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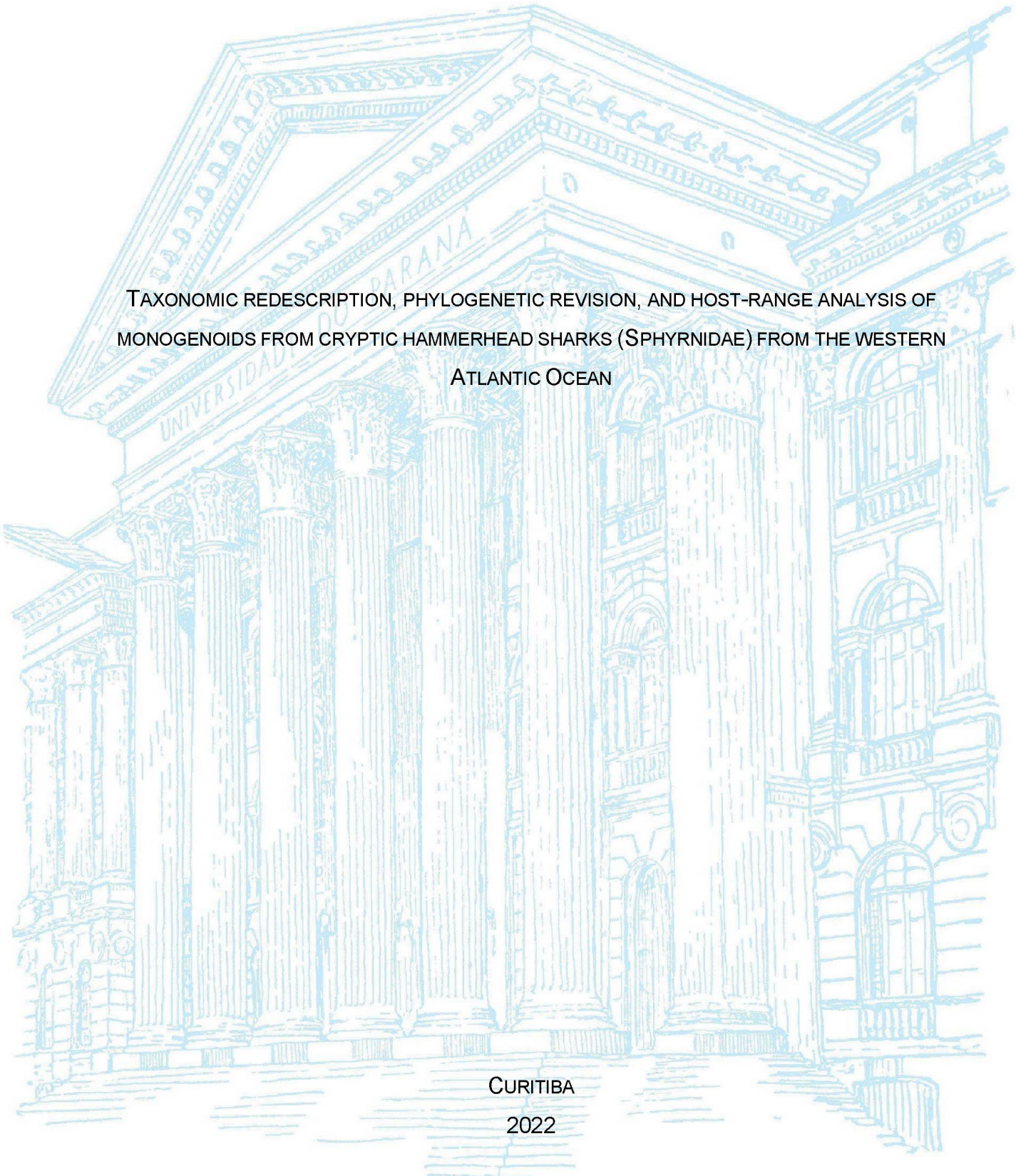
KAITLYN MARIE DALRYMPLE



TAXONOMIC REDESCRIPTION, PHYLOGENETIC REVISION, AND HOST-RANGE ANALYSIS OF MONOGENOIDS FROM CRYPTIC HAMMERHEAD SHARKS (SPHYRNIDAE) FROM THE WESTERN ATLANTIC OCEAN

CURITIBA

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Dissertação apresentada ao curso de Pós-Graduação em Ecologia e Conservação, Setor de Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Ecologia e Conservação.

Orientador: Prof. Dr. Walter A. Boeger

Coorientador: Dr. Emanuel L. Razzolini

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ECOLOGIA E CONSERVAÇÃO da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **KAITLYN MARIE DALRYMPLE** intitulada: **TAXONOMIC REDESCRIPTION, PHYLOGENETIC REVISION, AND HOST-RANGE ANALYSIS OF MONOGENOIDS FROM CRYPTIC HAMMERHEAD SHARKS (SPHYRNIDAE) FROM THE WESTERN ATLANTIC OCEAN**, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
A outorga do título de mestra está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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JOSE LUIS FERNANDO LUQUE ALEJOS
Avaliador Externo (UNIVERSIDADE FEDERAL RURAL DO RIO DE JANEIRO)

Assinatura Eletrônica
30/09/2022 13:59:06.0
STEPHEN ASH BULLARD
Avaliador Externo (AUBURN UNIVERSITY)

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Everything in nature depends on everything else, so it's interconnected. And so the many many patterns of interconnections lock it all together into a unity, which is much too complicated for us to think about, except in very simple, crude ways… In the same way that you see a flower in the field, it's really the whole field that is flowering, because the flower couldn't exist in that particular place without the special surroundings of the field that it has. You only find flowers in surroundings which will support them. (Alan Watts,1971, "*Conversations with Myself*")

RESUMO

Estima-se que menos de 10% da biodiversidade total da Terra seja conhecida e que os parasitos representem pelo menos um terço da biodiversidade global. Métodos moleculares modernos têm descoberto muitas espécies crípticas, o que significa que os estudos da fauna parasitária dessas espécies permanecem inexistentes ou incompletos. Este trabalho determinou e redescreveu monogenóides parasitas de brânquias de neonatos de duas espécies crípticas de *Sphyrna* spp. (Sphyrnidae) e seus híbridos da costa leste do Oceano Atlântico Norte dos EUA. Duas espécies de *Erpocotyle* Van Beneden & Hesse, 1863 (Hexabothriidae Price, 1942) infectaram os três tipos de hospedeiros. Uma filogenia atualizada da família construída usando sequências de 18S e 28S rDNA produzidas a partir deste estudo e obtidas do GenBank, e em combinação com dados morfológicos de Boeger & Kritsky (1989), produziu uma nova hipótese filogenética da família sendo composta por 4 a 5 clados. Outra espécie monogenóide, *Loimosina wilsoni* Manter, 1944 (Monocotylidae Taschenberg, 1879) foi encontrada restrita apenas a *S. lewini* e seus híbridos. As sequências de 28S coincidiram com as de *Loimosina* sp. do litoral sul do Brasil, e *L. parawilsoni* Bravo-Hollis, 1970 foi proposto como sinônimo júnior com base em uma análise morfológica limitada. Nas considerações finais, uma breve análise dos repertórios de hospedeiros da família é discutida usando uma filogenia baseada nas sequências 28S e otimização de Liebermann. Propõe-se que *Loimosina* tenha uma gama de hospedeiros restrita devido a um efeito fundador ancestral, explicado pelo ciclo taxon-pulse, um componente do Paradigma de Estocolmo.

Palavras-chave: Monocotylidae, Hexabothriidae, monogenóides, repertório de hospedeiros, taxonomia, Sphyrnidae, Paradigma de Estocolmo

ABSTRACT

It is estimated that less than 10% of Earth's total biodiversity is known, and that parasites represent at least a third of overall global biodiversity. Modern molecular methods have uncovered many cryptic species, which means that studies of the parasitic fauna of these species remain inexistent or incomplete. This work determined and redescribed parasitic monogenoids of gills of neonates of two cryptic *Sphyrna* spp. (Sphyrnidae) and their hybrids from the eastern North Atlantic Ocean coast of the USA. Two species of *Erpocotyle* Van Beneden & Hesse, 1863 (Hexabothriidae Price, 1942) infected all three types of hosts. An updated phylogeny of the family constructed using sequences of 18S and 28S rDNA produced from this study and obtained from GenBank, and in combination with morphological data from Boeger & Kritsky (1989), produced a new phylogenetic hypothesis of the family being composed of 4 to 5 clades. Another monogenoid species, *Loimosina wilsoni* Manter, 1944 (Monocotylidae Taschenberg, 1879) was found to be restricted to only *S. lewini* and the hybrids. Sequences of 28S matched those of *Loimosina* sp. from the southern coast of Brazil, and *L. parawilsoni* Bravo-Hollis, 1970 was proposed as a junior synonym based on a limited morphological analysis. In the final remarks, a brief analysis of host repertoires in the family is discussed using a phylogeny based on the 28S sequences and Liebermann optimization. It is proposed that *Loimosina* has a restricted host-range due to an ancestral Founder effect, explained by the taxon-pulse cycle, a component of the Stockholm Paradigm.

Key words: Monocotylidae, Hexabothriidae, monogenoids, host repertoire, taxonomy, Sphyrnidae, Stockholm Paradigm

OUTLINE

1 INTRODUCTION

It has been almost 300 years since the "father of modern taxonomy" Carl Linneaus first published his Systema Naturae, wherein the process of formally classifying organisms based on their morphological characteristics became a field of major interest. Since then, about 1.7 million species have been named, mostly insects, then plants, then other organisms, yet most experts agree that this likely represents less than 10% of Earth's total biodiversity. For parasites, like most microorganisms that reproduce rapidly and in large numbers, rapid evolutionary rates drive greater speciation, leading to estimations of parasites comprising at least a third of overall global biodiversity (see Dobson *et al.,* 2008 for a synthesis)

Biodiversity, a measure of richness and abundance of species, is a result of responses to density-dependent factors (e.g., competition between species or populations) and environmental filtering, or the effect local environmental conditions have on growth and survival, or even in response to neutral processes such as stochastic extinction events, or by random opportunity. For example, migratory species such as ungulates, which travel long-distance in response to seasonal changes in food availability, often have greater diversity in parasite fauna than in non-migratory species (Teitelbaum *et al.,* 2018). Not only do the parasites experience optimal environmental conditions for transmission year-round, they also are provided more opportunities for transmission given their social and migratory hosts. This ability to explore and exploit novel resources is known as ecological fitting (Agosta & Klemens, 2008), and can often lead to more rapid speciation and diversification.

If there truly are many more species yet undiscovered and thus understudied, this leaves us with the possibility of millions more undiscovered species of parasites, and this affects everything from taxonomy to phylogenetic placements for the species that we currently do know. With more recent advances in technology, especially in the use of molecular methods such as DNA barcoding and high-throughput sequencing, the resolution in identification and classification of species within taxonomic and phylogenetic groups has improved exponentially. Most significantly is the ability to identify cryptic species or the development of distinct lineages within populations, a feat not quite achievable using traditional morphological identification techniques alone (Poulin, 2014). Genetic markers such as mitochondrial cytochrome *c* oxidase 1 (COI) are frequently used for species delimitation, due to its greater resolution of the

delineation between closely related species (Hebert *et al.* 2003a, 2003b). COI is certainly not the only marker that suits this purpose, however, improvements in species delimitation methods have led to the discovery of many hidden, cryptic species, which could be harboring their own specific fauna of parasites.

This includes the case of the Carolina hammerhead (*Sphyrna gilberti* Quattro *et al.*, 2013) and the scalloped hammerhead (*S. lewini* (Griffith & Smith, 1834)) (Sphyrnidae), where the present study begins. These cryptic species were delimited in 2012 (Pinhal *et al.,* 2012), and the former was formally described by Quattro *et al.* (2013). Being recently described, and given the difficult nature of obtaining charismatic megafauna such as hammerhead sharks for research, data on parasites of *S. gilberti* is nonexistent. However, the present work was given the opportunity to perform such a study of parasitic fauna of this species. In the following chapters, we identified parasitic monogenoids molecularly and morphologically from the gills of these two *Sphyrna* spp., analyzed the phylogenies using maximum evidence datasets, and discussed the host repertoires of the parasites found. We conclude our work with suggested hypotheses for future work in this area.

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3 CHAPTER I: HEXABOTHRIIDAE AND MONOCOTYLIDAE (MONOGENOIDEA) FROM THE GILLS OF NEONATE HAMMERHEAD SHARKS (SPHYRNIDAE) *SPHYRNA GILBERTI, SPHYRNA LEWINI*, AND THEIR HYBRIDS FROM THE WESTERN NORTH ATLANTIC OCEAN

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HEXABOTHRIIDAE AND MONOCOTYLIDAE (MONOGENOIDEA) FROM THE GILLS OF NEONATE HAMMERHEAD SHARKS (SPHYRNIDAE) *SPHYRNA GILBERTI, SPHYRNA LEWINI*, AND THEIR HYBRIDS FROM THE WESTERN NORTH ATLANTIC OCEAN

ABSTRACT

Neonates of hammerhead sharks (Sphyrnidae), *Sphyrna lewini* (Griffith and Smith, 1834), the sympatric cryptic species, *Sphyrna gilberti* Quattro et al., 2013, and their hybrids were captured in the western North Atlantic, along the coast of South Carolina, USA, between 2018 and 2019 and examined for gill monogenoids. Parasites were identified and redescribed from the gills of 79 neonates, and DNA sequences from partial fragments of the nuclear 28S ribosomal RNA (rDNA) and cytochrome c oxidase I mitochondrial DNA (COI) genes were generated to confirm species identifications. Three species of monogenoids from Hexabothriidae Price, 1942 and Monocotylidae Taschenberg, 1879 were determined and redescribed. Two species of Hexabothriidae, *Erpocotyle microstoma* (Brooks, 1934) and *Erpocotyle sphyrnae* (MacCallum, 1931), infecting both species of *Sphyrna* and hybrids; and 1 species of Monocotylidae, *Loimosina wilsoni* Manter, 1944, infecting only *S. lewini* and hybrids. *Loimosina wilsoni* 28S rDNA sequences matched those of *Loimosina* sp. from the southern coast of Brazil. Based on limited morphological analysis, *Loimosina parawilsoni* is likely a junior synonym of *L. wilsoni*. This is the first taxonomic study of monogenoids infecting *S. gilberti* and hybrids of *S. gilberti* and *S. lewini.*

Key words: Monocotylidae, Hexabothriidae, host repertoire, taxonomy, Sphyrnidae, Carolina hammerhead, scalloped hammerhead, hybrids

3.1 INTRODUCTION

Two cryptically similar and sister species of hammerhead sharks (Sphyrnidae), *Sphyrna lewini* (Griffith and Smith, 1834) and *Sphyrna gilberti* Quattro *et al.*, 2013 occur along the coast of South Carolina (SC), USA (Quattro *et al.,* 2013). These species are commonly known as the scalloped and Carolina hammerhead, respectively. They can be distinguished only by a difference in the number of precaudal vertebrae *(S. gilberti* has about 10 fewer than *S. lewini)* and genetic data. Their divergence was estimated to have occurred 4.5 million years ago (Pinhal *et al.*, 2012; Quattro *et al.,* 2013). In addition, these species are capable of hybridization (Barker *et al.,* 2019). Because of the morphological similarity, *S. gilberti* was described only recently from the Carolina coasts (South and North Carolina states, USA) in the western North Atlantic Ocean (Quattro *et al.,* 2013). Hence, knowledge about its biology and distribution is limited, and adults' range remains uncertain. However, specimens of *S. gilberti* have been found along the Atlantic coast of Florida (FL), prior to being officially recognized as a distinct species (Abercrombie *et al.*, 2005; Duncan *et al.,* 2006; Quattro *et al.,* 2006). Barker *et al.* (2021) showed that neonates of *S. gilberti* are most common along coastal nurseries in SC, with abundances decreasing latitudinally to be at its lowest in southern FL, and were not found in the Gulf of Mexico. Furthermore, at least 3 adult specimens of *S. gilberti* were captured in southeast Brazil (Pinhal *et al.*, 2012). Knowledge of the parasite fauna of *S. gilberti* is sparse. Presently, the only parasite known for the Carolina hammerhead is a nematode in the spiral valve (Moravec *et al.*, 2020), although it is possible that specimens of *S. gilberti* have been unknowingly included in previous parasitic fauna studies of *S. lewini* in geographic locales where both species occur, and vice versa.

