6.3 (n = 26)
Number of proglottids	60–97 (n = 18)	6–11 (n = 37)	13–23 (n = 26)
Scolex length	369–498 (n = 17)	347–456 (n = 36)	392–512 (n = 23)
Scolex width	376–511 (n = 17)	239–321 (n = 36)	266–346 (n = 22)
Muscular pad length	81–143 (n = 14)	62–97 (n = 20)	74–119 (n = 11)
Muscular pad width	147–188 (n = 14)	109–158 (n = 20)	128–164 (n = 11)
Apical sucker length	26–44 (n = 11)	16–29 (n = 22)	16–25 (n = 8)
Apical sucker width	30–61 (n = 11)	18–42 (n = 22)	24–39 (n = 8)
Anterior loculus length	137–231 (n = 15)	165–238 (n = 36)	170–235 (n = 21)
Middle loculus length	45–76 (n = 15)	41–85 (n = 36)	60–105 (n = 21)
Posterior loculus length	53–82 (n = 15)	54–101 (n = 36)	68–135 (n = 21)
Loculus ratio (A:M:P)	1:0.2–0.5:0.2–0.5 (n = 15)	1:0.2–0.4:0.2–0.5 (n = 36)	1:0.3–0.5:0.3–0.7 (n = 21)
Hooks measurements			
Lateral AC	58–85 (n = 17)	40–57 (n = 36)	44–74 (n = 26)
Lateral CD	104–122 (n = 17)	80–120 (n = 36)	83–109 (n = 26)
Lateral CB	63–83 (n = 17)	71–125 (n = 36)	73–97 (n = 26)
Lateral AD	150–186 (n = 17)	106–170 (n = 36)	115–153 (n = 26)
Medial AC'	62–85 (n = 17)	37–57 (n = 36)	39–66 (n = 26)
Medial CD'	101–122 (n = 17)	83–129 (n = 36)	86–114 (n = 26)
Medial CB'	63–86 (n = 17)	71–121 (n = 36)	67–107 (n = 26)
Medial AD'	159–188 (n = 17)	113–169 (n = 36)	125–152 (n = 26)
Number of imature proglottids	59–92 (n = 18)	5–9 (n = 37)	10–20 (n = 26)
Number of mature proglottids	1–6 (n = 18)	1–2 (n = 37)	2–4 (n = 26)

TABLE 4. (Continued).

Mature/Terminal proglottid length	349–689 (n = 18)	551–948 (n = 34)	840–1,414 (n = 23)
Mature/Terminal proglottid width	153–298 (n = 18)	160–299 (n = 34)	203–299 (n = 26)
Proglottid length-width ratio	1.6–3.3 (n = 18)	2.3–5 (n = 34)	3.1–6.7 (n = 23)
Genital pore position (%)	29–40 (n = 18)	36–53 (n = 34)	44–55 (n = 23)
Total number of testes	24–36 (n = 14)	23–42 (n = 22)	43–63 (n = 26)
Number of pre-poral testes	6–11 (n = 15)	8–16 (n = 23)	13–23 (n = 27)
Number of post-poral testes	3–6 (n = 15)	3–7 (n = 22)	4–9 (n = 27)
Number of anti-poral testes	12–19 (n = 14)	12–23 (n = 25)	22–34 (n = 26)
Testes length	30–52 (n = 14)	34–67 (n = 27)	27–69 (n = 27)
Testes width	18–35 (n = 14)	26–40 (n = 27)	23–43 (n = 27)
Cirrus sac length	36–59 (n = 13)	47–81 (n = 33)	66–107 (n = 27)
Cirrus sac width	75–114 (n = 13)	59–120 (n = 33)	93–131 (n = 27)
Ovary length	150–330 (n = 13)	192–400 (n = 34)	294–764 (n = 24)
Ovary width	62–120 (n = 13)	59–123 (n = 34)	75–134 (n = 27)
Vitelaria length	8–18 (n = 11)	8–24 (n = 34)	10–24 (n = 22)
Vitelaria width	5–12 (n = 11)	6–18 (n = 34)	9–18 (n = 22)

TABLE 5. Morphometric data for species of *Acanthobothrium* from the estern Pacific Ocean described on this study. All measurements are in micrometers, unless otherwise indicated. TL, total length; # prog, number of proglottids; # loculi, number of loculi; # testes, number of testes. Marine ecoregions provided according to Spalding *et al.* (2007).

Species	Authority	TL	# prog	# testes	Type Host	Locality	Marine Ecoregions
A. atahualpai	Marques, Brooks & Barriga, 1997	>2.1	>17	20–30	*Gymnura afuerae*	Puerto Bolivar Equador	Tropical East Pacific/Guayaquil
A. bullardi	Ghoshroy & Caira, 2001	4.1–8.6	15–26	30–47	*Dasyatis brevis*	Puertecitos and Santa Rosalia, Gulf od California, Mexico	Warm Temperate Northeast Pacific /Cortezian
A. campbelli	Marques, Brooks & Monks, 1995	1–1.8	3–6	15–23	*Urotrygon chilensis*	Costa de Pajaros, Costa Rica	Tropical East Pacific/Nicoya
A. cimari	Marques, Brooks & Monks, 1995	5.8–10.2	14–33	42–62	*Dasyatis longus*	Punta Morales, Costa Rica	Tropical East Pacific/Nicoya
A. coquimbensis	Carvajal & Jeges, 1980	3–10	10–35	22–40	*Myliobatis chilensis*	Antofagasto and Coquimbo, Chile	Warm Temperate Southeastern Pacific/Humboldtian
A. costarricense	Marques, Brooks & Monks, 1995	6–10.8	15–35	37–62	*Dasyatis longus*	Punta Morales, Costa Rica	Tropical East Pacific/Nicoya
A. dasi	Ghoshroy & Caira, 2001	1.7–3.1	6–12	24–41	*Dasyatis brevis*	Gulf of California, Mexico	Warm Temperate Northeast Pacific /Cortezian
A. vargasi	Marques, Brooks & Monks, 1995	2–3	5–7	22–29	*Dasyatis longus*	Punta Morales, Costa Rica	Tropical East Pacific/Nicoya
Acanthobothrium n. sp. 6	present study	4.2–9	60–97	24–36	*Himantura pacifica*	PNp	Tropical East Pacific/Nicoya
Acanthobothrium n. sp. 7	present study	1.7–3	6–11	23–42	*Himantura pacifica*	PNp	Tropical East Pacific/Nicoya
Acanthobothrium n. sp. 8	present study	3.7–6.3	13–23	43–63	*Himantura pacifica*	PNp	Tropical East Pacific/Nicoya

*PNp = Playa Caleta, Montijo, Panama.

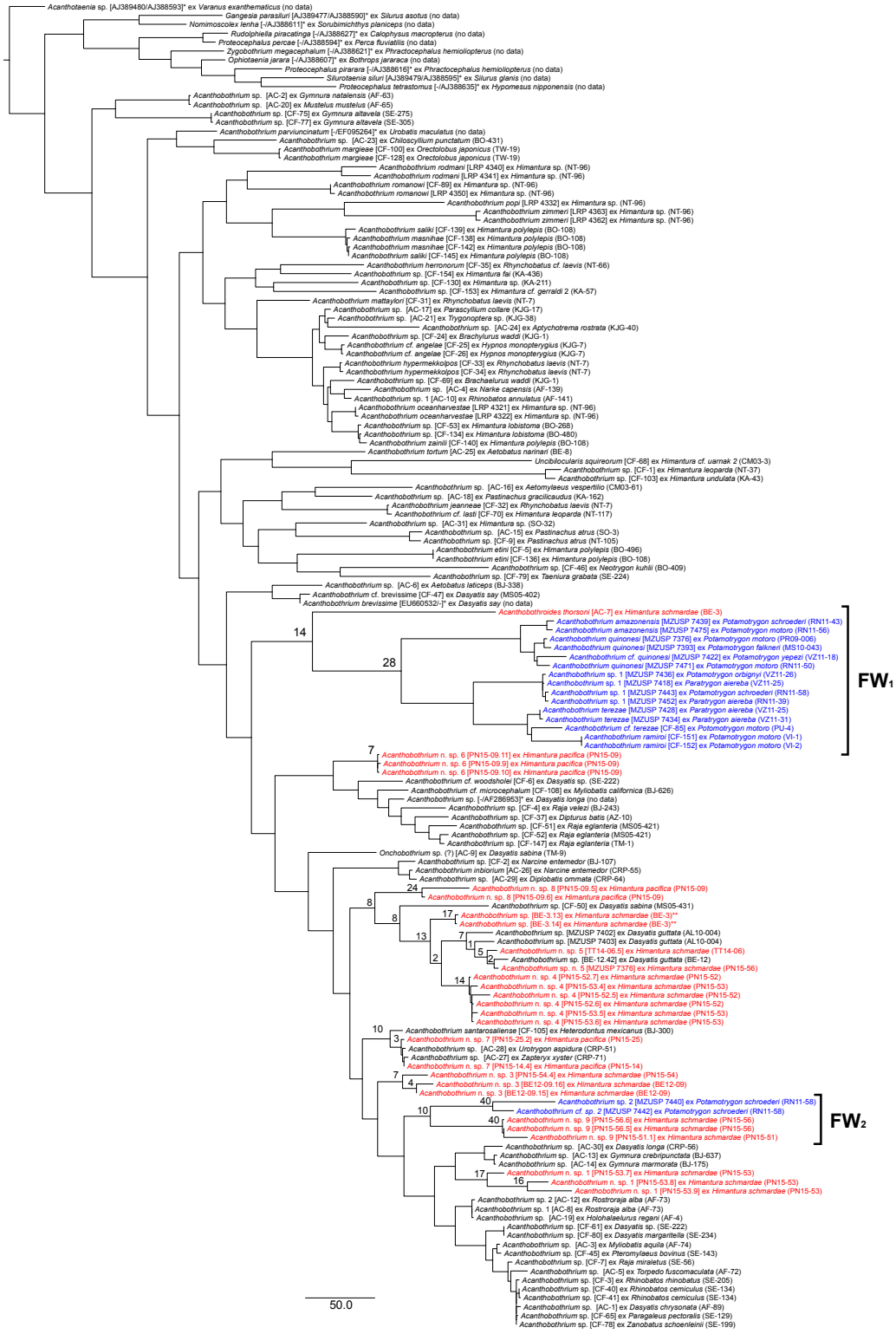

FIGURE 1. Sister-group relationships based on the simultaneous analysis of 16S and 28S rDNA regions using parsimony as the optimality criteria. Contents between brackets represent molecular codes and/or accession number for vouchers, contents between parentheses refer to host accession code. Freshwater taxa are in blue, and marine taxa from the present study are in red. FW_1 and FW_2 represent the close relation between marine and freshwater lineages.