By contrast, the scalloped hammerhead, *S. lewini,* has been formally recognized

as a species since the 19th century. It has a global range (Compagno, 1984). Five species of monogenoids were described from this shark. Two of these species are members of Hexabothriidae Price, 1942: *Erpocotyle microstoma* (Brooks, 1934), originally described in *Sphyrna zygaena* (Linnaeus, 1758) (type host) from the coast of Beaufort, North Carolina (NC), USA and reported in *S. lewini* from the western South Atlantic Ocean (Uruguay; Suriano and Labriola, 1998); and *Erpocotyle sphyrnae* (MacCallum, 1931), also described from *S. zygaena* off Woods Hole, Massachusetts (MA), USA and reported in *S. lewini* from the eastern North Atlantic Ocean (Senegal; Euzet and Maillard, 1967) and the Pacific Ocean (Hawaii, USA; Yamaguti, 1968). The other 3 species are members of Monocotylidae Taschenberg, 1879: *Cathariotrema selachii* (MacCallum, 1916), originally described from *S. zygaena* (exact locale unknown, but is likely off Woods Hole, MA – see Bullard *et al.*, 2021) and reported in *S. lewini* from the Northern Gulf of Mexico off Mississippi, Alabama and Louisiana in Bullard *et al.* (2021); *Loimosina wilsoni* Manter, 1944, originally described from *S. zygaena* from Montego Bay, Jamaica and reported in *S. lewini* from Alligator Harbor, FL, USA in Hargis (1955); and *Loimosina parawilsoni* Bravo-Hollis, 1970, described in *S. lewini* from the eastern tropical Pacific Ocean (Sinaloa, Mexico; Bravo-Hollis, 1970) (the latter 2 were formerly considered Loimoidae – see Boeger *et al.*, 2014).

In the present study, monogenoids infecting the gills of neo- nates of *S. gilberti*, *S. lewini* and their hybrids were identified, sequenced, illustrated and redescribed based on specimens collected from the western North Atlantic Ocean (SC, USA) and their types.

3.2 MATERIALS AND METHODS

3.2.1 Sampling and Collection

ETHICS STATEMENT:

All sharks were collected and sampled by authorized staff under official permits or scientific exemptions of U.S. state government agencies (SCDNR Scientific Permit no. 2212). A total of 87 neonates, all moribund upon capture, were collected. Because the shark species identification could not be determined at time of capture, the number of sharks sampled was necessary to gain a sufficient sample size for both species of hammerheads, *S. lewini* and *S. gilberti*, and their hybrids.

Sharks were captured using a 231 m long, 3 m deep gillnet with a stretched mesh of 10.3 cm in Bulls Bay, SC (Five Fathom Creek, 33.0095/-79.4853), a nursery area where both species of hammerhead and their hybrids are found in sympatry (Barker et al., 2019). Fresh carcasses were kept on ice, individually labeled, and fin clips from each specimen preserved in 20% salt-saturated DMSO and sent to the Marine Genomics Laboratory at Texas A&M University - Corpus Christi, USA, for molecular identification following the methods of Barker *et al.* (2019).

Monogenoids from each hammerhead specimen were identified before the host species identifications were determined. Gills were resected from each host within 10 h post-capture, flooded and shaken rapidly in hot water (68 °C) to relax, kill, and detach worms from the gill filaments. Some monogenoids were processed immediately – the haptor was fixed in 95% ethanol (EtOH) and the anterior end in 10% neutral buffer formalin (NBF) to generate hologenophores sensu Pleijel *et al.* (2008); the remaining specimens were fixed with either 10% NBF or 100% EtOH to obtain final concentrations of 5% NBF and 70% EtOH, respectively. Other hologenophores were

generated via some EtOH-fixed specimens by removing a small lateral part of their body.

3.2.2 Morphology

Parasites were stained with either acetocarmine or Gomori's trichrome (Humason, 1979) and mounted using Canada balsam or Permount Mounting Medium (Fisher Chemical, Fairlawn, New Jersey, USA). The haptor of some specimens were mounted separately in Hoyer's medium to examine sclerite and anchor morphology. Drawings were made using an Olympus BX50 differential interference contrast (DIC) compound microscope mounted with a camera lucida. Measurements of the haptoral hook-like sclerites and anchors were obtained using ImageJ software (www.nih.org) following measurements schemes of MacCallum (1931), Euzet & Maillard (1967), and Bullard & Dippenaar (2003) (Figs. 1A - E). Anchors were measured as indicated in Figure 2. Measurements are in micrometers; the range is presented followed by the average and the number of measured structures (n) in parentheses. Haptoral sucker pairs and their respective sclerites are numbered 1 - 3, with 1 being closest to the point of attachment for the haptoral appendix.

Vouchers and hologenophores are deposited at the National Museum of Natural History, Smithsonian Institution (USNM) in Washington D.C., USA, and the Harold W. Manter Laboratory of Parasitology (HWML) in Nebraska, USA.

For comparison, micrographs of the following specimens from USNM, HWML, and Nacional Collection of Helminths, Institute of Biology, National Autonomous University of Mexico (CNHE), Mexico (available at http://unibio.unam.mx) were examined: *E. microstoma* (syntypes: USNM 132155, HWML 1437), *E. sphyrnae* (syntypes: USNM 1320885 and USNM 1320884), *L. wilsoni* (syntypes: USNM

1337561, USNM 1337564, HWML 1425), and *L. parawilsoni* (holotype: CNHE 153, syntype CNHE 154).

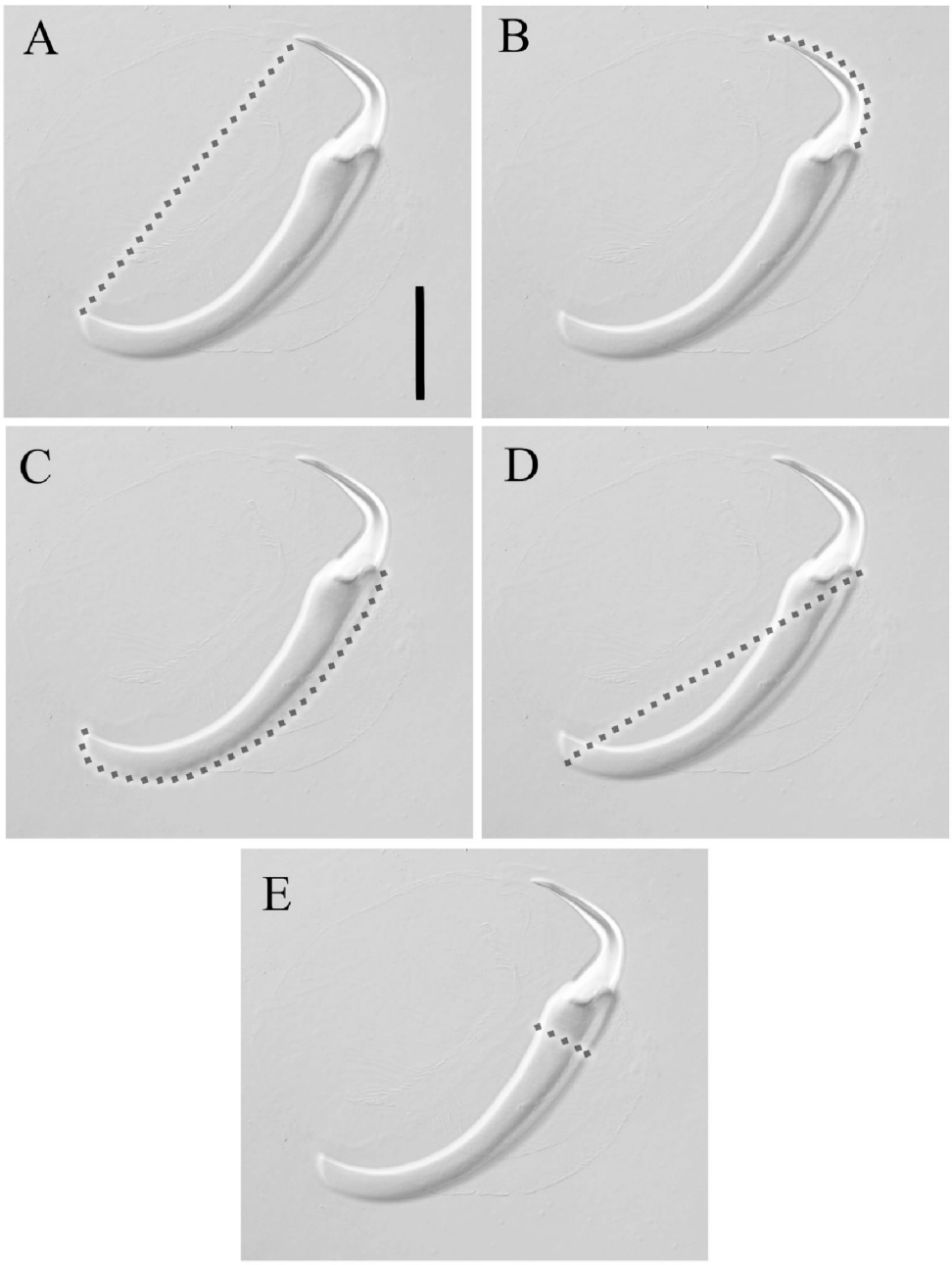


Figure 1: Measurements of sclerite curvature were taken using ImageJ software (www.nih.org): A) Tip-to-tip (MacCallum, 1931), B) Perimeter hook length (Euzet & Maillard, 1967), C) Perimeter shaft length (Euzet & Maillard, 1967), D) Shaft length (Bullard & Dippenaar, 2003), and E) Max shaft width (Bullard & Dippenaar, 2003). Scale bar 80 µm.

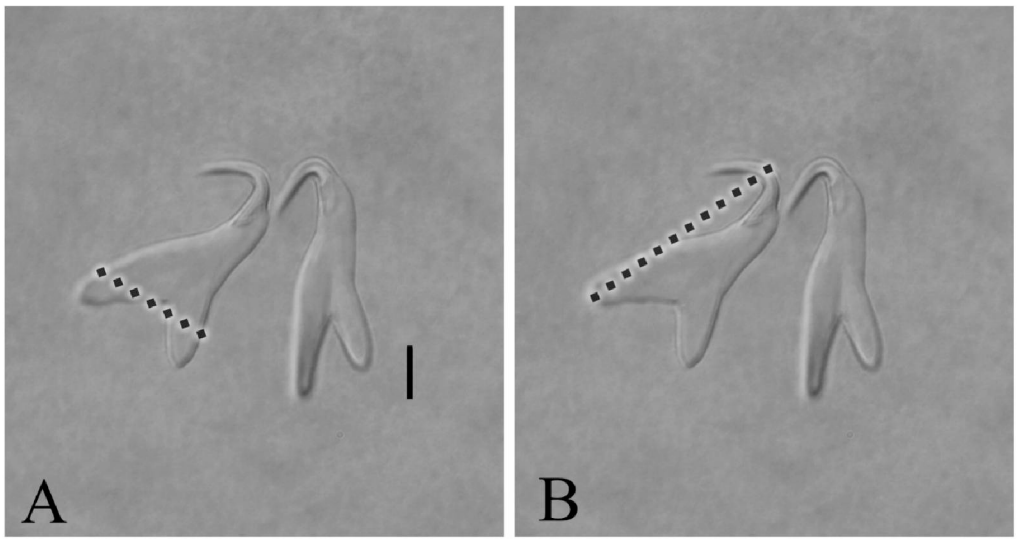


Figure 2: Width (A) and length (B) measurements of anchors of the haptoral appendix. Scale bar 13 µm.

3.2.3 Molecular Analyses

DNA was extracted from parasite tissue using a DNeasyBlood and Tissue kit (Qiagen, Valencia, California, USA) and manufacturer's protocol. Sequences of DNA from portions of the nuclear-encoded 28S ribosomal RNA (rDNA; 28S) and the mitochondrially encoded cytochrome *c* oxidase I (COI) genes were amplified and sequen- ced for species comparisons and confirmations. Primers LSU5 (5′-TAGGTCGACCCGCTGAAYTTAAGCA-3′; Jensen and Bullard, 2010) and 28S_ECD2 (5′-CTTGGTCCGTGTTTCAAGACGGG-3′; Tkach *et al.*, 2003) were used to amplify a1100 base pair portion of the 28S gene: a 25 µL total volume reaction contained 1× polymerase chain reaction (PCR) buffer (Promega, Madison, Wisconsin, USA), 3 mM $MgCl_2$, 0.4 mM deoxynucleotide triphosphates (dNTPs) (Promega), 0.4× Rediload gel loading buffer (Invitrogen, Waltham, MA, USA), 0.6 µM of each primer, 1 U GoTaq® Flexi DNA Polymerase (Promega) and 3 µL template DNA. Cycling was as follows: 5 min initial denaturation at 94°C, followed by 40 cycles

of denaturation at 94°C for 45 s, annealing at 62°C for 45 s, exten- sion at 72°C for 45 s, then a final extension at 72°C for 5 min. Primers JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAACATAATGAAAATG-3′; Bowles *et al.*, 1995) were used to amplify a portion of COI: a 25 μL total volume reaction contained 1× PCR buffer (Promega), 3 mM $MgCl_2$, 0.2 mM dNTPs (Promega), 0.4× Rediload gel loading buffer (Invitrogen), 0.2 μM of each primer, 1 U GoTaq® Flexi DNA Polymerase (Promega) and 1 μL template DNA. Cycling was as follows: 4 min at 94°C, followed by 40 cycles at 94°C for 30 s, annealing at 48°C for 40 s, extension at 72°C for 50 s and final extension at 72°C for 7 min.

All products were electrophoresed through 1% agarose gels stained with GelRed (Biotium, Fremont, California, USA) and visualized under ultraviolet light. Samples that produced a faint band were subjected to another round of PCR, done as above, except the template was the product from the initial PCR. Products were purified using ExoSAP-IT (Affymetrix, Santa Clara, California, USA) or by gel extraction using a QIAquick Gel Extraction kit (Qiagen), following the manufacturer's proto- col for both methods and then sent to Eurofins MWG Operon LLC (Louisville, Kentucky, USA) for bi-directional Sanger sequencing. Complementary sequences were assembled, and their chromatograms were assessed and edited by eye using Sequencher version 5.3 (Gene Codes Corp., Ann Arbor, Michigan, USA). Resulting sequences were compared with sequences available in the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST – Altschul *et al.*, 1990) and were deposited into GenBank (Table 1).

COI sequences were translated to confirm the absence of prema- ture stop codons,

which if present were corrected using Open Reading Frame Finder (RRID:SCR_016643) by NCBI and Geneious software version 4.8.5 (Kearse *et al.*, 2012). Sequences produced in this study and sequences of species of Hexabothriidae and Monocotylidae obtained from GenBank (Table 1) were aligned by hand in MEGA X (Kumar *et al.*, 2018), and distance analyses were done using the same program. The maximum composite likelihood method (Tamura *et al.*, 2004) was used to calculate the number of base substitutions per site between pairs of sequences (*p*-distance), and phylograms were generated using the neighbour-joining (NJ) method (Saitou and Nei, 1987), implementing pairwise deletions and support values obtained by 1000 bootstrap replicates (Felsenstein, 1985). Resulting trees represent intraspecific and interspecific distances between sequences but not phylogenetic relationships (Figs 3 and 4).

A maximum likelihood phylogenetic tree for Monocotylidae was constructed using 28S sequences obtained herein and from GenBank (see Table 1 for accession numbers) – the only fragment available for the group at the moment for species previously allo- cated in Loimoidae. Sequences were aligned using GUIDANCE 2 (Penn *et al.*, 2010*a*, 2010*b*) using the multiple sequence alignment algorithm MAFFT (Katoh *et al.*, 2002) and set to 100 alternative guide trees. The phylogeny was generated using raxmlGUI 2.0 (version 2.0.6) (Silvestro and Michalak, 2012; Stamatakis, 2014) with the general time reversible model with gamma rates (GTR + G) for 1000 bootstrap repetitions. The final tree was opened and edited in MEGA X (Fig. 5).

Genomic DNA was extracted from hosts using a Mag-Bind Blood and Tissue DNA Kit (Omega Bio-Tek, Norcross, Georgia, USA). Double-digest restriction site-associated DNA sequencing libraries were prepared following the methods of Barker

et al. (2019) and sequenced on an Illumina HiSeq 4000 DNA sequencer. Subsequent data processing and species identification was performed following the methods of Barker *et al*. (2021). Briefly, the dDocent pipeline (Puritz *et al*., 2014) was used to trim reads, map reads and call single-nucleotide poly- morphisms (SNPs). Hosts were identified as *S. lewini* or *S. gilberti* using a panel of 1491 diagnostic SNPs, and a match of at least 95% to 1 species was required for identification. The program NewHybrids (Anderson and Thompson, 2002) was used to deter- mine if ambiguous individuals could be assigned into a hybrid category [first-generation hybrid (F1), *S. lewini* backcross (BX), *S. gilberti* backcross].

3.2.4 Prevalence determination

Upon receipt of shark identifications, prevalence of infection per parasite species, including coinfections, as well as per family, were calculated. Coinfections included instances where specimens of multiple species were found to infect the same host individual. Monogenoid prevalence is the percent of infected sharks, regardless of the parasite species; Hexabothriidae and Monocotylidae prevalence include all species from their respective families. In some instances, only fragments of specimens were collected but could not be identified to species or even family level, in which case these were included within the category of highest taxonomic classification possible.

3.3 RESULTS

3.3.1 Species determination and prevalence of infection

Of the 87 neonates examined, 44 were determined molecularly as *S. gilberti*, 20 as *S. lewini*, and 15 as hybrids of the two species (six F1 hybrids, five *S. lewini* BX, and four *S. gilberti* BX). Eight sharks could not be determined at the species level (i.e., no DNA

or morphology available could ascertain *Sphyrna* species, as only the head of these specimens were provided and attempts to sequence were unsuccessful). Thus, only the remaining 79 neonates with confirmed species identities were used to report prevalence (Table 3, in Tables). In total, three species of monogenoids were determined as *E. microstoma*, *E. sphyrnae,* and *L. wilsoni* (see redescriptions below). Both species of *Erpocotyle* were found to infect both species and hybrids of the studied hammerhead sharks, while *L. wilsoni* was found only on specimens of *S. lewini* and hybrids.

3.3.2 Molecular species identity

In total, 22 COI and four 28S sequences were generated (accession numbers in Table 1; sequence composition in Table 2; see Tables section). The 28S sequences from the Monocotylidae specimens (n = 4) were 100% identical to that of a *Loimosina* sp. (Table 1; 99% query coverage).

There is no GenBank sequence available for *Erpocotyle*, however, neighbor-joining analysis using COI sequences obtained herein supported the finding of two distinct species of *Erpocotyle* in the sharks examined (Fig. 3). *Erpocotyle sphyrnae* and *E. microstoma* sequences formed two distinct groups each with 100% bootstrap support. Likewise, the two available sequences of species of *Loimosina* formed a clade (100% bootstrap support) distinct from all available Monocotylidae sequences from GenBank (Fig. 4). Intraspecific variation for *E. sphrynae* was 0.00 - 0.02 and 0.01 - 0.04 for *E. microstoma*; for *L. wilsoni*, these values were 0.01 - 0.02 (Tables 4 and 5, respectively, in Tables section).

The maximum likelihood phylogeny using 28S sequences of Monocotylidae supports the proximity of *L. wilsoni* and *L.* sp. (of Boeger *et al.,* 2014), which comprise

a monophyletic clade to the paraphyletic *Neoheterocotyle* and its sister *Troglocephalus* (Fig. 5). *Cathariotrema* (another monocotylid from hammerheads) is much further distanced in the tree, forming a sister-clade to *Triloculotrema*.

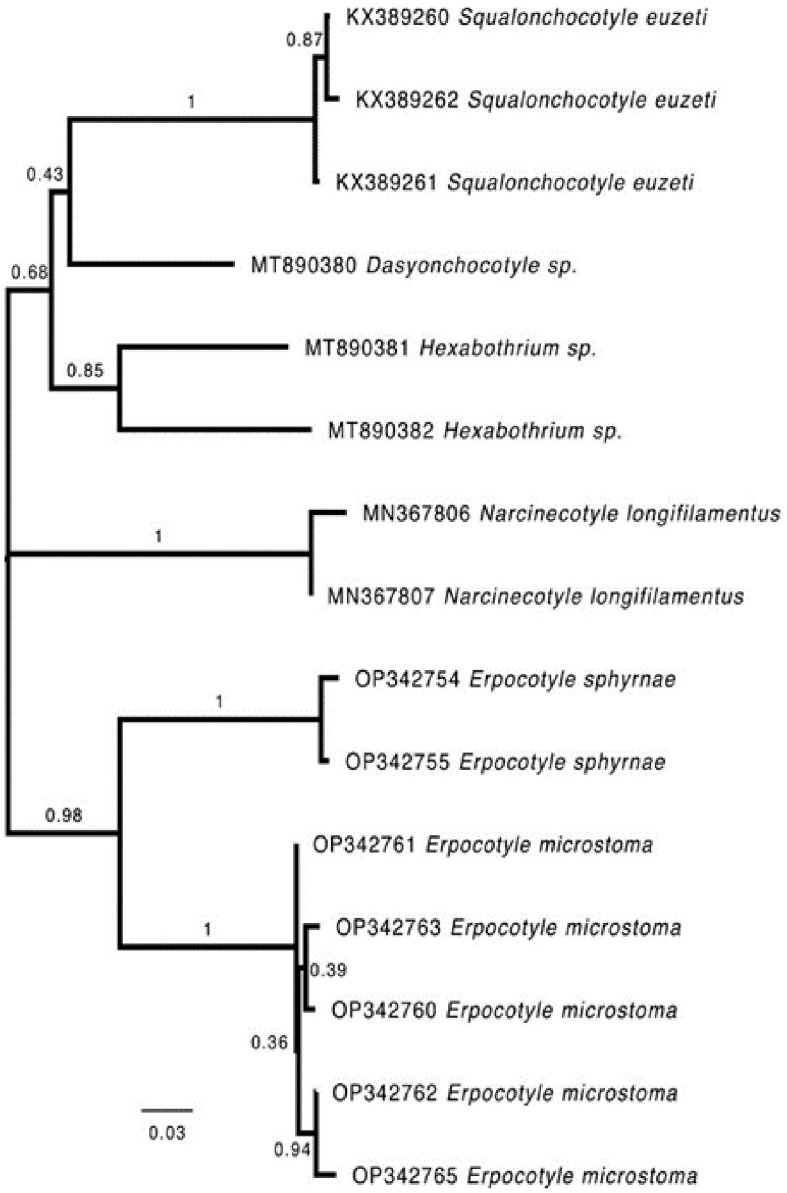
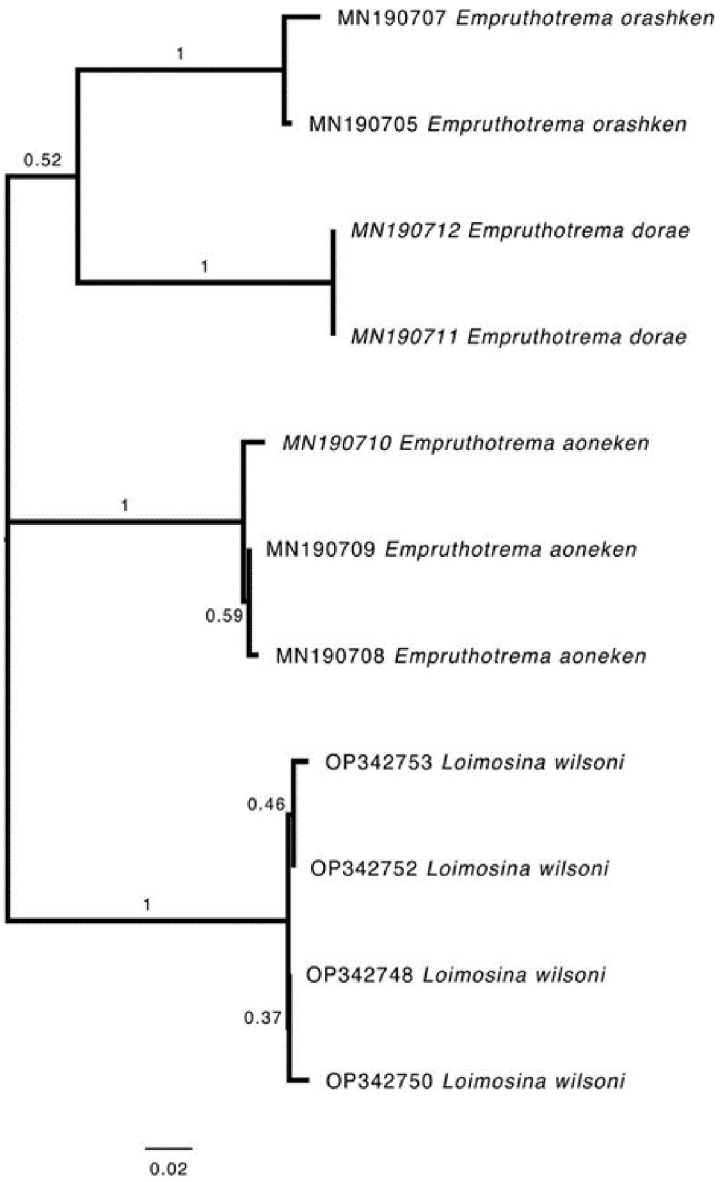


Figure 3: Neighbor-Joining tree based on Tamura-Nei distances (1993) for Hexabothriidae COI sequences from this study and from the GenBank database conducted in MEGA X (Kumar *et al.*, 2018). Sum of branch length = 1.11 and bootstrap support is shown, for 15 nucleotide sequences representing six species

with a total of 821 positions in the final dataset. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances (number of base substitutions per site, Table 4). Codon positions included were 1st+2nd+3rd.

Figure 4: Neighbor-Joining tree based on Tamura-Nei distances (1993) for Monocotylidae COI sequences from this study and from GenBank conducted in MEGA X (Kumar *et al.*, 2018). Sum of branch length = 0.532 and bootstrap support is



shown, for fourteen nucleotide sequences representing four species with a total of 348 positions in the final dataset. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances (number of base substitutions per site, Table 5). Codon positions included were 1st+2nd+3rd.