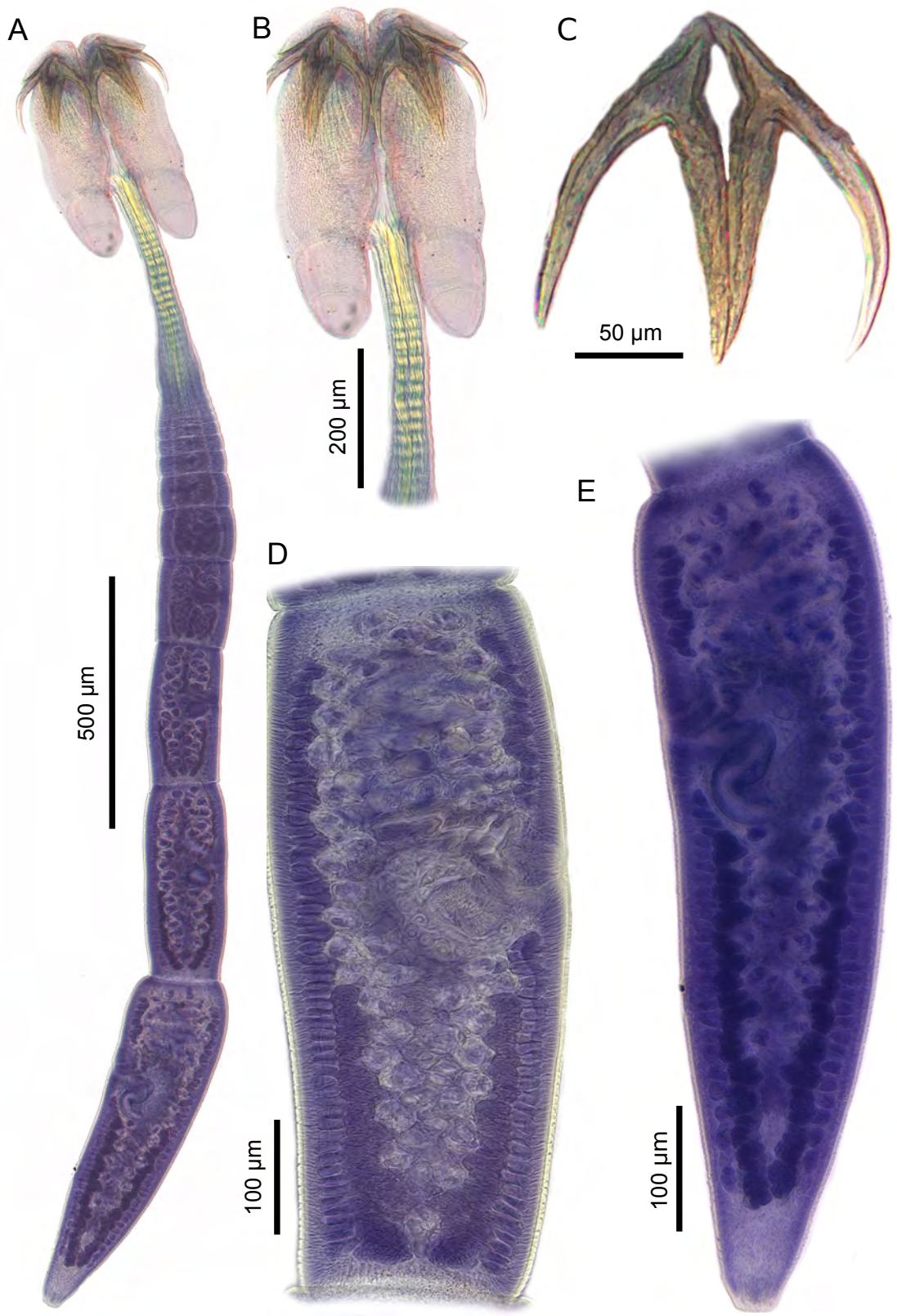

FIGURE 2. Light micrograph of *Acanthobothrium* n. sp. 1 from *Himantura schmardae* (Werner). A. whole worm; B. scolex; C. hooks; D. subterminal mature proglottid; E. terminal mature proglottid.

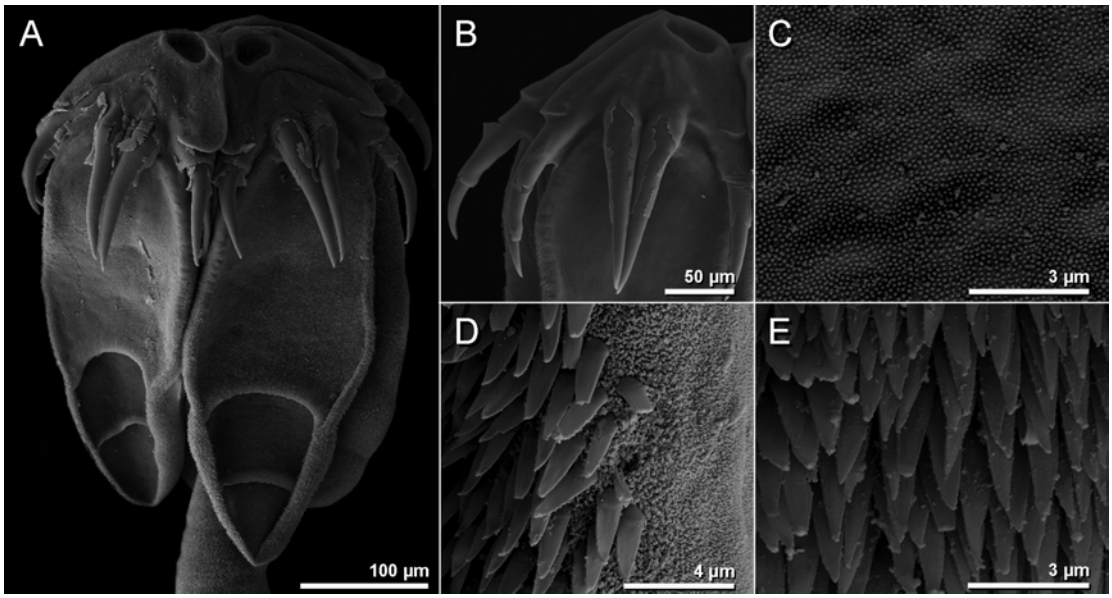

FIGURE 3. Scanning electron micrographs of *Acanthobothrium* n. sp. 1. A. scolex; B. detail of hooks and apical pad; C. surface of distal bothridia; D. surface of lateral margin from anterior loculus; E. cephalic peduncle.

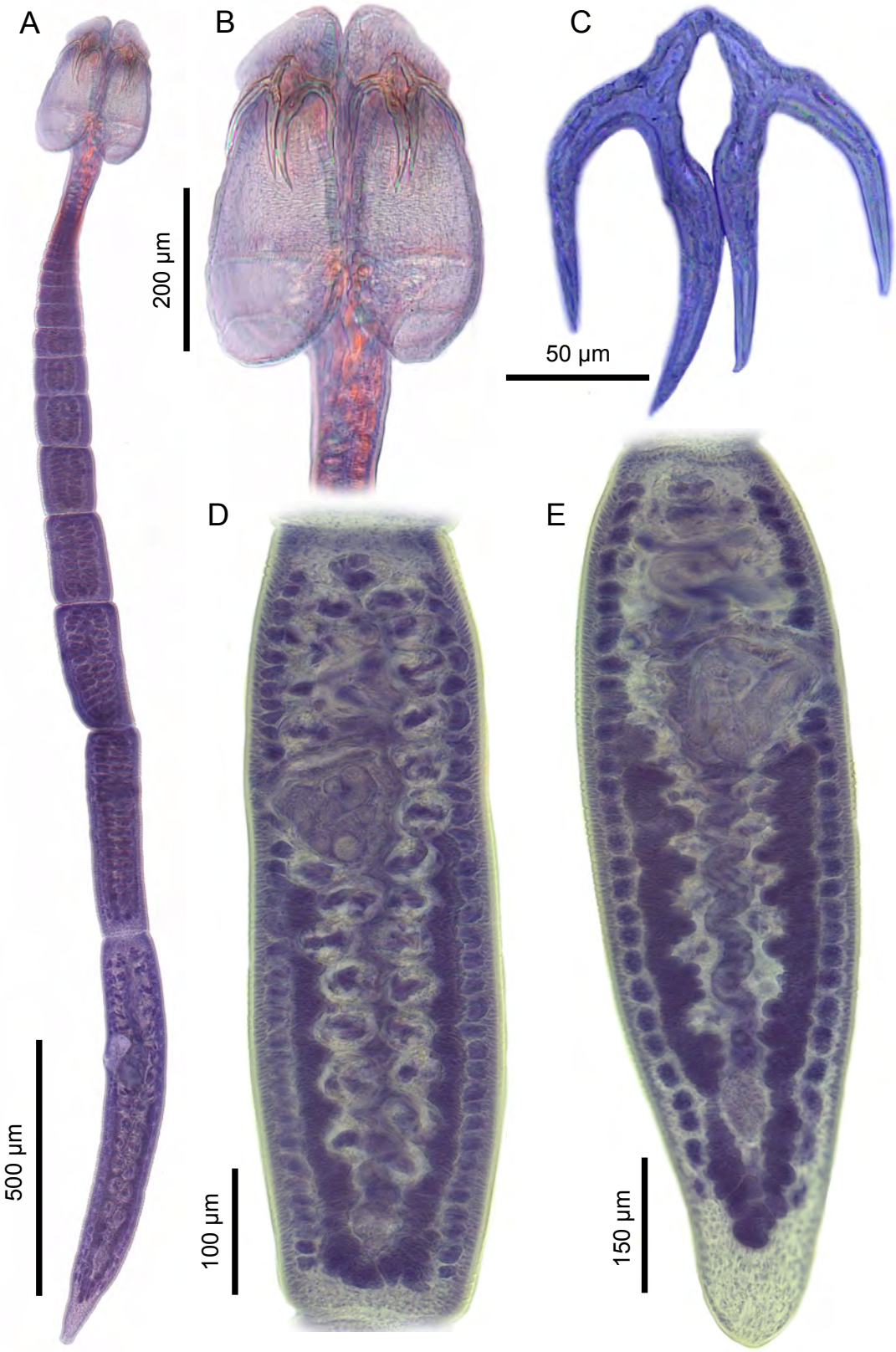

FIGURE 4. Light micrograph of *Acanthobothrium* n. sp. 2 from *Himantura schmardae* (Werner). A. whole worm; B. scolex; C. hooks; D. subterminal mature proglottid; E. terminal mature proglottid.