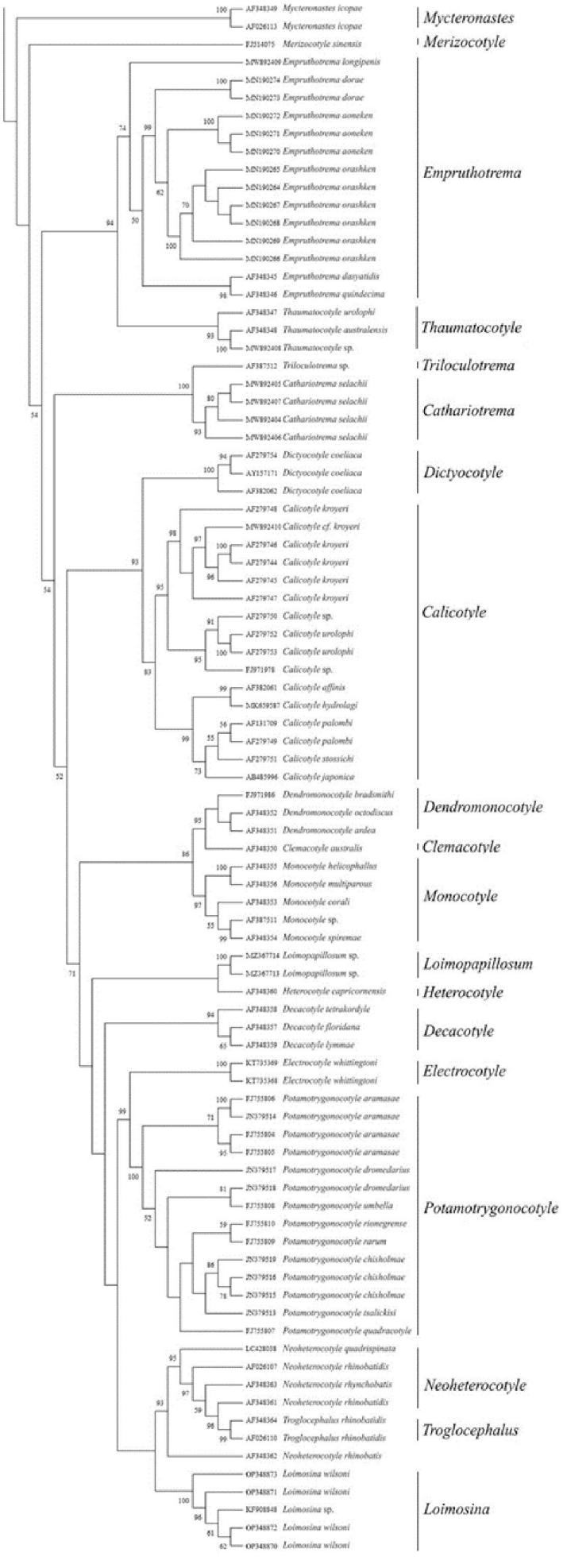

AF348349 Mycteronastes icopae
AF026113 Mycteronastes icopae
Mycteronastes
FJ514075 Merizocotyle sinensis
Merizocotyle
MW892409 Empruthotrema longipenis
MN190274 Empruthotrema dorae
MN190273 Empruthotrema dorae
MN190272 Empruthotrema aoneken
MN190271 Empruthotrema aoneken
MN190270 Empruthotrema aoneken
MN190265 Empruthotrema oraskken
MN190264 Empruthotrema oraskken
MN190267 Empruthotrema oraskken
MN190268 Empruthotrema oraskken
MN190269 Empruthotrema oraskken
MN190266 Empruthotrema oraskken
AF348345 Empruthotrema dasyatidis
AF348346 Empruthotrema quindecima
Empruthotrema
AF348347 Thaumatocotyle urolophi
AF348348 Thaumatocotyle australensis
MW892408 Thaumatocotyle sp.
Thaumatocotyle
AF387512 Triloculotrema sp.
Triloculotrema
MW892405 Cathariotrema selachii
MW892407 Cathariotrema selachii
MW892404 Cathariotrema selachii
MW892406 Cathariotrema selachii
Cathariotrema
AF279754 Dictyocotyle coeliaca
AY157171 Dictyocotyle coeliaca
AF382062 Dictyocotyle coeliaca
Dictyocotyle
AF279748 Calicotyle kroyeri
MW892410 Calicotyle cf. kroyeri
AF279746 Calicotyle kroyeri
AF279744 Calicotyle kroyeri
AF279745 Calicotyle kroyeri
AF279747 Calicotyle kroyeri
AF279750 Calicotyle sp.
AF279752 Calicotyle urolophi
AF279753 Calicotyle urolophi
FJ971978 Calicotyle sp.
AF382061 Calicotyle affinis
MK859587 Calicotyle hydrolagi
AF131709 Calicotyle palombi
AF279749 Calicotyle palombi
AF279751 Calicotyle stossichi
AB485996 Calicotyle japonica
Calicotyle
FJ971986 Dendromonocotyle bradsmithi
AF348352 Dendromonocotyle octodiscus
AF348351 Dendromonocotyle ardea
Dendromonocotyle
AF348350 Clemacotyle australis
Clemacotyle
AF348355 Monocotyle helicophallus
AF348356 Monocotyle multiparous
AF348353 Monocotyle corali
AF387511 Monocotyle sp.
AF348354 Monocotyle spiremae
Monocotyle
MZ367714 Loimopapillosum sp.
MZ367713 Loimopapillosum sp.
Loimopapillosum
AF348360 Heterocotyle capricornensis
Heterocotyle
AF348358 Decacotyle tetrakordyle
AF348357 Decacotyle floridana
AF348359 Decacotyle lymmae
Decacotyle
KT735369 Electrocotyle whittingtoni
KT735368 Electrocotyle whittingtoni
Electrocotyle
FJ755806 Potamotrygonocotyle aramasae
ZS379514 Potamotrygonocotyle aramasae
FJ755804 Potamotrygonocotyle aramasae
FJ755805 Potamotrygonocotyle aramasae
ZS379517 Potamotrygonocotyle dromedarius
ZS379518 Potamotrygonocotyle dromedarius
FJ755808 Potamotrygonocotyle umbella
FJ755810 Potamotrygonocotyle rionegrense
FJ755809 Potamotrygonocotyle rarum
ZS379519 Potamotrygonocotyle chisholmae
ZS379516 Potamotrygonocotyle chisholmae
ZS379515 Potamotrygonocotyle chisholmae
ZS379513 Potamotrygonocotyle tsalickisi
FJ755807 Potamotrygonocotyle quadracotyle
Potamotrygonocotyle
LC428038 Neoheterocotyle quadrispinata
AF026107 Neoheterocotyle rhinobatidis
AF348363 Neoheterocotyle rhynchobatis
AF348361 Neoheterocotyle rhinobatidis
Neoheterocotyle
AF348364 Troglocephalus rhinobatidis
AF026110 Troglocephalus rhinobatidis
Troglocephalus
AF348362 Neoheterocotyle rhinobatis
OP348873 Loimosina wilsoni
OP348871 Loimosina wilsoni
KF908848 Loimosina sp.
OP348872 Loimosina wilsoni
OP348870 Loimosina wilsoni
Loimosina

Figure 5: Maximum Likelihood tree (GTR+G substitution model, 1000 bootstraps) for Monocotylidae 28S sequences from this study and GenBank conducted in raxmlGUI 2.0 (version 2.0.6) (Stamatakis, 2014; Silvestro, 2012) using 92 sequences representing 54 species with a total of 4022 positions in the final dataset. The consensus tree is shown with bootstraps as support; values lower than 50% have been removed.

3.3.3 Taxonomy

Subclass Heteronchoinea Boeger & Kritsky, 2001

Hexabothriidae Price, 1942

Erpocotyle microstoma (Brooks, 1934)

Figures 6A-H

Redescription (based on 9 whole- and 9 partially- mounted specimens, including hologenophores, 2 syntypes; measurements based on specimens collected in present study): Body 3,623 - 7,425 (5,562; n = 7) long, 800 - 1,575 (1,172; n = 10) at greatest width, tapering anteriorly (Fig. 5A). Oral sucker 160 - 283 (224; n = 9) long, 200 - 375 (278; n = 10) wide, multiple papillae present within mouth. Pharynx bulbous, 73 - 150 (98; n = 7) long, 63 - 150 (88; n = 7) wide. Esophagus short. Caeca double, diverticulated, fusing just anterior to haptor, extends as non-diverticulated branches into haptor and haptoral appendix. Haptor symmetrical, squared-oval shaped 950 - 1,550 (1,257; n = 7) long, 800 - 1,075 (938; n = 6) wide, armed with two parallel symmetrical rows of bell-shaped suckers, and haptoral appendix; haptoral suckers in 3 pairs; pairs 1 - 3 measuring 380 - 550 (464; n = 7), 425 - 550 (493; n = 6), and 375 - 500 (451; n = 6) in diameters, respectively; hook-shaped sclerites embedded within

haptoral suckers (Figs. 6E-H), composed by long slightly-curved shaft, short recurved hook, measurements are included in Table 6; haptoral appendix 1,013 - 1,875 (1,515; n = 8) long, 255 - 500 (407; n = 10) wide, bearing two distal bell-shaped suckers composed of a small proximal and distal bulb and pair of anchors (Fig. 5D): proximal bulb 36 - 65 (51; n = 8) long, 65 - 85 (73; n = 8) wide; distal bulb 95 - 225 (164; n = 8) long, 125 - 185 (151; n = 8) wide; anchors 50 - 75 (62; n = 9) in total length, 25 - 40 (31; n = 3) wide. Testes numerous (40 - 55; n = 3), irregular-shaped, located in posterior third of the body; vas deferens winding from testes to base of male copulatory organ (MCO). Germarium (ovary) J-shaped (Fig. 6C), 554 - 670 (612; n = 2) long, 268 - 430 (349; n = 2) wide, pre-testicular, proximally poorly lobate, descending branch straight, ascending branch straight. Ootype smooth (Fig. 6C); seminal receptacle present, 225 - 340 (278; n = 3) long, 105 - 125 (117; n = 3) wide; uterus ventral to vas deferens, containing few eggs; eggs 138 - 190 (168; n = 4) long, 40 - 65 (53; n = 4) wide, with polar filaments 70 - 120 (98; n = 3). Common genital pore posterior to caeca bifurcation. MCO unarmed (Fig. 6B), distally bulbous, ovate, 18 - 63 (35; n = 5) in diameter; prostatic region 33 - 120 (77; n = 5) long, 13 - 65 (36; n = 5) wide; distal portion of vas deferens with thick walls. Two latero-ventral vaginal apertures; vagina parallel, proximally connected to vitelline commissure; distal vaginal duct expanded with glands along entire length.

Taxonomic summary:

Type host and locality: *Sphyrna zygaena* Beaufort, NC, USA (western North Atlantic Ocean)

Present hosts and localities: *S. lewini, S. gilberti*, hybrid (*S. lewini/S. gilberti*) Bulls Bay, Awendaw, SC, USA (western North Atlantic Ocean)

Other reported localities: *Sphyrna mokarran* Panama Canal, Panama (Pacific Ocean); *S. tudes* Punta del Este, Uruguay (western South Atlantic Ocean)

Site of infection: gill filaments

Specimens studied: syntypes USNM 132155, HWML 1437; vouchers: USNM 1666647, USNM 1666648, USNM 1666649, USNM 1666651, USNM 1666652, USNM 1666653, USNM 1666654, HWML 216856, HWML 216857, HWML 216858, HWML 216859, HWML 216860, HWML 216861, HWML 216863, HWML 216864; hologenophores: USNM 1666642, USNM 1666643, USNM 1666644, USNM 1666645, USNM 1666646, USNM 1666650, HWML 216851, HWML 216852, HWML 216853, HWML 216854, HWML 216855, HWML 216861, HWML 216862, HWML 216863, HWML 216864, HWML 216865, HWML 216866

Representative sequences: COI – Genbank OP342755, OP342756, OP342757, OP342758, OP342759, OP342760, OP342761, OP342762, OP342763, OP342764, OP342765, OP342766, OP342767, OP342768, OP342769, OP342770, OP342771

Remarks: Brooks (1934) indicated the absence of the MCO in his original description of this species. It thus far has been either considered missed or assumed absent in all other redescriptions (Price, 1942; Caballero *et al.*, 1956; Suriano & Labriola, 1998). However, the MCO is clearly present in available syntypes and specimens collected in the present study, and it corresponds to the morphology of MCO of *Erpocotyle* species as diagnosed by Boeger & Kritsky (1989), except for the relatively expanded prostatic region, which can represent either an artifact or an autapomorphic feature of the species. The vaginae, which were never previously described in detail (see Price, 1942; Caballero *et al.*, 1956; Suriano & Labriola, 1998),

also conform with the general morphology for species of the genus as diagnosed by Boeger & Kritsky (1989) – the vaginae are parallel, non-muscular, differentiated into two segments, and distally expanded with basal glands. The generic diagnosis for *Erpocotyle* spp. includes glando-muscular terminal vaginae, a homologous character. However, compared to those from USNM, our specimens displayed a more dilated distal vagina in *E. microstoma* than in *E. sphyrnae*. This feature, along with the shape and size of the sucker sclerites, may potentially be used to distinguish *E. microstoma* from the remaining species of the genus.

Available sequences for hexabothriids are limited (only nine COI, fourteen Cytb, thirteen 28S, and seven 18S). The five COI sequences of *E. microstoma* used in this analysis form a group in the NJ tree, with high bootstrap support (Fig. 3; Table 5). Conspecific distances among sequences of *E. microstoma* did not exceed 0.04 while interspecific distances varied between 0.29 - 0.40. Although sequences generated were short (~ 340 bp), such short sequences have been demonstrated to still be useful in species delimitation of other organisms (Hajibabaei *et al.*, 2006). The distances (0.23 - 0.25; Table 4) between sequences of the two morphologically distinguishable species observed in this study support the delimitation between *E. microstoma* and *E. sphyrnae*.

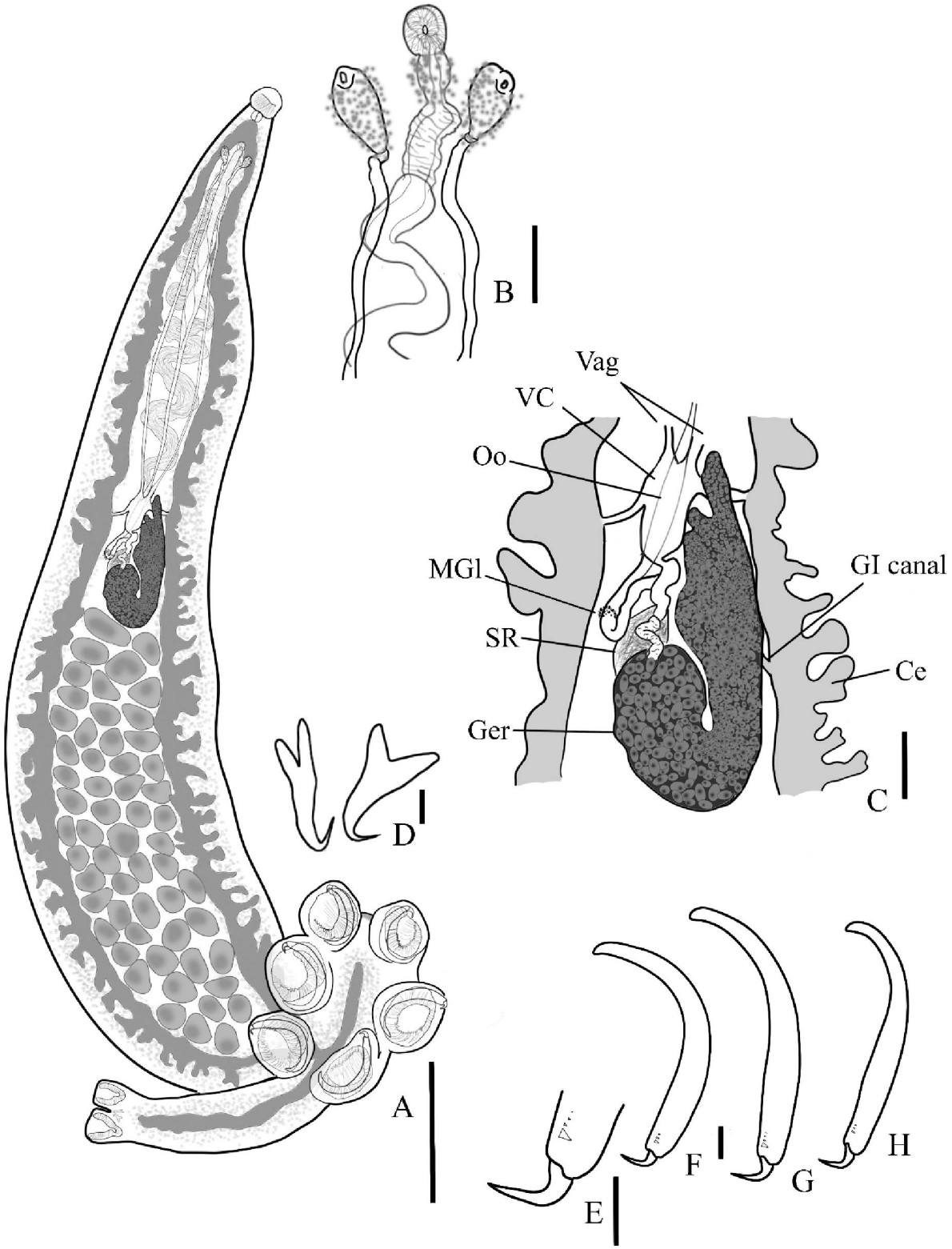


Figure 6 (A-H): *Erpocotyle microstoma* (A) whole body, scale bar 1000 µm; (B) male copulatory organ (MCO) and parallel vaginae, scale bar 50 µm; (C) midsection of

specimen containing the Ce (cecae), GI (gastrointestinal) canal, reproductive organs Ger (germarium), MGl (Mehlis gland), Oo (ootype), SR (seminal receptacle), and Vag (vaginae), and VC (vitelline canal), scale bar 100 µm; (D) anchors of the haptoral appendix, scale bar 60 µm; (E) curvature of the terminal hook of a sucker sclerite, scale bar 60 µm; (F) - (H) sucker sclerites 1 - 3, respectively, scale bar 60 µm.

Erpocotyle sphyrnae (MacCallum, 1931)

Figures 7A-H

Redescription (based on 7 whole- and 5 partially- mounted specimens, including hologenophores, 5 syntypes; measurements are from specimens collected in present study): Body fusiform, 1,425 - 4,550 (2,719; n = 7) long, 263 - 890 (521; n = 9) wide at posterior third of body (Fig. 7A). Oral sucker 140 - 445 (258; n = 9) long, 190 - 550 (350; n = 8) wide, multiple papillae present within mouth. Pharynx bulbous, 48 - 80 (62; n = 6) in diameter. Esophagus short. Caeca double, diverticulated, fusing just anterior to haptor, extend as non-diverticulated branches into haptor and haptoral appendix. Vitellaria extending posteriolaterally, branching into haptor and haptoral appendix. Haptor symmetrical, in the shape of a squared-oval 480 - 970 (704; n = 8) long and 375 - 562 (478; n = 4) wide, armed with two parallel symmetrical rows of bell-shaped suckers. Haptoral appendix projecting marginally between first sucker-sclerite pair; hook-shaped sclerites embedded within haptoral suckers, comprises long shaft and long recurved hook (Figs. 7E - H). Haptoral suckers 1 - 3 measure 180 - 325 (252; n = 7), 180 - 330 (258; n = 7), and 180 - 325 (250; n = 7) in diameter measurements of sucker sclerites in Table 7. Haptoral appendix 410 - 1,030 (740; n = 8) long, 140 - 320 (222; n = 8) wide with two terminal bell-shaped suckers, each containing a muscular proximal bulb 13 - 53 (31; n = 7) long, 25 - 63 (46; n = 7) wide, and a distal bulb, 48 -

145 (107; n = 8) long, 58 - 148 (107; n = 8) wide, and a pair of anchors between suckers (Fig. 7D), 45 - 58 (52; n = 10) long and 13 - 33 (22; n = 5) wide. Genital pore common. Testes multiple (60 - 98; n = 3), post-germarium, each subovate. Vas deferens distally wide with thick walls, winding from anterior testes to base of MCO; MCO composed by a distal unarmed, ovate muscular bulb 25 - 50 (40; n = 4) in diameter and a proximal elongate prostatic region 36 - 100 (79; n = 4) long and 29 - 45 (37; n = 4) wide (Fig. 7B). Two parallel vaginae (Fig. 7B), located on either side of the MCO, differentiated into two segments: distal segment expanded with glands along entire length and a thin muscular layer on the most distal segment. Vaginal pores opening at level of genital pore, with each vaginal duct connecting proximally to the vitelline reservoir/commissure. Ootype smooth (Fig. 7C); uterus with up to 3 eggs; eggs fusiform, 125-235 (197; n = 3) long, 35-50 (42; n = 3) wide, with two polar filaments 25-95 (53; n = 3) long. Germarium J-shaped, 180 - 648 (357; n = 5) long by 95 - 348 (185; n = 5) wide, adjacent to testes to the left of the medial line; proximal germarium lobate, straight descending and ascending germarium branches. Seminal receptacle ovate, 85 - 188 (139; n = 4) long and 40 - 90 (70; n = 4) wide.

Taxonomic summary:

Type host and locality: *Sphyrna zygaena* Woods Hole, MA, USA (western North Atlantic Ocean)

Present host and localities: *S. lewini, S. gilberti,* hybrid (*S. lewini/S. gilberti*) Bulls Bay, Awendaw, SC, USA (western North Atlantic Ocean)

Other hosts and localities: *S. zygaena, S. lewini* Dakar, Senegal (eastern North Atlantic Ocean); *S. lewini* Hawaii, USA (Pacific Ocean); *S. mokarran* Nueweiba, Egypt (Gulf of Aqaba)

Site of infection: gill filaments

Specimens studied: syntypes USNM 1320884, USNM 1320885; vouchers: USNM 1666656, USNM 1666657, USNM 1666659, USNM 1666660, USNM 1666661, HWML 216867, HWML 216868, HWML 216869; hologenophores: USNM 1666655, USNM 1666658, HWML 216870, HWML 216871

Representative sequences: COI-Genbank OP342754, OP342755

Remarks: Yamaguti (1968) presented the latest redescription of this species, which provides its morphology in detail, but indicated the presence of six irregular muscular pits, two median and four submedian, within the anterior sucker. These structures were not present in any of the specimens examined herein nor were they noted in any of the previous descriptions/redescriptions (MacCallum, 1931; Price, 1942; Euzet & Maillard, 1967).

Compared to the syntypes, our specimens depicted a protruding bulge on one of the proximal ends of the anchors (Fig. 7D). However, this feature was not visible in the museum specimens likely because their anchors were not properly flattened. Anchors have been shown to have high morphological variation among hexabothriids (Teo *et al.*, 2013; Khang *et al.*, 2016), thus, the presence of this bulge may be a possible differential diagnostic between *E. sphrynae* and *E. microstoma*.

The two COI sequences generated for *E. sphyrnae* formed a group in the NJ tree (Fig. 3). This group is adjacent to the group composed by *E. microstoma* sequences, presenting a distance of 0.23 - 0.25 among these species, compared to distances between 0.35 - 0.41 with other, non-*Erpocotyle* Hexabothriidae sequences (Table 4).

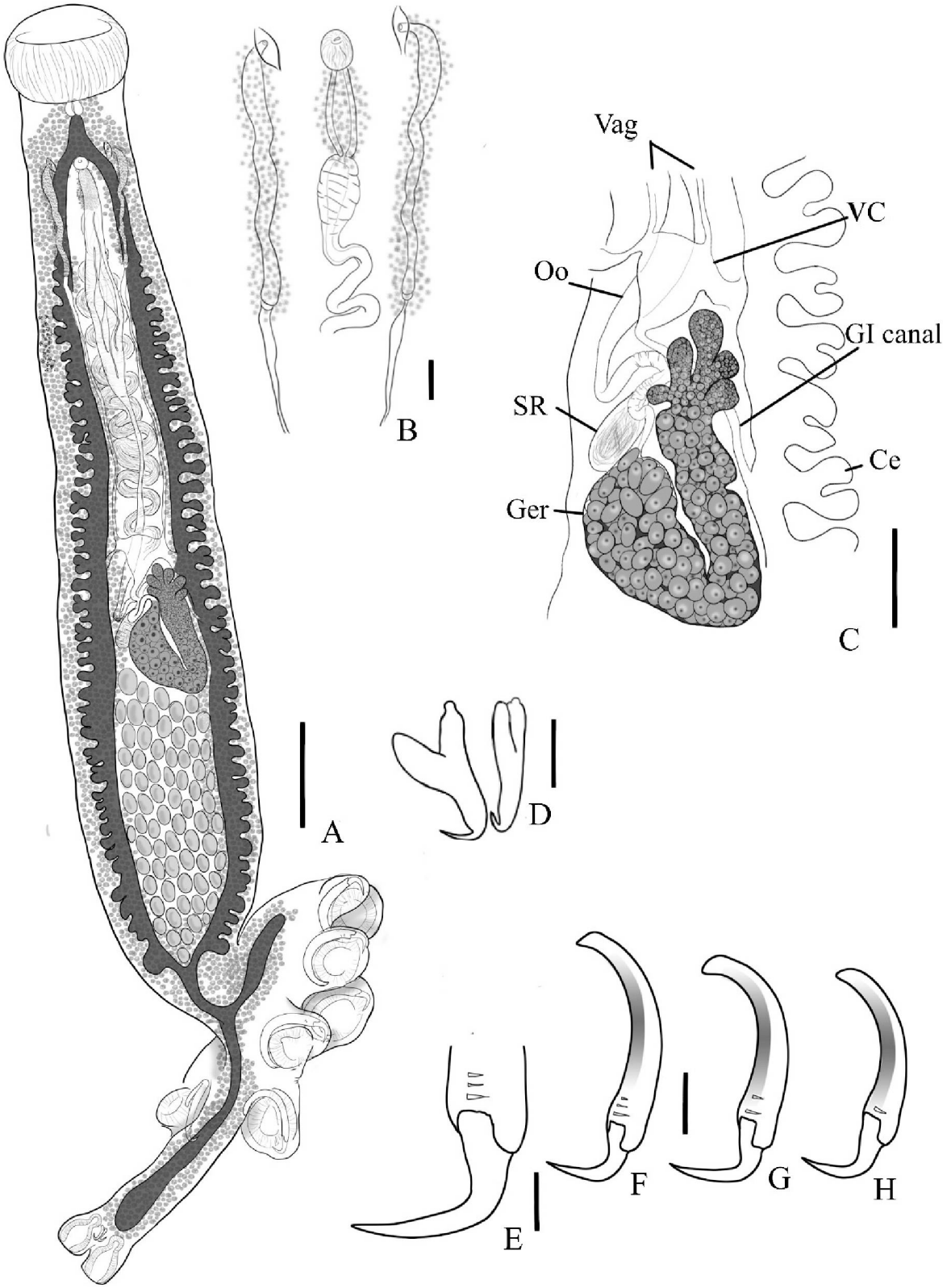


Figure 7 (A-H): *Erpocotyle sphyrnae* (A) whole body, scale bar 500 µm; (B) male

copulatory organ (MCO) and parallel vaginae, scale bar 60 µm; (C) midsection of specimen (abbreviations as in Figure 6), scale bar 200 µm; (D) anchors of the haptoral appendix, scale bar 25 µm; (E) curvature of the terminal hook of a sucker sclerite, scale bar 30 µm; (F) - (H) sucker sclerites 1 - 3, respectively, scale bar 60 µm.

Subclass Polyonchoinea Bychowsky, 1937

Monocotylidae Taschenberg, 1879

Loimosina wilsoni (Manter, 1944)

Figures 8A-F

Redescription (based on 9 whole- and 2 partially- mounted specimens, including hologenephores and 3 syntypes; measurements are from specimens collected in present study): Body 1,415 - 3,226 (2,089; n = 4) long by 229 - 506 (371; n = 5) wide at mid-length (Fig. 8A). Head tapering anteriorly bearing three pairs of symmetrical pits. Mouth subterminal, ventral with prepharynx; large bulbous pharynx, 104 - 221 (166; n = 8) long, 103 - 255 (155; n = 8) wide, large excretory vesicle on each side (Fig. 8D); esophagus inconspicuous; caeca lacking diverticulae, extending posterolaterally, terminating blind just anterior to haptor. Haptor subcircular, 184 - 393 (281; n = 8) long, 378 - 673 (504; n = 8) wide, with an external row of small and often inconspicuous loculi, a single latero-posterior pair of anchors (Fig. 8F), seven pairs of smaller hooks organized evenly and symmetrically around the rim of the haptor (Fig. 8E). Anchors 27 - 60 (44; n = 10) long, 9 - 16 (12; n = 5) wide, evenly curved point and shaft, truncated superficial root, elongate deep root 16 - 37 (28; n = 7). Hooks similar in shape and size, 8 (n = 2) long, with short, recurved point, slightly curved shaft, erected truncate thumb, shank about as long as shaft; filamentous hook loop reaching ½ of shank. Common

genital pore overlaps pharynx, common genital atrium expanded. Single, large, heavily lobed testis, 55 - 105 (85; n = 3) long, 21 - 28 (24; n = 3) wide, post-germarium, occupying middle third of body; vas deferens intercaecal, convoluted, several distal loops expanding into seminal vesicle; ejaculatory bulb pear-shaped with short, cone-shape sclerotized MCO (Fig. 8B). Germarium strongly lobate (especially in larger organisms) (Fig. 8D); uterus expanded; vaginal opening ventro-lateral; vagina a sinuous duct distally expanded, glandular. Vitellaria follicular, coextensive with cecae; vitelline commissure overlapping with branch of germarium; ootype, seminal receptacle not observed. Egg ovate, 67 - 108 (94; n = 3) long, 46 - 87 (71; n = 3) wide, with a short proximal filament (Fig. 8C).