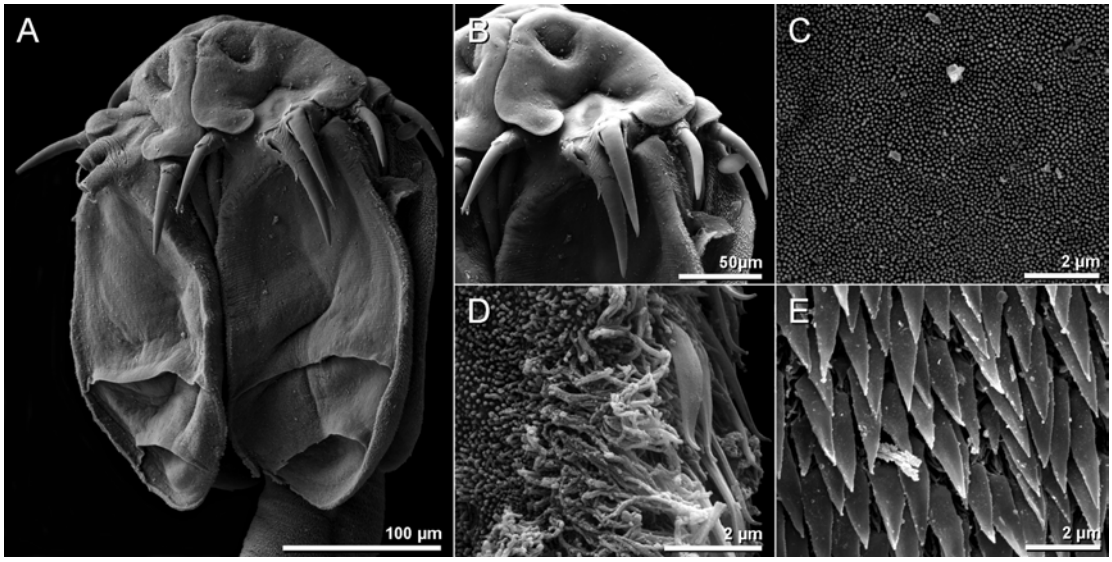

FIGURE 5. Scanning electron micrographs of *Acanthobothrium* n. sp. 2. A. scolex; B. detail of hooks and apical pad; C. surface of distal bothridia; D. surface of lateral margin from anterior loculus; E. cephalic peduncle.

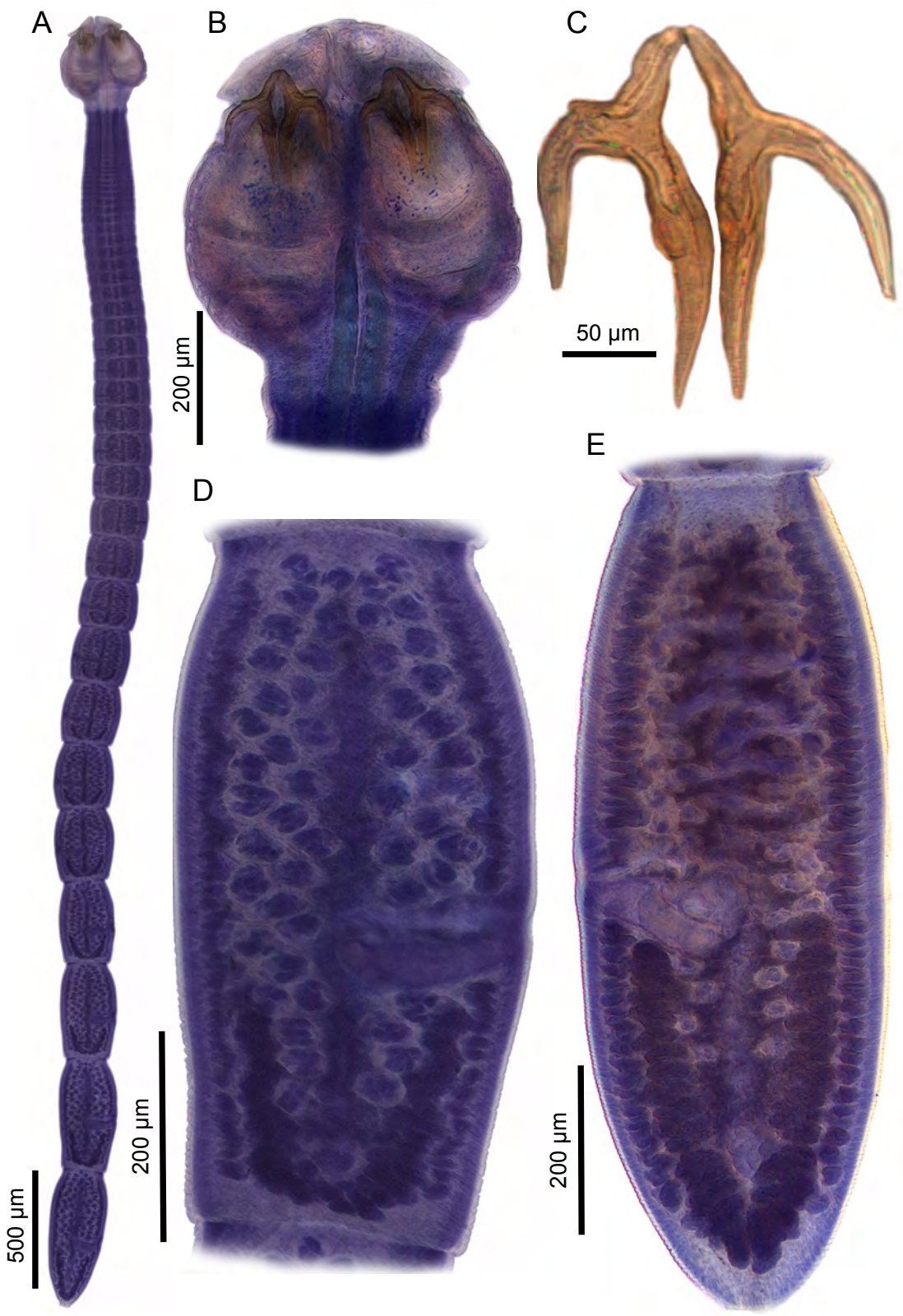

FIGURE 6. Light micrograph of *Acanthobothrium* n. sp. 3 from *Himantura schmardae* (Werner). A. whole worm; B. scolex; C. hooks; D. subterminal mature proglottid; E. terminal mature proglottid.

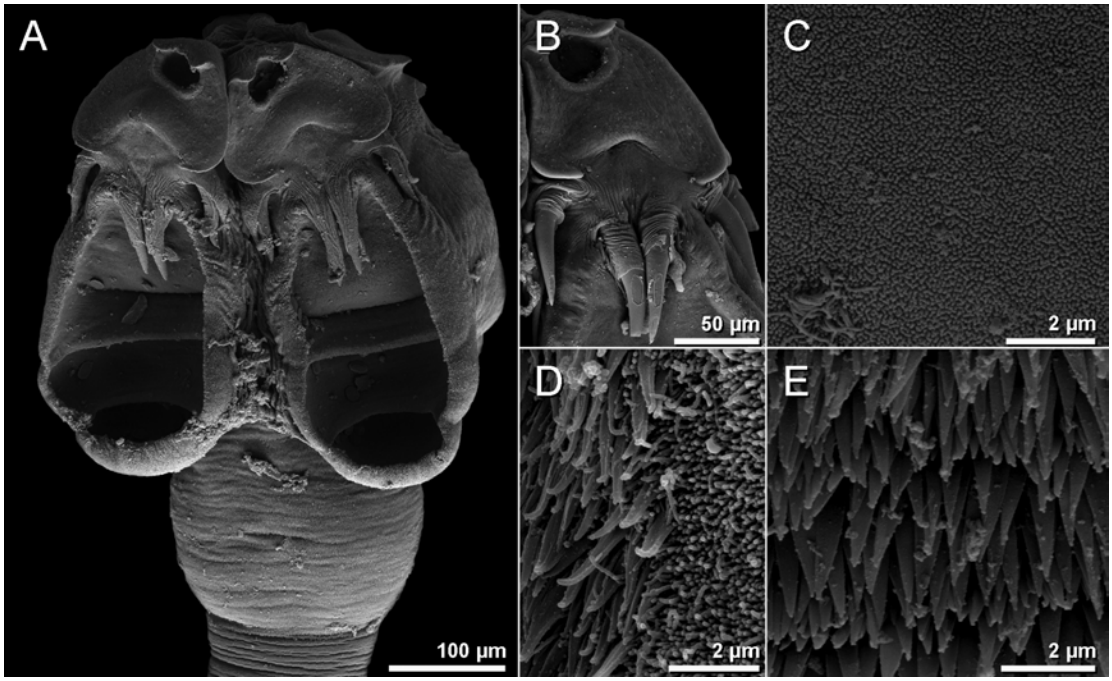

FIGURE 7. Scanning electron micrographs of *Acanthobothrium* n. sp. 3. A. scolex; B. detail of hooks and apical pad; C. surface of distal bothridia; D. surface of lateral margin from anterior loculus; E. cephalic peduncle.