Taxonomic summary:

Type host and locality: *Sphyrna zygaena* Montego Bay, Jamaica (Caribbean Sea)

Present hosts and localities: *Sphyrna lewini* and hybrids (*S. lewini/S. gilberti*) Bulls Bay, Awendaw, SC, USA (western North Atlantic Ocean)

Other hosts and localities: *S. lewini* Dakar, Senegal (eastern North Atlantic Ocean)

Site of infection: gill filaments

Specimens studied: syntypes USNM 1337561, USNM 1337564, HWML 1425; vouchers: USNM 1666662, USNM 1666663, USNM 1666664, USNM 1666665, USNM 1666666, USNM 1666668, USNM 1666669, USNM 1666670, HWML 216873, HWML 216874, HWML 216875, HWML 216876, HWML 216878, HWML 216879, HWML 216880, HWML 216881; holo- genophores: USNM 1666667, HWML 216872, HWML 216877

Representative sequences: COI-Genbank OP342748, OP342749, OP342750,

OP342751, OP342752, OP342753; 28S-Genbank OP348870, OP348871, OP348872, OP348873

Remarks: Bravo-Hollis (1970) noted that the main differences between *L. parawilsoni* and *L. wilsoni* were geographical location (*L. parawilsoni* described from *S. lewini* in the Pacific Ocean), and few morphological features. *Loimosina parawilsoni* depicts a sub-circular haptor with hooks embedded in the papillae, a "different [sic]" shape of the anchors, the presence of a common genital pore, and a MCO with sclerotized, blade-like lateral walls. Manter (1944) interpreted the MCO of *L. wilsoni* as "rudimentary, consisting of a very short, very thinly chitinized tube near the male pore [sic]", uncertain of its position within or external to the ejaculatory bulb. Euzet & Maillard (1967) did not observe a MCO, instead considered this as the sclerotized distal wall of the ejaculatory bulb, protruding slightly ventrally. Syntypes from Manter (1944) were not mounted flat; structures were difficult to study and no MCO was visible in the syntypes. Unfortunately, there is no specimen from Euzet & Maillard (1967) in museum collections. Our specimens presented a short conical, sclerotized MCO at the distal extremity of the ejaculatory bulb (Fig. 8B) – thus, the presence of a sclerotized MCO projecting from the ejaculatory bulb is not a distinguishable diagnostic between the two species of *Loimosina*. Likewise, Bravo-Hollis (1970) mentions a difference in the shape of the anchors, but a difference between those of the syntype of *L. parawilsoni* (CNHE 154) and our specimens was not evident. Molecular sequences from specimens of *Loimosina* collected from *S. lewini* from the Pacific Ocean would allow clarification of this classification. However, as morphological descriptions stand, specimens of both *Loimosina* species are greatly similar morphologically, which leaves the only differential criterion to be the shape of the haptor. However, the shape of non-sclerotized structures are often affected by fixation and mounting. Hence, given that

both species are described from the same host, we strongly suggest that *L. parawilsoni* is a junior synonym of *L. wilsoni.* However, a definitive decision should await adequate restudy of specimens of *L. parawilsoni* and, preferably, a molecular delimitation of the species.

The same nucleotide composition of 28S from species of *Loimosina* redescribed in this study and that of Boeger *et al.* (2014) also strongly supports that *L. wilsoni*, is present in at least one species of *Sphyrna* in the South Atlantic Ocean (see discussion).

Available partial sequences of the monocotylids *Empruthotrema orashken* Irigoitia *et al.*, 2019, *Em. dorae* Irigoitia *et al.*, 2019, and *Em. aoneken* Irigoitia *et al.*, 2019 of the COI mtDNA gene grouped with the sequences produced herein. Sequences of *L. wilsoni* cluster together with significant bootstrap support and present short intraspecific distances (0.01 - 0.02, Table 5) and significantly greater interspecific distances (0.22 - 0.32; Fig. 4).

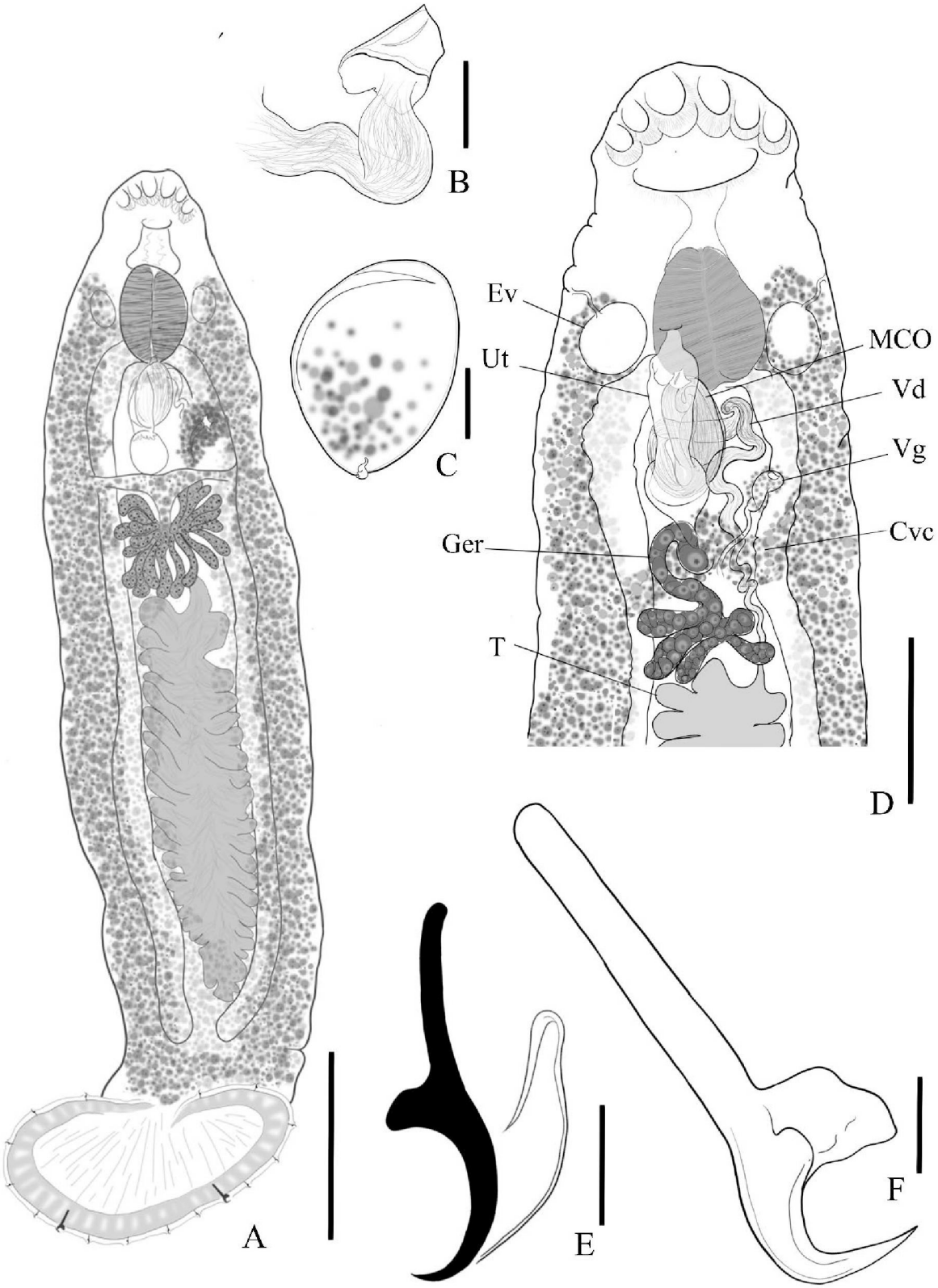

B
C
Ev
Ut
MCO
Vd
Vg
Cvc
Ger
T
D
A
E
F

Figure 8 (A-F): *Loimosina wilsoni* (A) whole body, scale bar 500 µm; (B) sclerotized, cone shaped male copulatory organ (MCO), scale bar 16 µm; (C) egg, scale bar 25 µm; (D) mid- to terminal portion of specimen containing the Cvc (common vitelline commissure), Ev (excretory vesicle), Ger (germarium), MCO, T (teste), Ut (uterus), Vd (vas deferens), and Vg (vagina), scale bar 150 µm; (E) haptoral hook, scale bar 10 µm; (F) haptoral anchor, scale bar 10 µm.

3.4 DISCUSSION

Age-dependent parameters can produce high variability when averaging measurements (or providing ranges), and it would aid in the validation of measurements and resolve diagnostic issues related to genera of Hexabothriidae if these variables were identified (see Vaughan & Christison, 2012). For example, the extension of the vitellaria into the haptoral appendix is disputed in the literature as to whether it is a reliable character of a species or genera, or if it is age-dependent for individuals (Price, 1942; Euzet & Maillard, 1967). Originally used by Price (1942) as a diagnostic in genera of Hexabothriidae, the extension of the vitellaria was noted to vary with the maturity of the specimen by Cerfontaine (1899), Sproston (1946), and by Euzet & Maillard (1967) in their respective redescriptions of *E. sphyrnae*. Accordingly, Boeger & Kritsky (1989) did not mention this aspect of the vitellaria distribution in their revision of Hexabothriidae. In our specimens, the vitellaria visibly extended into the haptor and the haptoral appendix in all intact mature specimens of both species of *Erpocotyle* (n = 16), but was not observed to do so in three juvenile representatives of *E. sphyrnae*, indicating that this character is age-dependent and not a reliable generic or specific diagnosis.

This study also revealed differences in the host repertoires of monogenoids – indicating different abilities to infect the spectrum of available hosts. Monogenoids typically demonstrate narrow host repertoires being either monoxenous – infecting a single host species – or stenoxenous – infecting a few related host species (Euzet & Combes, 1980; Rohde, 1994; Whittington *et al.*, 2000). In this study, *L. wilsoni* presented a narrow host repertoire, as it was found only on *S. lewini* and the hybrids and did not infect any of the 44 *S. gilberti* examined. host repertoire is typically determined by the opportunity of encounter and the compatibility of the involved host/parasite species (Combes, 2001; Araujo *et al.*, 2015; Brooks *et al.*, 2019). Factors involved in the success of the establishment of a parasite may depend on biological and behavioral aspects of the host, such as season of reproduction, stage of maturation, physiology, feeding behavior, and immunity, to list a few (MacDonald, 1975; Tinsley & Jackson, 2002; Glennon *et al.*, 2006; Ohashi *et al.*, 2007; Whittington & Kearn, 2011).

Hosts for *L. wilsoni* have been identified as *S. zygaena* and *S. lewini* (syns: *S. diplana* (Springer, 1941) and *S. couardi* (Cadenat, 1950)) (Manter, 1944; Hargis, 1955; Euzet & Maillard, 1967). However, there remains some uncertainty in the literature over host identity. Hargis (1955) first suggested that the type host, *S. zygaena*, was possibly misidentified by Manter (1944), a doubt also mentioned by Euzet & Maillard (1967), and that the host could have been *S. diplana* (syn. *S. lewini*). This is supported by the fact that Manter (1944) never observed first-hand the shark specimens as they were collected and provided by Dr. C. B. Wilson (for whom *L. wilsoni* was named). Euzet & Maillard (1967) also indicated the morphological similarities of *S. couardi* and *S. lewini* in their own report, which have been later synonymized in McEachran & Séret (1987). Paradoxically, as advances in molecular research have improved species delimitation,

cryptic host species are often uncovered, such as the case of *S. gilberti* and *S. lewini*. Thus, it becomes even more intriguing that in this study that *L. wilsoni* remained specific to *S. lewini* and admixed individuals with at least 50% *S. lewini* DNA (*S. lewini* F1 and BX) and that no *S. gilberti* or *S. gilberti* backcrosses (*S. gilberti* BX) were infected by *L. wilsoni.* The exclusion of *S. gilberti* BX from the host repertoire of *L. wilsoni* remains to be verified, however, given the small number of specimens of *S. gilberti* BX (n = 4). The exclusion of species *S. gilberti,* in contrast, from the host repertoire of *L. wilsoni* appears concrete given the large number of specimens of this species (n = 44).

Boeger *et al*. (2014) reported a specimen of *Loimosina* sp. from an unidentified hammerhead individual *Sphyrna* sp. off the coast of southeast Brazil. Although these authors were unable to identify this parasite to the species level based on morphology, its 28S rDNA sequence (GB Accession KF908848) was 98 - 100% identical to our specimens, suggesting that it was *L. wilsoni*. As *S. gilberti* appears not to be in the host repertoire of *L. wilsoni*, but only in *S. lewini* and hybrids, it is likely that the *Sphyrna* host species from Brazil was *S. lewini,* which is known from those coasts (Compagno, 1984), or yet another species, such as *S. media* Springer, 1940, *S. mokarran* (Rüppell, 1837), *S. tiburo* (Linnaeus, 1758), *S. tudes* (Valenciennes, 1822), or *S. zygaena,* all reported from south Brazil (Compagno, 1984). Whereas it is not known if *L. wilsoni* can infect other *Sphyrna* species, its presence on hybrids of *S. lewini* and *S. gilberti* indicates the possibility of it being stenoxenous and thus potentially using any of these hammerheads as host. Previous studies of the molecular phylogeny of family Sphyrnidae has found the globally distributed *S. lewini* to be more closely related to smaller, range-restricted (eastern Pacific and western Atlantic) sharks *S. media, S. tudes,* and *S. tiburo* than to other globally distributed large sharks *S. mokarran* and *S.*

zygaena (Lim *et al.,* 2010). This is further supported by Pinhal *et al.* (2012) which showed the cryptic Atlantic lineage, now known as *S. gilberti*, to be more closely related to *S. lewini* than the other smaller sharks. This further draws doubt to *S. zygaena* being included in the host repertoire of this parasite.

The hexabothriids *E. microstoma* and *E. sphyrnae* have wider host-ranges than *L. wilsoni.* Both have been originally described from specimens of *S. zygaena* from the western North Atlantic along the US coast (Woods Hole, MA and Beaufort, NC, respectively) and included in their repertoire are other hammerheads: *E. sphyrnae* has been reported in *S. zygaena* and *S. lewini* from Dakar, Senegal (Euzet & Maillard, 1967), *S. lewini* in Hawaii (Yamaguti, 1968), *S. mokarran* from Nuweibaa, Egypt (Maillard & Paperna, 1978), and now in *S. lewini, S. gilberti*, and their hybrids from the SC coast of the USA (present study). *Erpocotyle microstoma* has been reported also in *S. mokarran* but from the Panama Canal (Pacific Ocean; Caballero *et al.*, 1956), *S. tudes* from Punta del Este, Uruguay (Suriano & Labriola, 1998), and now in *S. lewini, S. gilberti*, and their hybrids from the SC coast of the USA (present study). Maillard & Paperna (1978) were the first to recognize the wider repertoires of both these *Erpocotyle* species, and the vast geographic range of *E. sphyrnae*. Here, we further expand the host repertoire and geographic ranges of these two species.