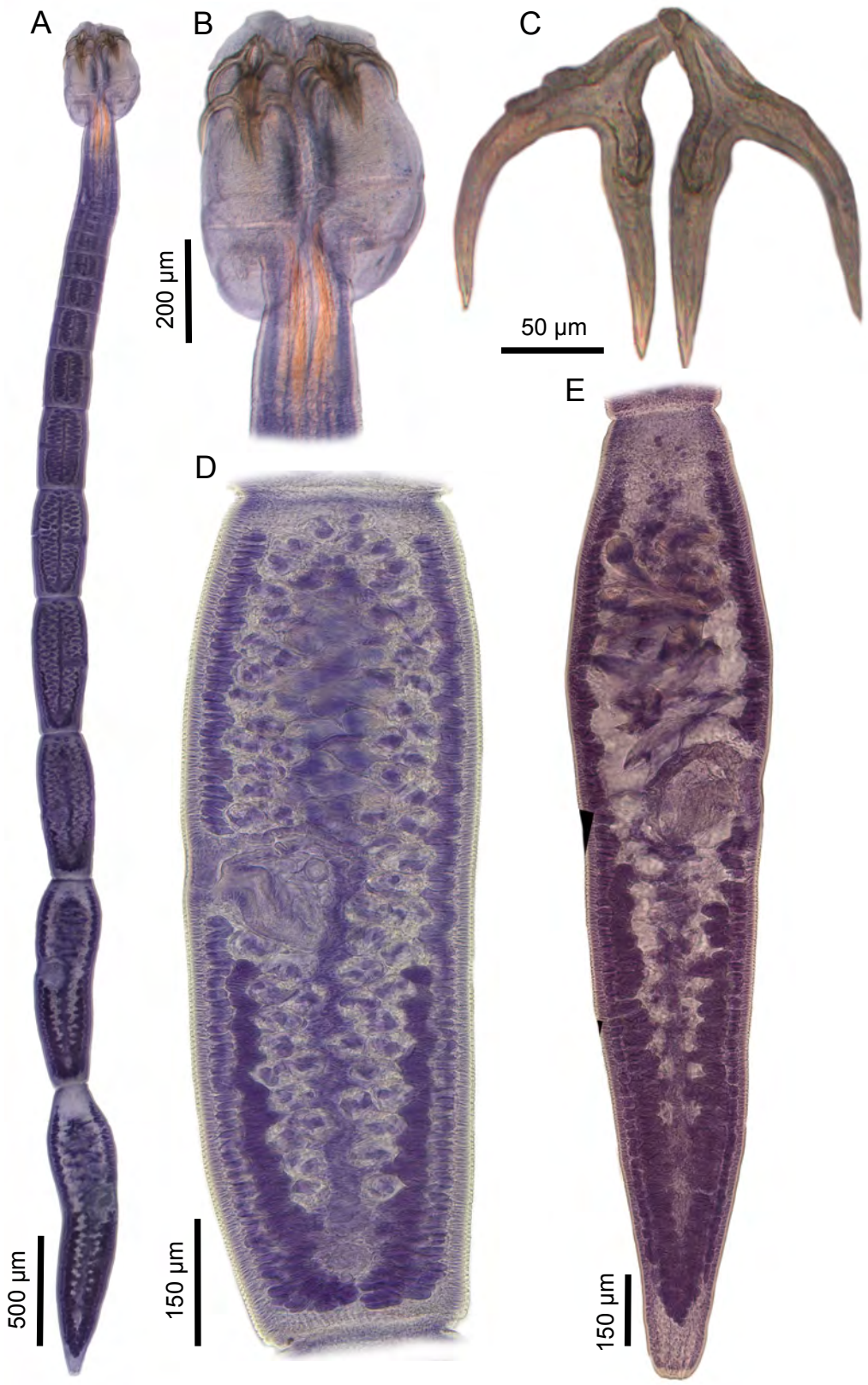

FIGURE 8. Light micrograph of *Acanthobothrium himanturi* from *Himantura schmardae* (Werner). A. whole worm; B. scolex; C. hooks; D. subterminal mature proglottid; E. terminal mature proglottid.

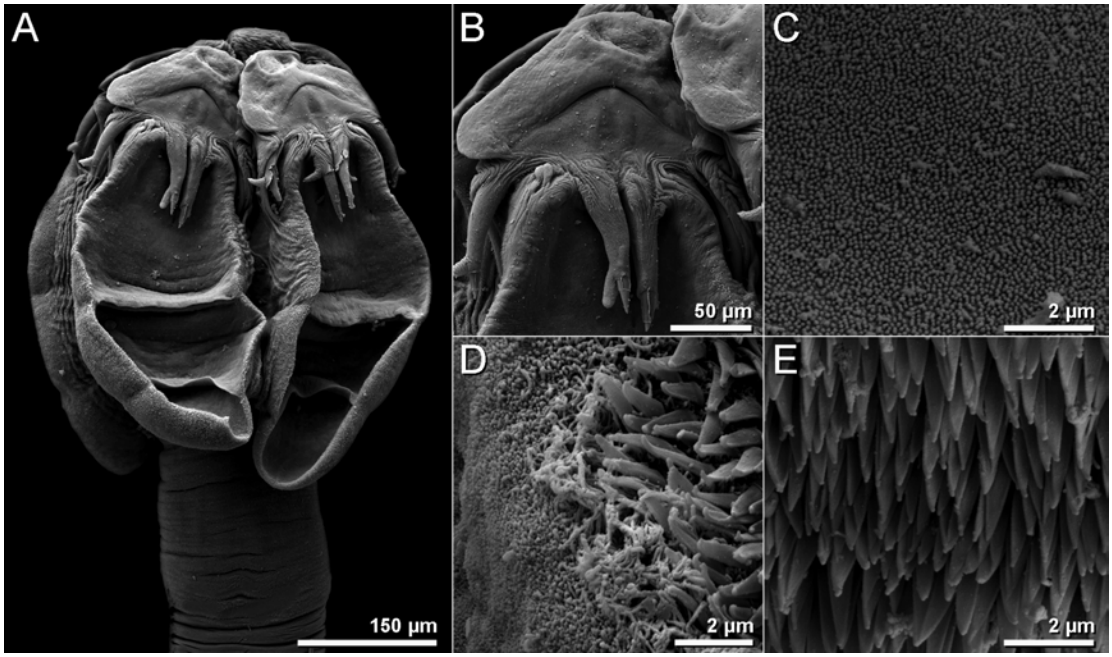

FIGURE 9. Scanning electron micrographs of *Acanthobothrium himanturi*. A. scolex; B. detail of hooks and apical pad; C. surface of distal bothridia; D. surface of lateral margin from anterior loculus; E. cephalic peduncle.

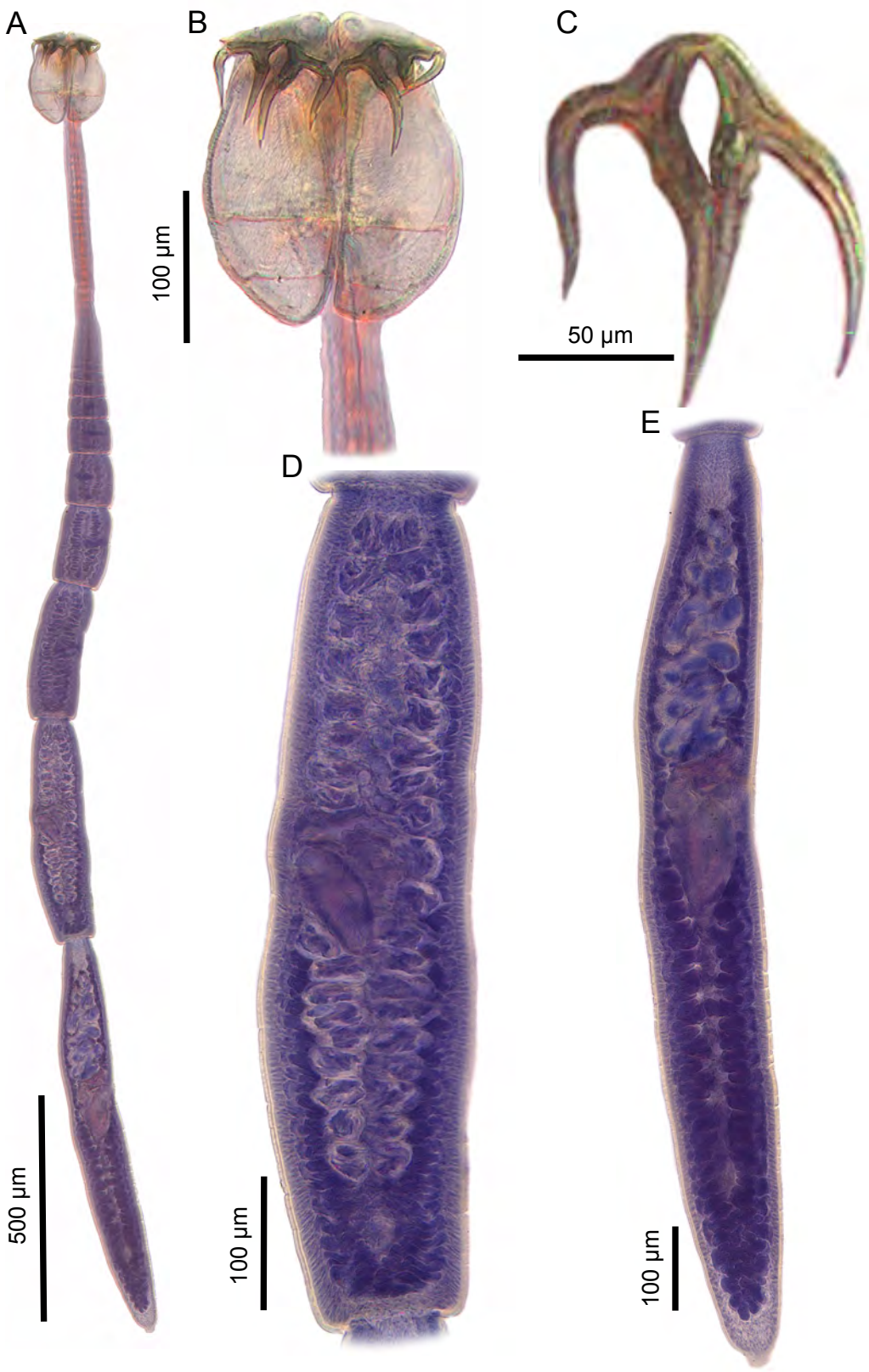

FIGURE 10. Light micrograph of *Acanthobothrium* n. sp. 5 from *Himantura schmardae* (Werner). A. whole worm; B. scolex; C. hooks; D. subterminal mature proglottid; E. terminal mature proglottid.