Bullard *et al.* (2021) updated the list of monocotylids in *S. lewini* to include *Cathariotrema selachii*, which they found infecting the olfactory cavities of *S. lewini* in the northern Gulf of Mexico. The present study did not investigae the olfactory cavities of the specimens of *S. lewini, S. gilberti,* and hybrids collected from the western North Atlantic. However, *C. selachii*, the only described species and thus the type for the genus, is noted as having a very broad host repertoire; it has been found to infect several *Sphyrna* spp., including *S. lewini*, as well as species of *Carcharhinus* Blainville,

1816, *Rhizoprionodon* Whitley, 1929 (both Carcharhinidae), and *Alopias* Rafinesque, 1810 (Alopiidae), in the Gulf of Mexico and in the western North Atlantic. Thus, it is entirely possible that *C. selachii* infects *S. lewini* and/or *S. gilberti* in the western North Atlantic.

In conclusion, this is the first study to compare monogenoid fauna between cryptic *Sphyrna* species and their hybrids in the western North Atlantic Ocean, and the first report of monogenoids collected from the gills of individuals of *S. gilberti* and hybrids with *S. lewini*. Genetic sequencing using nuclear ribosomal ITS2 sequences showed that species *S. lewini* and *S. gilberti* diverged around 4.5 million years ago (Pinhal *et al.*, 2012) yet can be found sympatrically and can produce reproductive and viable hybrids. This study demonstrated that these two hammerheads have differences in monogenoid fauna of the gills, which in addition to the number of vertebrae, further supports a distinction between species.

Lastly, Loimoidae or Loimoinae Price, 1936 - a subfamily of Monocotylidae - was previously composed of species of three genera: *Loimos* McCallum, 1917, *Loimosina*, and *Loimopapillosum* Hargis, 1955. The more comprehensive morphological analysis of the specimens provided in this study and the recognition that the sequence of the 28S rDNA fragment of *L. wilsoni* presented herein is identical to that of *Loimosina* sp. published by Boeger *et al.* (2014). This corroborates the decision that species of these genera are allocated within the Monocotylidae and do not compose a monophyletic assemblage within the family, following the suggestion of Boeger *et al.* (2014) and Chero *et al.* (2021). Although there is no sequence currently available for species of *Loimos*, molecular phylogenetic analyses using sequences of *Loimosina* from the present study and from Boeger *et al.* (2014), and *Loimopapillosum* (see Chero *et al.*, 2021), strongly indicate that these are members of distinct clades

within the Monocotylidae. The classification of *Loimos* and a more robust decision on the validity of Loimoidae/Loimoinae, however, should await sequencing of corresponding DNA fragments and phylogenetic analysis. Likewise, sequences of *Loimosina* specimens from the Pacific Ocean (in combination with morphological examination) would clarify whether *L. parawilsoni* is in fact a synonym for *L. wilsoni*, as suggested in the present study.

3.5 TABLES

Table 1. Sequences generated and used in the present study from families Monocotylidae and Hexabothriidae. COI sequences were used for distance analysis (species delimitation) and 28S sequences were used for phylogenetic analysis of the family Monocotylidae.

	GenBank No.			
	COI	28S		
Monocotylidae Taschenberg, 1879			Reference(s)	Notes
Calicotyle affinis Scott, 1911	-	AF382061	Chisolm *et al.* (2001a)	
Calicotyle hydrolagi Ñacari *et al.*, 2019	-	MK659587	Ñacari *et al.* (2020)	
Calicotyle japonica Kitamura *et al.*, 2010	-	AB485996	Kitamura *et al.* (2010)	
Calicotyle kroyeri Diesing, 1850	-	AF279748 AF279744 AF279745 AF279746 AF279747, MW892410	Chisolm *et al.* (2001a); Bullard *et al.* (2021)	
Calicotyle palombi Euzet & Williams, 1960	-	AF279749 AF131709	Chisolm *et al.* (2001a); Mollaret *et al.* (2000)	
Calicotyle sp.	-	AF279750 FJ971978	Chisolm *et al.* (2001a); Perkins *et al.* (2009)	
Calicotyle stossichi Braun, 1899	-	AF279751	Chisolm *et al.* (2001a)	
Calicotyle urolophi Chisolm et al., 1991	-	AF279753 AF279752	Chisolm *et al.* (2001a)	
Cathariotrema selachii (MacCallum, 1916)	-	MW892407 MW892406 MW892405	Bullard *et al.* (2021)	

		MW892404		
Clemacotyle australis Young, 1967	-	AF348350	Chisolm *et al.* (2001b)	
Decacotyle floridana (Pratt, 1910)	-	AF348357	Chisolm *et al.* (2001b)	
Decacotyle lymmae Young, 1967	-	AF348359	Chisolm *et al.* (2001b)	
Decacotyle tetrakordyle Young, 1967	-	AF348358	Chisolm *et al.* (2001b)	
Dendromonocotyle ardea Chisholm & Whittington, 1995	-	AF348351	Chisolm *et al.* (2001b)	
Dendromonocotyle bradsmithi Chisolm *et al.*, 1995	-	FJ971986	Perkins *et al.* (2009)	
Dendromonocotyle octodiscus Hargis, 1955	-	AF348352	Chisolm *et al.* (2001b)	
Dictyocotyle coeliaca Nybelin, 1941	-	AF279754 AF382062, AY157171	Chisolm *et al.* (2001b), Lockyer *et al.* (2003)	
Electrocotyle whittingtoni Vaughan *et al.*, 2016	-	KT735369 KT735368	Vaughan *et al.* (2016)	
Empruthotrema aoneken Irigoitia *et al.*, 2019	MN190708, MN190709	MN190270 MN190271 MN190272	Irigoitia *et al.* (2019)	
Empruthotrema dasyatidis Whittington & Kearn, 1992	-	AF348345	Chisolm *et al.* (2001b)	
Empruthotrema dorae Irigoitia *et al.*, 2019	MN190712, MN190711	MN190274 MN190273	Irigoitia *et al.* (2019)	
Empruthotrema longipenis Kritsky *et al.*, 2017	-	MW892409	Bullard *et al.* (2021)	
Empruthotrema orashken Irigoitia *et al.*, 2019	MN190702, MN190704, MN190705	MN190265 MN190266 MN190269 MN190268 MN190267 MN190264	Irigoitia *et al.* (2019)	
Empruthotrema quindecima Chisholm & Whittington, 1999	-	AF348346	Chisolm *et al.* (2001b)	
Heterocotyle capricornensis Chisholm & Whittington, 1996	-	AF348360	Chisolm *et al.* (2001b)	
Loimopapillosum pascuali Chero *et al.*, 2021	-	MZ367714 MZ367713	Chero *et al.* (2021)	
Loimosina wilsoni Manter, 1944	OP342748, OP342749, OP342750, OP342751, OP342752, OP342753	OP348870, OP348871, OP348872, OP348873	Present study	
Loimosina sp.	-	KF908848	Boeger *et al.* (2014)	
Thaumatocotyle australensis Beverley-Burton & Williams, 1989	-	AF348348	Chisolm *et al.* (2001b)	Recorded in GenBank as *Merizocotyle australensis*
Mycteronastes icopae (Beverley-Burton & Williams, 1989)	-	AF026113, AF348349	Mollaret *et al.* (1997); Chisolm *et al.* (2001b)	Recorded in GenBank as *Merizocotyle icopae*

Merizocotyle sinensis Timofeeva, 1984	-	FJ514075	Unpublished	
Thaumatocotyle urolophi (Chisholm & Whittington, 1999)	-	AF348347	Chisolm *et al.* (2001b)	Recorded in GenBank as *Merizocotyle urolophi*
Monocotyle corali Chisholm, 1998	-	AF348353	Chisolm *et al.* (2001b)	
Monocotyle helicophallus Measures, Beverley-Burton & Williams, 1990	-	AF348355	Chisolm *et al.* (2001b)	
Monocotyle multiparous Measures, Beverley-Burton & Williams, 1990	-	AF348356	Chisolm *et al.* (2001b)	
Monocotyle sp.	-	AF387511	Chisolm *et al.* (2001b)	
Monocotyle spiremae Measures, Beverley-Burton & Williams, 1990	-	AF348354	Chisolm *et al.* (2001b)	
Neoheterocotyle quadrispinata Nitta, 2019	-	LC428038	Nitta (2019)	
Neoheterocotyle rhinobatidis (Young, 1967)	-	AF026107, AF348361 AF348362	Mollaret *et al*. (1997); Chisolm *et al.* (2001b)	
Neoheterocotyle rhynchobatis (Tripathi, 1959)	-	AF348363	Chisolm *et al.* (2001b)	
Potamotrygonocotyle aramasae (Young, 1967)	-	FJ755806 FJ755804 FJ755805, JN379514	Unpublished; Fehlauer-Ale & Littlewood (2011)	
Potamotrygonocotyle chisholmae Domingues & Marques, 2007	-	JN379519 JN379516 JN379515	Fehlauer-Ale & Littlewood (2011)	
Potamotrygonocotyle dromedarius Domingues & Marques, 2007	-	JN379518 JN379517	Fehlauer-Ale & Littlewood (2011)	
Potamotrygonocotyle quadracotyle Domingues, Pancera & Marques, 2007	-	FJ755807	Unpublished	
Potamotrygonocotyle rara Domingues, Pancera & Marques, 2007	-	FJ755809	Unpublished	Recorded as *Potamotrygonocotyle rarum* in GenBank
Potamotrygonocotyle rionegrensis Domingues, Pancera & Marques, 2007	-	FJ755810	Unpublished	Recorded as *Potamotrygonocotyle rionegrense* in GenBank
Potamotrygonocotyle tsalickisi Mayes, Brooks & Thorson, 1981	-	JN379513	Fehlauer-Ale & Littlewood (2011)	
Potamotrygonocotyle umbella Domingues, Pancera & Marques, 2007	-	FJ755808	Unpublished	
Thaumatocotyle sp.	-	MW892408	Bullard *et al.* (2021)	
Triloculotrema sp.	-	AF387512	Unpublished	Boudaya & Neifar 2016 reported this sequence corresponded to their new species *T. euzeti* Boudaya & Neifar, 2016.
Troglocephalus rhinobatidis Young, 1967	-	AF026110, AF348364	Mollaret *et al.* (1997); Chisolm *et al.* (2001b)	

Gyrodactylidae Cobbold, 1864			
Bothitrema bothi (MacCallum, 1913)	-	AF387508	Justine *et al.* (2002)
Gyrodactylus ticuchi Pinacho-Pinacho *et al.,* 2021	-	MT879662.1	Pinacho-Pinacho *et al.* (2021)
Gyrodactylus tobala Pinacho-Pinacho *et al.,* 2021	-	MT879660, MT879661	Pinacho-Pinacho *et al.* (2021)
Tetraonchus monenteron (Wagener, 1857)	-	MK881304	Unpublished
Hexabothriidae Price, 1942			
Dasyonchocotyle sp. Hargis, 1955	MT890380	-	Unpublished
Erpocotyle microstoma (Brooks, 1934)	OP342755, OP342756, OP342757, OP342758, OP342759, OP342760, OP342761, OP342762, OP342763, OP342764, OP342765, OP342766, OP342767, OP342768, OP342769, OP342770, OP342771	-	Present study
Erpocotyle sphyrnae (MacCallum, 1931)	OP342754, OP342755	-	Present study
Hexabothrium sp. (Kuhn, 1829)	MT890381, MT890382	-	Unpublished
Narcinecotyle longifilamentus Torres-Carrera *et al.,* 2020	MN367806, MN367807	-	Torres-Carrera *et al.* (2020)
Squalonchocotyle euzeti Kheddam *et al.,* 2016	KX389260, KX389261, KX389262	-	Kheddam *et al.* (2016)

Table 2: Sequence compositions, with no gaps, per monogenoid species collected from gills of *Sphyrna* spp.: length in base pairs (bp), reported as a range; variable sites (V); parsimony-informative sites (PI); percent composition of each nucleotide. Variable and PI sites are compared between both species of *Erpocotyle*. *Loimosina wilsoni* was the only monocotylid identified in the present study, and was not compared to the other species of Hexabothriidae.

	Length (bp)	**V**	**PI**	**A%**	**T%**	**C%**	**G%**
COI							
L. wilsoni	347 - 370	9	1	28.5	44.2	7.5	19.9
E. microstoma	332 - 347	71	63	28.8	35.2	14.8	21.3
E. sphyrnae	332 - 364			27.7	35.0	14.6	22.7
28S							
L. wilsoni	642-950	0	0	28.4	24.9	28.3	18.5

Table 3: Summary of prevalence of infection and coinfection (%) per monogenoid species (*L. wilsoni, E. microstoma,* and *E. sphyrnae*) and per monogenoid family (Hexabothriidae is represented by the combined prevalence of both *Erpocotyle* species; *L. wilsoni* was the only monocotylid found, thus its prevalence is representative of the family in the present study) successfully identified shark individuals (n = 79); BX = backcross. The shaded boxes represent the numbers of each species of host sampled.

	Number of specimens per species	*L. wilsoni*	*E. microstoma*	*E. sphyrnae*	Coinfections	Hexabothriid	Monogenoid
S. gilberti	44	0	27	14	2	45	50
S. lewini	20	60	30	15	30	40	75
F1 hybrid	6	33	0	17	0	17	50
S. lewini BX	5	40	20	20	20	40	80
S. gilberti BX	4	0	25	0	0	50	50
	Total Prevalence	22	25	14	10	42	58

Table 4: Tamura-Nei (1993) distances between Hexabothriidae COI sequences from this study and from GenBank based on a 821 base pair alignment. The number of base substitutions per site between sequences were calculated in MEGA X (Kumar et al., 2018) using the maximum composite likelihood method (Tamura *et al.,* 2004). Standard error estimates are above the diagonal. This analysis involved six species with a total of 15 sequences. Codon positions included were 1st+2nd+3rd.