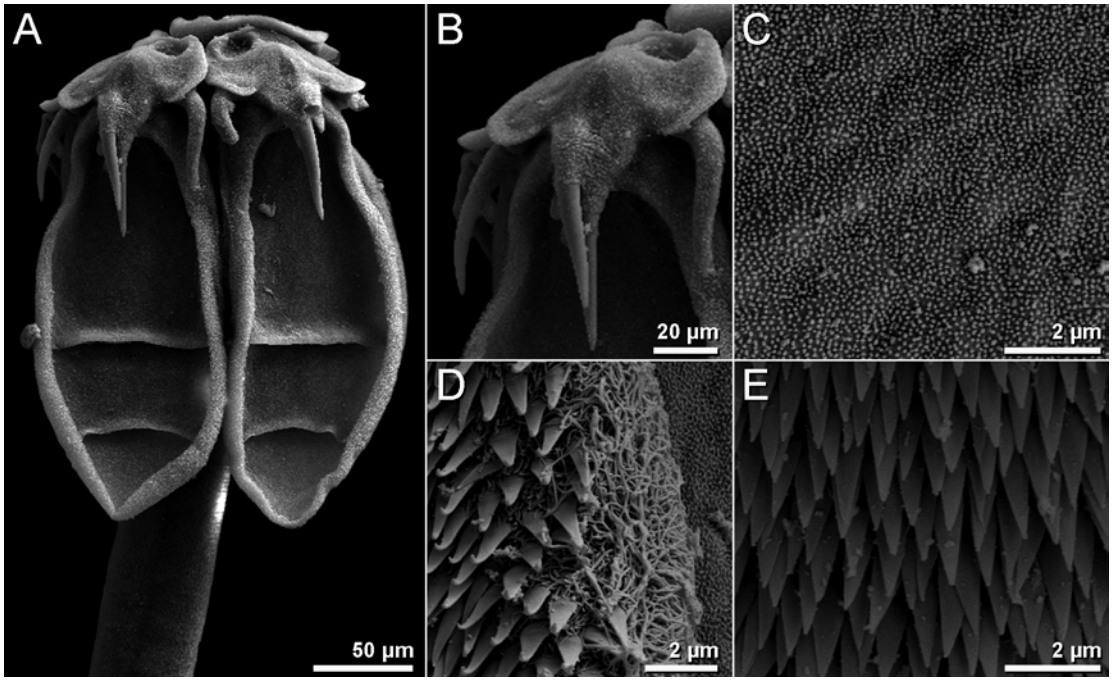

FIGURE 11. Scanning electron micrographs of *Acanthobothrium* n. sp. 5. A. scolex; B. detail of hooks and apical pad; C. surface of distal bothridia; D. surface of lateral margin from anterior loculus; E. cephalic peduncle.

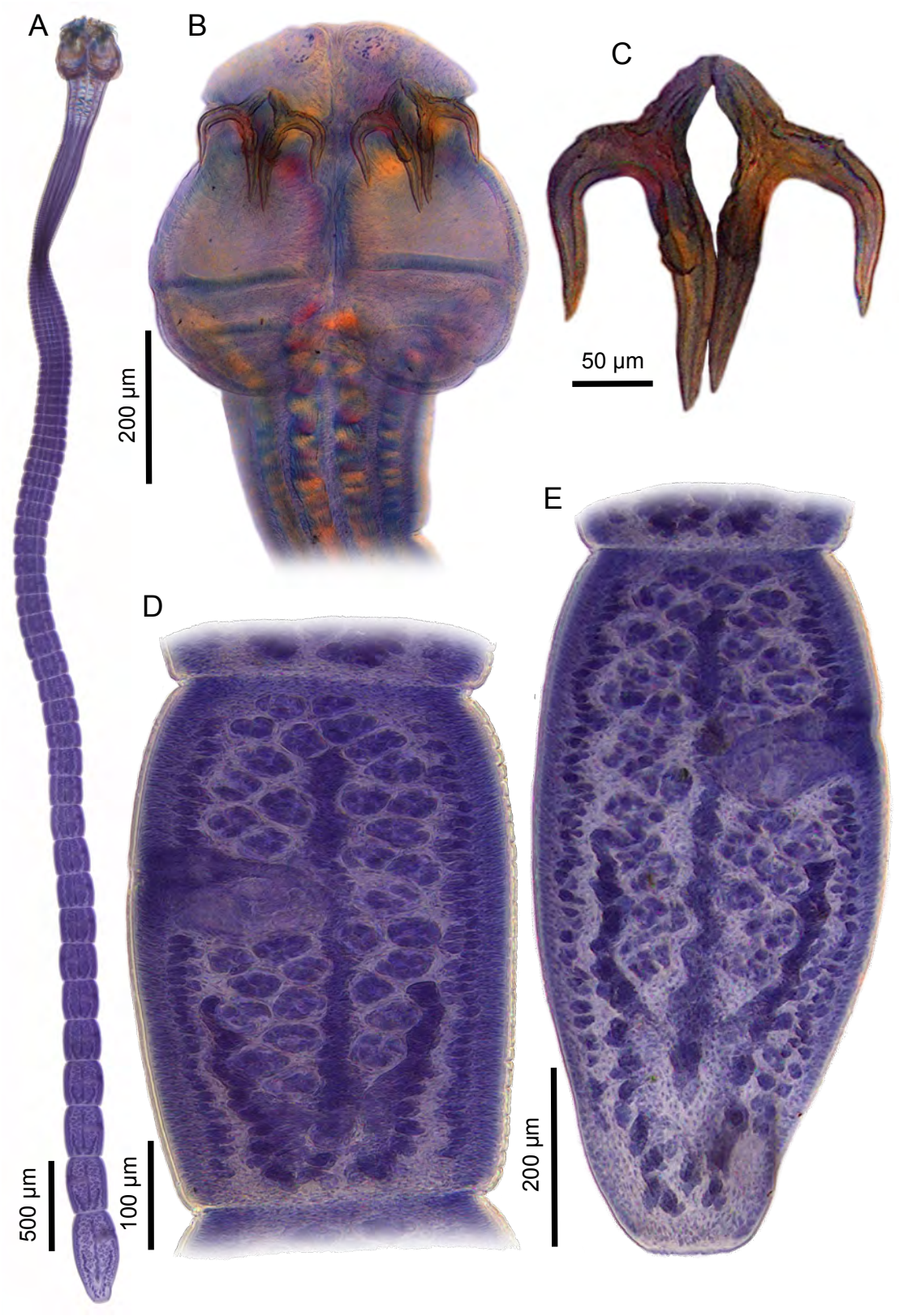

FIGURE 12. Light micrograph of *Acanthobothrium* n. sp. 6 from *Himantura pacifica* (Beebe & Tee-Van). A. whole worm; B. scolex; C. hooks; D. subterminal mature proglottid; E. terminal mature proglottid.

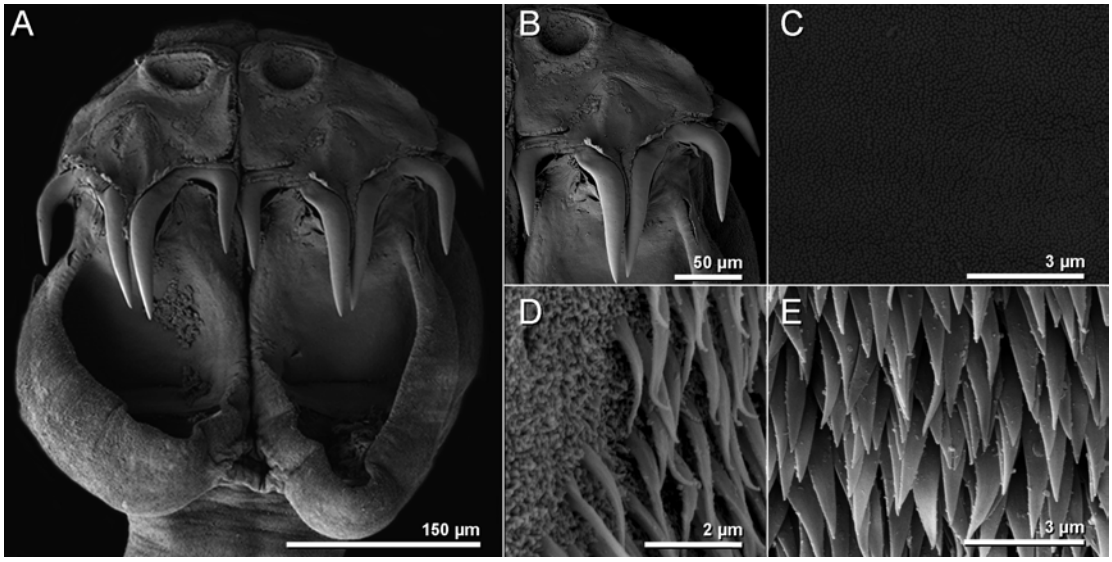

FIGURE 13. Scanning electron micrographs of *Acanthobothrium* n. sp. 6. A. scolex; B. detail of hooks and apical pad; C. surface of distal bothridia; D. surface of lateral margin from anterior loculus; E. cephalic peduncle.

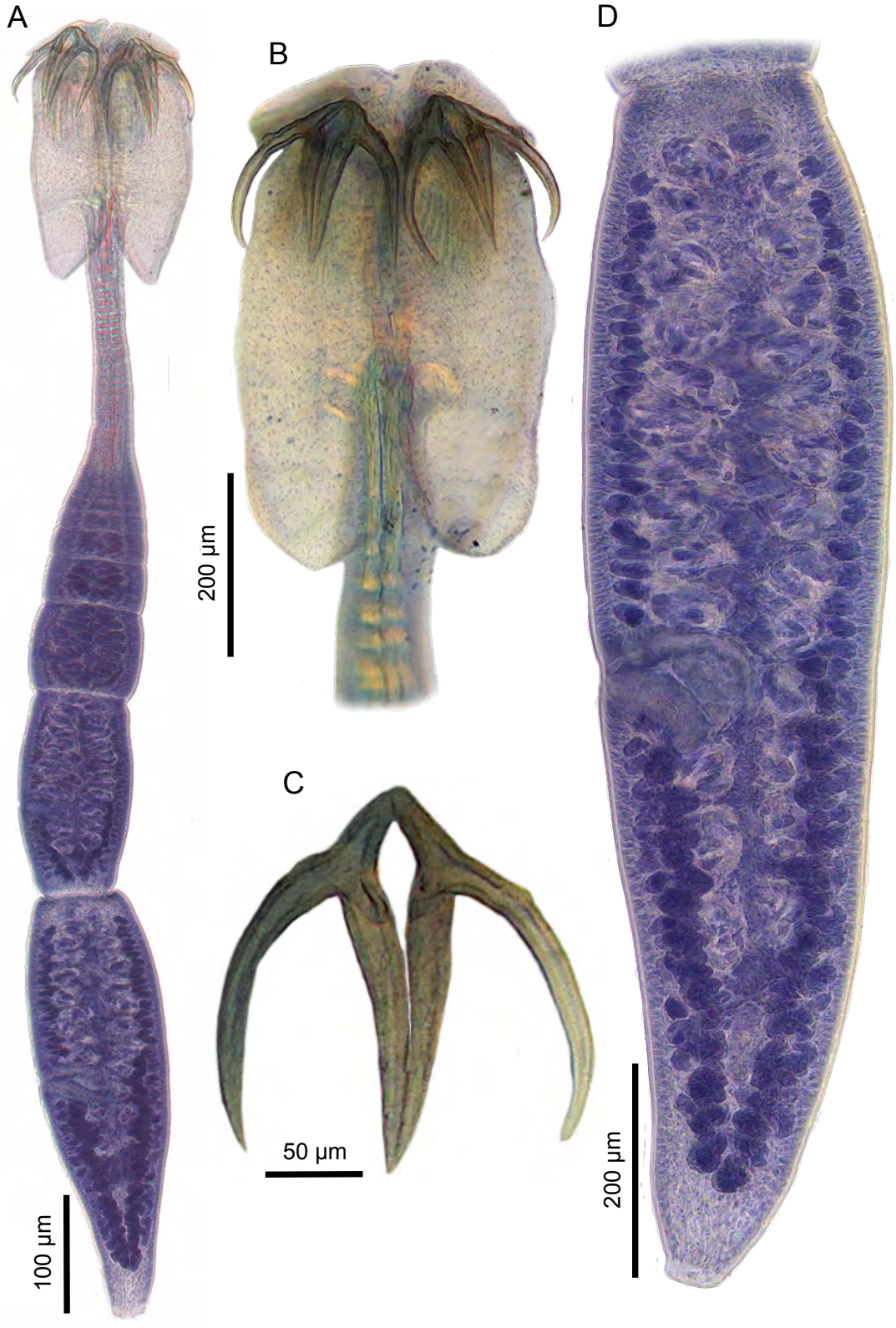

FIGURE 14. Light micrograph of *Acanthobothrium* n. sp. 7 from *Himantura pacifica* (Beebe & Tee-Van). A. whole worm; B. scolex; C. hooks; D. terminal mature proglottid.

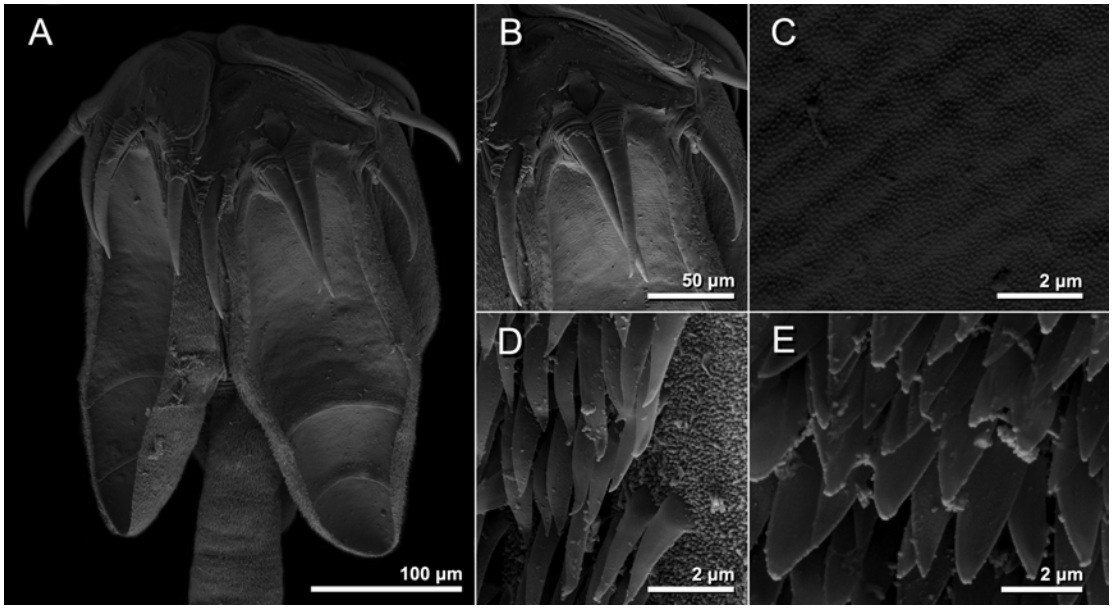

FIGURE 15. Scanning electron micrographs of *Acanthobothrium* n. sp. 7. A. scolex; B. detail of hooks and apical pad; C. surface of distal bothridia; D. surface of lateral margin from anterior loculus; E. cephalic peduncle.

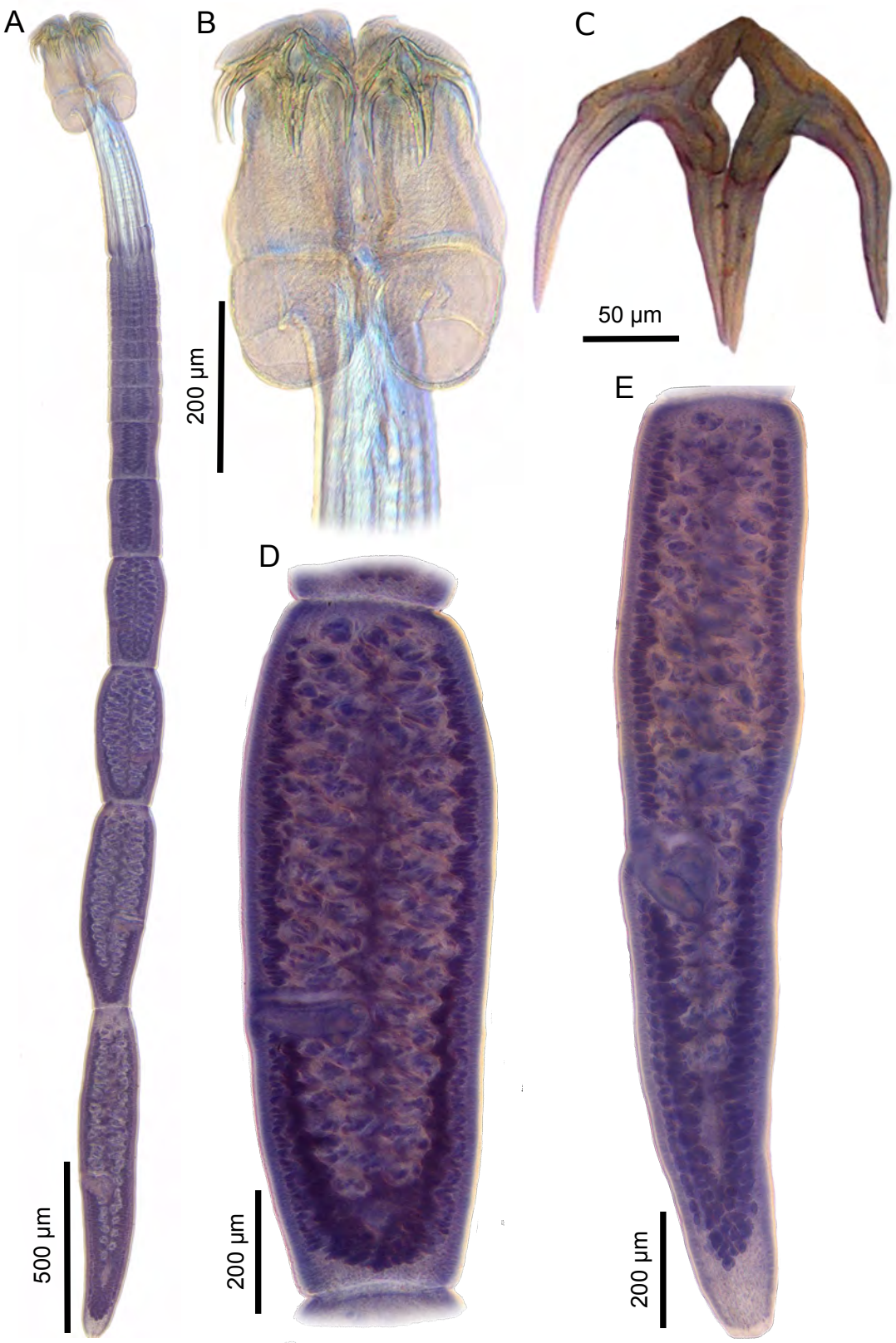

FIGURE 16. Light micrograph of *Acanthobothrium* n. sp. 8 from *Himantura pacifica* (Beebe & Tee-Van). A. whole worm; B. scolex; C. hooks; D. subterminal mature proglottid; E. terminal mature proglottid.

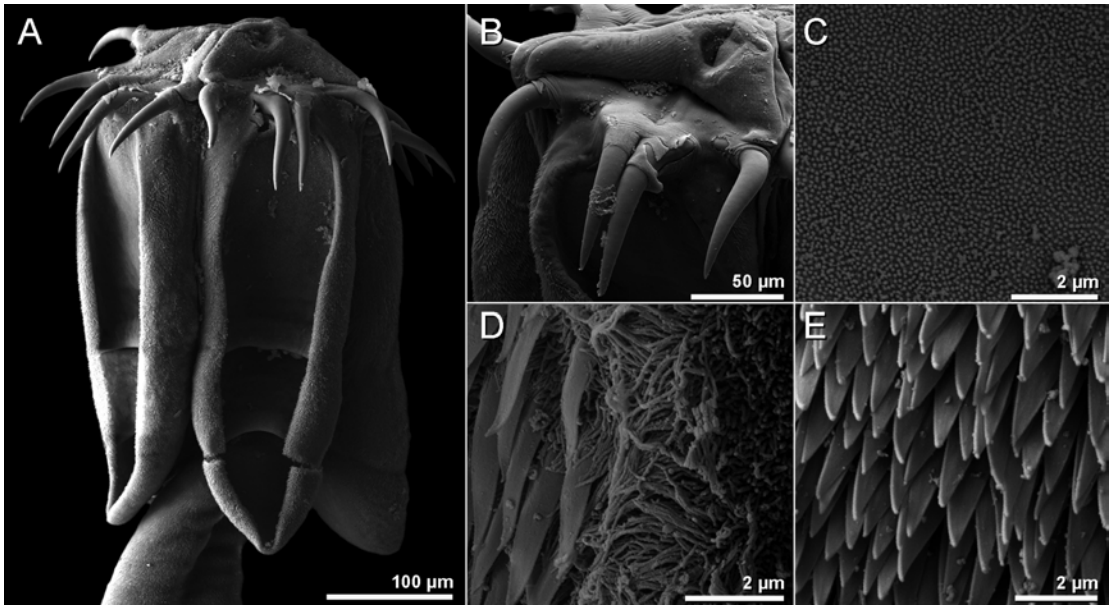

FIGURE 17. Scanning electron micrographs of *Acanthobothrium* n. sp. 8. A. scolex; B. detail of hooks and apical pad; C. surface of distal bothridia; D. surface of lateral margin from anterior loculus; E. cephalic peduncle.

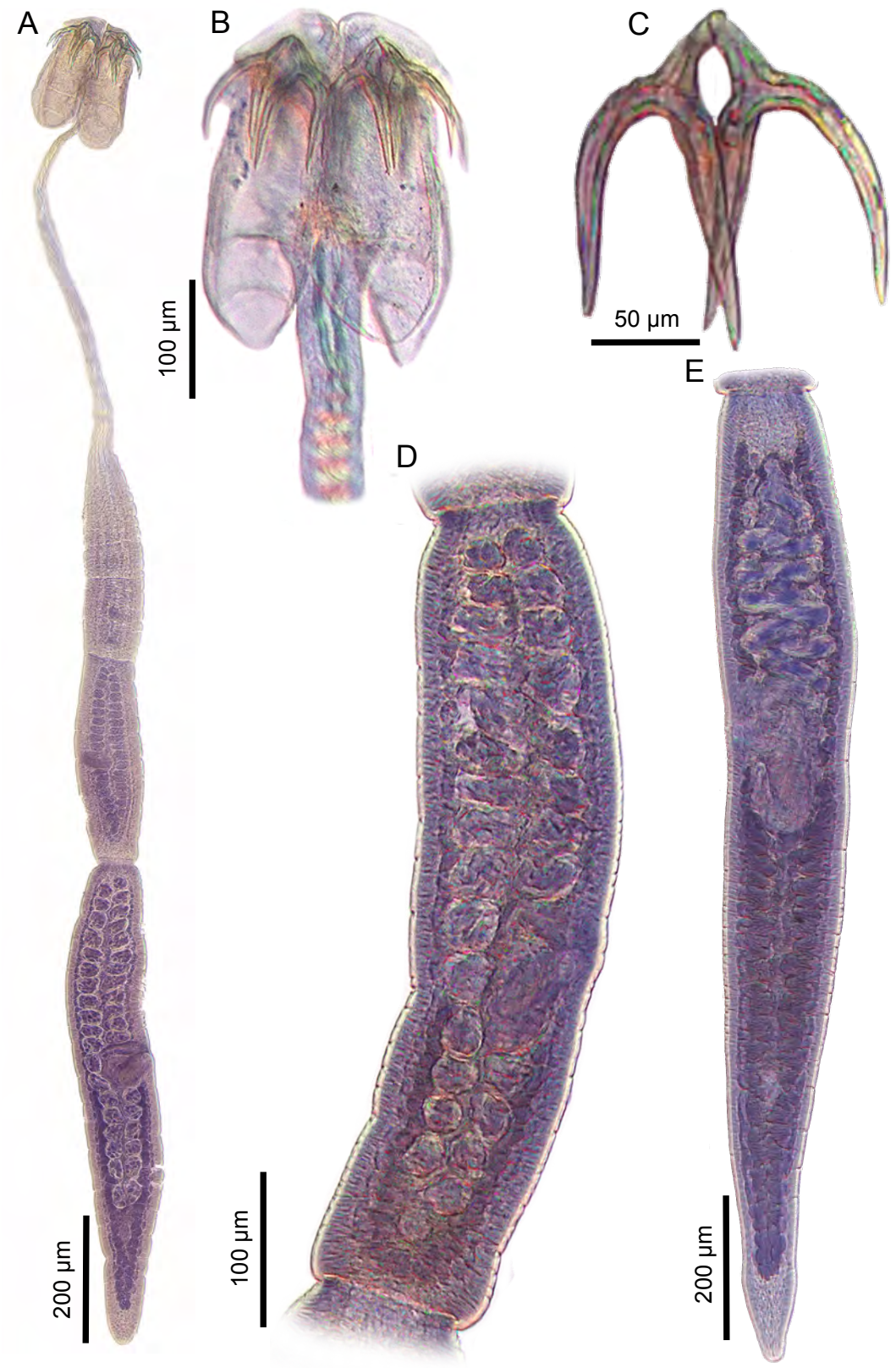

FIGURE 18. Light micrograph of *Acanthobothrium* n. sp. 9 from *Himantura schmardae* (Werner). A. whole worm; B. scolex; C. hooks; D. subterminal mature proglottid; E. terminal mature proglottid.

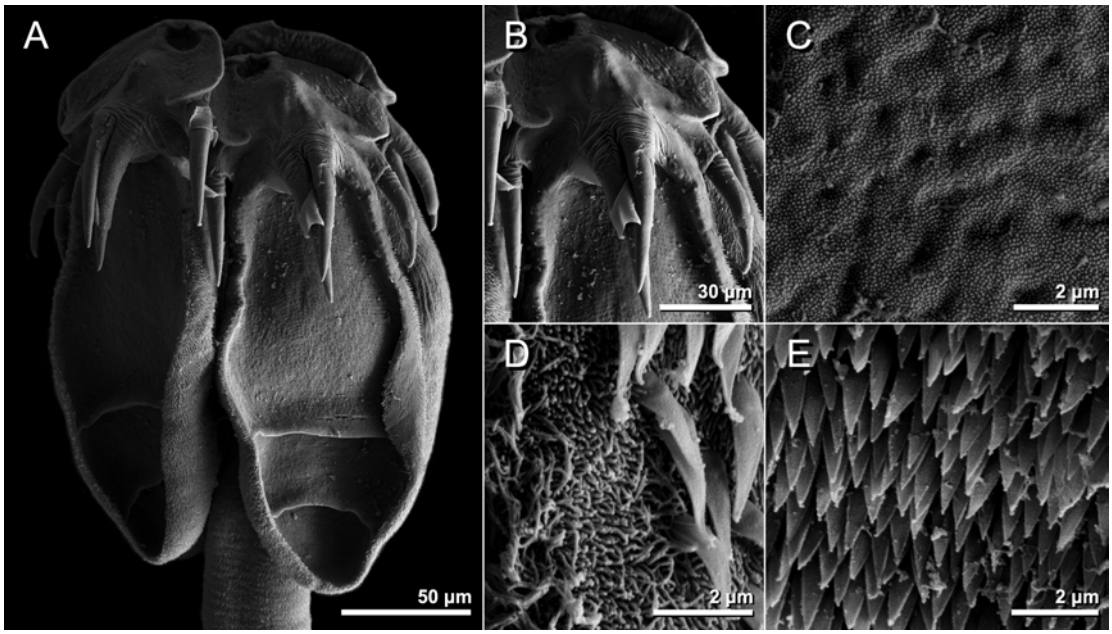

FIGURE 19. Scanning electron micrographs of *Acanthobothrium* n. sp. 9. A. scolex; B. detail of hooks and apical pad; C. surface of distal bothridia; D. surface of lateral margin from anterior loculus; E. cephalic peduncle.

Considerações Finais

Esta dissertação teve como objetivo principal identificar as linhagens de parasitas marinhos das espécies anfi-Americanas de *Himantura*, pertencentes a três gêneros: *Rhinebothrium* (Cap. 1), *Anindobothrium* (Cap. 2) e *Acanthobothrium* (Cap. 3). A escolha destes gêneros reside no fato de que eles também ocorrem nas arraias de água doce (Potamotrygonidae) e que potencialmente podem conter grupos-irmãos de linhagens que hoje residem em potamotrigonídeos. Para tanto, analisamos a fauna dos parasitas de *Himantura schmardae* e *H. pacifica* com o intuito de fornecer um refinamento taxonômico para estudos que visem esclarecer a evolução dos potamotrigonídeos e seus parasitas. Neste sentido, além de redescrever espécies com o uso de ferramentas taxonômicas modernas, buscou-se investigar a diversidade de parasitas marinhos claramente subestimada para estes hospedeiros. Tal suspeita residia principalmente no fato de que é comum identificar pares de espécies gêmeas de cada lado do istmo do Panamá. Essa principal hipótese de que a diversidade de parasitas para as espécies anfi-Americanas de *Himantura* estava subestimada foi corroborada pelos resultados apresentados nos três capítulos. Além da re-descrição de três espécies (*Acanthobothrium himanturi*, *Anindobothrium anacolum* e *Rhinebothrium tetralobatum*) parasitas de *H. schmardae*, foram reconhecidas 11 novas espécies, das quais sete são parasitas deste mesmo hospedeiro (*Anindobothrium inexpectatum* n. sp.; *Acanthobothrium* n. sp. 1, 2, 3, 5, 9 e *Rhinebothrium reydai* n. sp.) e quatro parasitas de *H. pacifica* (*Anindobothrium carioni* n. sp.; *Acanthobothrium* n. sp. 6, 7, 8).

O primeiro capítulo consistiu em avaliar a fauna de *Rhinebothrium*. Além da redescrição de *Rhinebothrium tetralobatum* que teve seu tipo descrito como parasita de *H. schmardae*, era esperado encontrar ao menos uma espécie nova para este gênero parasitando *H. pacifica*. Entretanto, os resultados foram surpreendentes. Primeiro pelo fato de que nenhuma espécie foi encontrada

parasitando *H. pacifica* (os 11 exemplares foram coletados na mesma localidade), e segundo pelo fato de que as espécies reconhecidas parasitando *H. schmardae* no mar do caribe parecem ter distribuição restrita às costas da Colombia e Panamá (*R. tetralobatum* e *R. reydai* n. sp., respectivamente). Ambos os resultados conduzem à hipotese de que a distribuição desses parasitas é heterogênea ao longo do registro de ocorrência dos hospedeiros, e que seria necessário determinar a curva de acumulação de espécies para se ter certeza de que toda a diversidade de parasitas foi amostrada. Considerando-se que em um estudo de co-evolução é imprescindível conhecer a fauna dos parasitas para correlacioná-la com a fauna dos hospedeiros conclui-se que seja necessário direcionar esforços no sentido de ampliar a amostragem. Isto porque, mais do que coletar um grande número de animais de uma mesma localidade seria preciso amostrar um número de hospedeiros ideal para cada localidade (baseado na sua curva de acumulação de espécies de parasitas), e considerar que se tenha amostrado este hospedeiro ao longo de sua distribuição geográfica.

O capítulo 2, além de avaliar a diversidade de *Anindobothrium*, identificou, ainda, o posicionamento filogenético deste gênero dentro da ordem Rhinebothriidea. Os resultados permitiram propor uma nova família de Rhinebothriidae para acomodar *Anindobothrium*. Anindobothriidae n. fam. possui suporte dentro de Rhinebothriidea assim como todas as famílias que fazem parte desta ordem. Entretanto, há instabilidade do arranjo interno desses clados, o que faz com que seu posicionamento mude de acordo com o critério de otimalidade utilizado. A estabilidade das famílias dentro de Rhinebothriidea só será alcançada com estudos que contemplem dados adicionais. A diversidade do gênero *Anindobothrium* contraria o que foi relatado para o gênero *Rhinebothrium* (Cap. 1) no Oceano Pacífico, mas corrobora os resultados para o Oceano Atlântico. Diferentemente do observado em relação a *Rhinebothrium*, foi reconhecida uma espécie de *Anindobothrium* parasita de *H. pacifica* (*A.*

carioni n. sp.), o que era esperado, haja vista que a espécie tipo (*A. anacolum*) foi descrita parasitando *H. schmardae* na Colômbia. Entretanto, assim como apresentado no Cap. 1, as duas espécies de *Anindobothrium* do mar do Caribe também aparentam ter distribuição restrita (*A. anacolum* – Colômbia e Trinidade & Tobago; e *A. inexpectatum* n. sp. – Belize e Panamá), o que sugere uma distribuição heterogênea do parasita ao longo da ocorrência do hospedeiro. No contexto taxonômico, as três espécies marinhas de *Anindobothrium* foram reconhecidas molecularmente, entretanto, não possuem um caráter diagnóstico que as diferencie morfologicamente. Neste sentido foi feita uma análise discriminante com os dados morfológicos, a fim de diferenciar essas espécies, a qual forneceu suporte para a identificação morfológica das mesmas como linhagens independentes. Estes resultados demonstram que o uso de métodos não tradicionais pode ser uma ferramenta útil em taxonomia, em concordância com o conceito atual de taxonomia integrativa.

O terceiro e último capítulo avaliou a fauna do gênero *Acanthobothrium* e forneceu, pela primeira vez, uma hipótese para a posição filogenética de suas linhagens. Foram descritas oito novas espécies, das quais cinco são parasitas de *H. schmardae* e três de *H. pacifica*. O fato de *H. pacifica* ter um menor número de espécies remete novamente ao que foi discutido no Cap. 1, para *Rhinebothrium*, e no Cap. 2, para *Anindobothrium*, no que concerne à distribuição heterogênea dos parasitas ao longo das regiões de ocorrência do hospedeiro. A hipótese filogenética criada a partir dos dados moleculares evidencia dois clados de parasitas de *H. schmardae* (*Acanthobothroides* sp. – que deverá ser transferido para *Acanthobothrium*, e *Acanthobothrium* n. sp. 9) como grupos-irmãos de dois clados representados por parasitas de potamotrigonídeos. Este resultado corrobora a hipótese mais recente sobre a derivação dos potamotrigonídeos, a qual também é evidenciada pelo Cap. 2 (*A. lisae* grupo-irmão das demais espécies marinhas de *Anindobothrium*).

Os resultados apresentados nessa dissertação indicam que a diversidade de parasitas de *Anindobothrium*, *Rhinebothrium* e *Acanthobothrium* estava subestimada. Correlacionado a esta constatação, este estudo também revela a importância da representatividade biogeográfica na documentação da fauna parasitária diante da aparente heterogeneidade apresentada na distribuição destas linhagens ao longo da distribuição do hospedeiro. Esta constatação é via de regra ignorada em estudos direcionados à elucidar associações históricas entre parasitas e hospedeiros e a qual deve ser considerada futuramente. Nossos resultados evidenciam, adicionalmente, a importância do uso da taxonomia integrativa na descrição de novas espécies, haja vista que algumas espécies reconhecidas neste estudo não seriam identificadas por métodos tradicionalmente utilizados na taxonomia destes grupos. As hipóteses filogenéticas apresentadas corroboram a próxima relação entre os parasitas das arraias anfi-Americanas de *Himantura* e os potamotrigonídeos e consequentemente a hipótese mais recente sobre a derivação das arraias de água doce. Finalmente, fica evidenciado a presença de algumas lacunas que deverão ser preenchidas no futuro, dentre elas a documentação mais detalhada de outros hospedeiros que residem na suposta área de derivação dos potamotrigonídeos dando ênfase na representatividade biogeográfica desta região, especialmente para o Pacífico oriental.

Resumo

Estudos co-evolutivos requerem uma base taxonômica e filogenética robusta para estabelecerem de forma inequívoca as relações entre as linhagens envolvidas. Neste sentido, o presente estudo identificou as linhagens de parasitas marinhos das espécies anfi-Americanas de *Himantura* Müller & Henle, considerado o suposto grupo-irmão dos potamotrigonídeos - arraias Neotropicais restritas ao sistemas fluviais da America do Sul. O objetivo foi contribuir com o alicerce taxonômico necessário para a elucidar as associações históricas entre as arraias de água doce, seu suposto grupo-irmão marinho, e suas faunas helmintológicas. Neste sentido, foi abordada a diversidade de três gêneros de cestóideos, cujas linhagens são compartilhadas entre arraias marinhas e potamotrigonídeos: *Acanthobothrium* Blanchard, 1948, *Anindobothrium* Marques, Brooks & Lasso, 2001 e *Rhinebothrium* Linton, 1890. Cada um destes grupos é abordado em um capítulo individualmente. Os resultados deste estudo incluem a descrição de 11 espécies novas, dentre as quais, sete são parasitas de *H. schmardae* (Werner) e 4 parasitas de *H. pacifica* (Beebe & Tee-Van), além da redescrição de três espécies previamente conhecidas para *H. schmardae*. Todas as descrições e redescrições foram baseadas em um número de indivíduos sem precedentes na taxonomia dos grupos e incluíram dados sobre microscopia eletrônica de varredura dentro do padrões atuais de descrições taxonômicas. Também foram abordadas as relações filogenéticas das linhagens de *Acanthobothrium* e *Anindobothrium*. Em ambos os casos, dados moleculares revelam congruência entre as relações de parentesco de seus membros e aqueles evidenciados para seus hospedeiros. Os dados parasitológicos apresentados corroboram hipóteses recentes que postulam que potamotrigonídeos compartilham um ancestral comum com as linhagens anfi-Americanas de *Himantura*. Por fim, algumas abordagens utilizadas ilustram os benefícios de integrar diferentes bases de dados no refinamento taxonômico destes grupos dentro do conceito do que hoje reconhecemos como taxonomia integrativa.

Abstract

Studies on the co-evolution require accurate taxonomic and phylogenetic information to unambiguously establish associations within the lineages involved. Therefore, the present study identified marine parasite lineages from amphi-American species of *Himantura* Müller & Henle, *H. schmardae* (Werner) and *H. pacifica* (Beebe & Tee-Van). These hosts are considered the sister-group of potamotrygonids, which are Neotropical freshwater stingrays restricted to river systems in South America. Our motivation was the contribution on sound taxonomic grounds, in order to elucidate the historical associations among freshwater batoids, their alleged marine sister-group and their cestode parasites. To achieve this goal, we documented the fauna of three genera of cestodes, whose lineages can be found both in marine and freshwater stingrays, namely *Acanthobothrium* Blanchard, 1948, *Anindobothrium* Marques, Brooks & Lasso, 2001 and *Rhinebothrium* Linton, 1890. Each chapter addresses each genus separately. Our results consist of descriptions of 11 species new to science, among which seven are found parasitizing *H. schmardae* and 4 infecting *H. pacifica*. Furthermore, redescriptions are provided for three species detected in *H. schmardae*. All descriptions and redescriptions were based on an unprecedented number of specimens and included data obtained from histology, ligth microscopy and scanning electron microscopy. In addition to the taxonomic approach, we evaluated the phylogenetic relationships of *Acanthobothrium* and *Anindobothrium*. Molecular data from both genera revealed the congruence between the known patterns of host relationships and their parasites. The parasitological data presented in this study supports the recent hypothesis that potamotrygonids and amphi-American species of *Himantura* share a common ancestor. Moreover, the combined approach applied in this study illustrates the benefits of integrating different data sources for the taxonomic refinement of these groups within the concept of integrative taxonomy.

Biografia

Bruna Trevisan Souza se formou em bacharel em Ciências Biológicas com habilitação em Biologia Marinha no ano de 2012 pela Universidade Estadual Paulista “Júlio de Mesquita Filho” - Campus do Litoral Paulista. Durante a graduação foi bolsista de iniciação científica da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP - IC), ao desenvolver um projeto sobre crescimento de anomuros no laboratório de crustáceos (CRUSTA) sob orientação do prof. Dr. Marcelo A. A. Pinheiro (novembro/2010 a outubro/2011). No ano de 2012, realizou estágio na Secretaria do Verde e Meio Ambiente da Prefeitura de São Paulo, na divisão de Fauna (DEPAVE–3), onde desenvolveu atividades de manejo, reabilitação, nutrição e reintrodução de animais silvestres. Em 2013 foi técnica do laboratório de Helmintologia Evolutiva (LHE) do departamento de Zoologia no Instituto de Biociências da Universidade de São Paulo (com bolsa FAPESP - TT–3). Em janeiro de 2014 ingressou na pós-graduação no mesmo laboratório, sob orientaçãoo do Prof. Dr. Fernando P. de L. Marques.