	OP342754 *Erpocotyle sphyrnae*	OP342755 *Erpocotyle sphyrnae*	OP342763 *Erpocotyle microstoma*	OP342762 *Erpocotyle microstoma*	OP342760 *Erpocotyle microstoma*	OP342759 *Erpocotyle microstoma*	OP342761 *Erpocotyle microstoma*	KX389260 *Squalonchocotyle euzeti*	KX389261 *Squalonchocotyle euzeti*	KX389262 *Squalonchocotyle euzeti*	MN367806 *Narcinecotyle longifilamentus*	MN367807 *Narcinecotyle longifilamentus*	MT890380 *Dasyonchocotyle sp.*	MT890381 *Hexabothrium sp.*	MT890382 *Hexabothrium sp.*
OP342754 *Erpocotyle sphyrnae*		0.01	0.22	0.22	0.22	0.22	0.22	0.32	0.31	0.32	0.30	0.30	0.34	0.30	0.29
OP342755 *Erpocotyle sphyrnae*	0.02		0.22	0.22	0.22	0.21	0.22	0.32	0.32	0.32	0.30	0.30	0.33	0.30	0.31
OP342763 *Erpocotyle microstoma*	0.25	0.24		0.01	0.01	0.01	0.01	0.33	0.32	0.33	0.33	0.32	0.25	0.30	0.32
OP342762 *Erpocotyle microstoma*	0.25	0.25	0.03		0.01	0.01	0.01	0.31	0.29	0.31	0.30	0.32	0.25	0.29	0.31
OP342760 *Erpocotyle microstoma*	0.25	0.25	0.01	0.02		0.01	0.01	0.33	0.32	0.33	0.33	0.32	0.25	0.29	0.32
OP342759 *Erpocotyle microstoma*	0.25	0.24	0.04	0.01	0.03		0.01	0.35	0.35	0.36	0.33	0.32	0.25	0.36	0.40
OP342761 *Erpocotyle microstoma*	0.24	0.23	0.01	0.01	0.01	0.02		0.32	0.32	0.32	0.32	0.32	0.25	0.29	0.31
KX389260 *Squalonchocotyle euzeti*	0.39	0.40	0.38	0.36	0.38	0.43	0.37		0.01	0.00	0.34	0.25	0.22	0.25	0.25
KX389261 *Squalonchocotyle euzeti*	0.38	0.39	0.36	0.35	0.37	0.42	0.36	0.01		0.01	0.34	0.25	0.21	0.25	0.25
KX389262 *Squalonchocotyle euzeti*	0.39	0.39	0.39	0.37	0.39	0.44	0.38	0.01	0.01		0.34	0.25	0.22	0.25	0.25
MN367806 *Narcinecotyle longifilamentus*	0.37	0.36	0.38	0.36	0.38	0.39	0.35	0.41	0.40	0.41		0.00	0.30	0.36	0.31
MN367807 *Narcinecotyle longifilamentus*	0.36	0.35	0.36	0.37	0.37	0.37	0.35	0.30	0.30	0.31	0.00		0.30	0.31	0.26
MT890380 *Dasyonchocotyle sp.*	0.41	0.39	0.29	0.30	0.31	0.31	0.31	0.26	0.24	0.26	0.30	0.30		0.25	0.24
MT890381 *Hexabothrium sp.*	0.35	0.36	0.32	0.32	0.32	0.40	0.32	0.30	0.31	0.31	0.43	0.35	0.29		0.17
MT890382 *Hexabothrium sp.*	0.37	0.37	0.35	0.36	0.36	0.47	0.35	0.28	0.29	0.29	0.39	0.31	0.26	0.22	

Table 5: Tamura-Nei distances (1993) between Monocotylidae COI sequences from this study and from the GenBank database based on a 348 base pair alignment. The number of base substitutions per site between sequences are shown and were calculated in MEGA X (Kumar et al., 2018) using the maximum composite likelihood method (Tamura et al., 2004). Standard error estimates are above the diagonal. This analysis involved four species with a total of fourteen sequences. Codon positions included were 1st+2nd+3rd.

	OP342753 *Loimosina wilsoni*	OP342748 *Loimosina wilsoni*	OP342752 *Loimosina wilsoni*	OP342750 *Loimosina wilsoni*	MN190712 *Empruthotrema dorae*	MN190711 *Empruthotrema dorae*	MN190710 *Empruthotrema aoneken*	MN190709 *Empruthotrema aoneken*	MN190708 *Empruthotrema aoneken*	MN190707 *Empruthotrema orashken*	MN190706 *Empruthotrema orashken*	MN190705 *Empruthotrema orashken*	MN190704 *Empruthotrema orashken*	MN190703 *Empruthotrema orashken*
OP342753 *Loimosina wilsoni*		0.00	0.00	0.01	0.29	0.29	0.21	0.21	0.21	0.25	0.25	0.23	0.24	0.24
OP342748 *Loimosina wilsoni*	0.01		0.00	0.01	0.30	0.30	0.21	0.21	0.21	0.24	0.24	0.22	0.24	0.24
OP342752 *Loimosina wilsoni*	0.01	0.01		0.01	0.29	0.29	0.21	0.20	0.20	0.24	0.24	0.22	0.24	0.24
OP342750 *Loimosina wilsoni*	0.01	0.01	0.01		0.30	0.30	0.21	0.21	0.21	0.24	0.24	0.22	0.24	0.24
MN190712 *Empruthotrema dorae*	0.32	0.32	0.32	0.32		0.00	0.19	0.19	0.19	0.19	0.19	0.20	0.19	0.19
MN190711 *Empruthotrema dorae*	0.32	0.32	0.32	0.32	0.00		0.19	0.19	0.19	0.19	0.19	0.20	0.19	0.19
MN190710 *Empruthotrema aoneken*	0.24	0.24	0.24	0.25	0.21	0.21		0.01	0.01	0.21	0.21	0.20	0.21	0.21
MN190709 *Empruthotrema aoneken*	0.23	0.23	0.22	0.24	0.20	0.20	0.01		0.00	0.21	0.21	0.20	0.21	0.21
MN190708 *Empruthotrema aoneken*	0.24	0.24	0.23	0.25	0.20	0.20	0.01	0.00		0.21	0.21	0.20	0.21	0.21
MN190707 *Empruthotrema orashken*	0.28	0.26	0.27	0.27	0.22	0.22	0.26	0.26	0.26		0.00	0.01	0.01	0.01
MN190706 *Empruthotrema orashken*	0.28	0.26	0.27	0.27	0.22	0.22	0.26	0.26	0.26	0.00		0.01	0.01	0.01
MN190705 *Empruthotrema orashken*	0.24	0.22	0.22	0.23	0.21	0.21	0.25	0.24	0.25	0.03	0.03		0.01	0.01
MN190704 *Empruthotrema orashken*	0.26	0.25	0.25	0.26	0.22	0.22	0.26	0.25	0.26	0.02	0.02	0.01		0.00
MN190703 *Empruthotrema orashken*	0.26	0.25	0.25	0.26	0.22	0.22	0.26	0.25	0.26	0.03	0.02	0.02	0.00	

Table 6: *Erpocotyle microstoma* sclerite measurements in micrometers. Sclerites are numbered 1 through 3, with 1 being closest to the point of attachment of the haptoral appendix. Ranges are given, with averages in parentheses and number of specimens measured. See Figure 1 for images of measurements: A) Tip-to-tip (MacCallum, 1931), B) Perimeter hook length (Euzet & Maillard, 1967), C) Perimeter shaft length (Euzet & Maillard, 1967), D) Shaft length (Bullard & Dippenaar, 2003), and E) Max shaft width (Bullard & Dippenaar, 2003)

Sclerite	A	B	C	D	E
1	230 - 710 (482; n =11)	60 - 91 (71; n = 7)	518 - 1006 (802; n = 7)	345 - 720 (538; n = 10)	50 - 110 (75; n = 11)
2	260 - 650 (477; n = 11)	66 - 91 (81; n = 7)	554 - 980 (824; n = 7)	410 – 680 (547; n = 11)	60 - 120 (81; n = 11)
3	240 - 610 (439; n = 9)	65 - 80 (75; n = 6)	491 - 803 (693; n = 6)	370 - 630 (488; n = 9)	50 - 95 (72; n = 10)

Table 7: *Erpocotyle sphyrnae* sclerite measurements in micrometers. Sclerites are numbered 1 through 3, with 1 being closest to the point of attachment of the haptoral appendix. Ranges are given with averages in parentheses and number of measured specimens. See Figure 1 for images of measurements: A) Tip-to-tip (MacCallum, 1931), B) Perimeter hook length (Euzet & Maillard, 1967), C) Perimeter shaft length (Euzet & Maillard, 1967), D) Shaft length (Bullard & Dippenaar, 2003), and E) Max shaft width (Bullard & Dippenaar, 2003)

Sclerite	A	B	C	D	E
1	82.5 - 257 (164; n = 10)	81 - 166 (122; n = 5)	386 - 444 (424; n = 5)	150 - 347 (253; n = 9)	25 - 50 (38; n = 9)
2	100 - 240 (177; n = 10)	87 - 172 (128; n = 6)	373 - 530 (425; n = 6)	170 - 310 (258; n = 10)	25 - 50 (41; n = 10)
3	75 - 225 (167; n =10)	80 - 141 (124; n = 5)	287 - 508 (375; n = 5)	145 - 305 (238; n = 10)	20 - 475 (80; n = 10)

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4 CHAPTER II: PHYLOGENY OF HEXABOTHRIIDAE PRICE, 1942 USING MAXIMUM EVIDENCE APPROACH (MOLECULAR AND MORPHOLOGICAL)

PHYLOGENY OF HEXABOTHRIIDAE PRICE, 1942 USING MAXIMUM EVIDENCE APPROACH (MOLECULAR AND MORPHOLOGICAL)

ABSTRACT

The phylogeny of Hexabothriidae has been largely based on Boeger & Kritsky's (1989) morphological hypothesis and subsequent revisions for the last 3 decades. However, this analysis excluded two genera for lack of data at the time and four more genera have since been proposed. Since then, the use of molecular data in phylogenetic analysis has grown rapidly. Using a "maximum evidence" dataset comprised of updated morphological characters and available 18S sequences, obtained from GenBank and from our own sequencing, we analyzed and compared the phylogeny of Hexabothriidae using parsimony, Bayesian Inference, and maximum likelihood. In doing so, we have evidenced consistent support for some clades and updated the phylogeny by including recently described and previously excluded genera.

Key Words: Hexabothriidae, phylogeny, maximum evidence, morphology, molecular

4.1 INTRODUCTION

Hexabothriidae Price, 1942 is a family of Monogenoidea (Platyhelminthes, Neodermata) infecting species of Chondrichthyes Huxley, 1880. These external aquatic parasites are found worldwide, have single-host life cycles, and may be diagnosed by the presence of six posterior haptoral suckers (originally referred to as *bothridia*), of which the name - Hexabothriidae - is a testament. There are currently 18 genera in the family, as follows: *Branchotenthes* Bullard & Dippenaar 2003, *Callorhynchocotyle* Suriano & Incorvaia 1982, *Dasyonchocotyle* Hargis 1955, *Epicotyle* Euzet & Maillard 1974, *Erpocotyle* Van Beneden & Hesse 1863, *Heteronchocotyle* Brooks 1934, *Hexabothrium* (Kuhn 1829), *Hypanocotyle* Chero *et al.* 2018, *Mobulicola* Patella & Bullard, 2013, *Narcinecotyle* Torres-Carrera *et al.* 2020, *Neonchocotyle* Ktari & Maillard 1972, *Paraheteronchocotyle* Mayes et al. 1981, *Pristonchocotyle* Watson & Thorson 1976, *Protocotyle* Euzet & Maillard 1974, *Pseudohexabothrium* Brinkmann 1952, *Rajonchocotyle* Cerfontaine 1899, *Rhinobatonchocotyle* Doran 1953, and *Squalonchocotyle* Cerfontaine 1899. The diversity of the family, however, is poorly known and there are certainly more species and supraspecific categories to be discovered.

The phylogeny of Hexabothriidae has been revised several times, with the most recent being 33 years old (Boeger & Kritsky, 1989). Before that, Euzet & Maillard (1974) completed a thorough review and summary of the history of the family, which began with the first description of a single representative specimen by Kuhn (1829) from the gills of two *Scyliorhinus* spp. (Scyliorhinidae Gill, 1862). Boeger & Kritsky (1989) conducted a thorough phylogenetic revision, studying available museum specimens at the time, determining phylogenetically informative character traits, and revising generic descriptions with confirmations of included species. This

phylogeny has been widely accepted and cited. Since then, however, four more genera have been described and added to the family, and two previously described genera, *Pristonchocotyle* Watson & Thorson 1976 and *Pseudohexabothrium* Brinkmann 1952, did not have adequate specimens available for study and were not included in the revision. Additionally, the study by Boeger and Kritsky (1989) was restricted to morphological data.

A search in the National Center for Biotechnology Information (NCBI) GenBank database currently yields 43 sequences from various regions representing 8 genera. Molecular sequences from Hexabothriidae have thus far been used in three studies of intrafamily relationships (see discussion, Chero *et al.,* 2018; Chero *et al.*, 2019; Torres-Carrera *et al.,* 2020). While none of these used a combined dataset, however, did discuss molecular relationships in the context of morphological relationships.

Chero *et al.* (2019) concluded that *Rhinobatonchocotyle* is morphologically similar to *Pristonchocotyle,* and that a phylogeny based on 18S sequences showed that *Rhinobatonchocotyle* shared a common ancestor with *Hypanocotyle,* and these two shared a common ancestor with *Pseudohexabothrium,* supporting their previous (2018) study using 28S sequences. Torres-Carrera *et al.* (2020) also supported *Rhinobatonchocotyle* as a sister group of *Hypanocotyle*. These authors also showed *Narcinecotyle* to be more distantly related to the previously mentioned genera using both 18S and 28S regions, while their species presented morphological similarity to species of *Dasyonchocotyle, Protocotyle, Rajonchocotyle, Hypanocotyle, Erpocotyle, Mobulicola, Branchotenthes, and Squalonchocotyle.*

In this study, we use all current and available morphological and molecular data in a concatenated dataset ("maximum evidence") to determine the phylogenetic relationships between genera of Hexabothriidae.

4.2 MATERIALS AND METHODS

4.2.1 Material collection

See previous chapter, section 3.2.1.

4.2.2 Processing morphological characters

Boeger & Kritsky (1989) argued that existing literature is inadequate when determining homologous series due to a prioritization of autapomorphies in traditional taxonomic descriptions. However, given the closure of most museums regarding sharing of collections due to the COVID-19 pandemic, this study relies on their conclusions from examined museum specimens to compile a morphological character trait matrix, some of which have since been lost from collections and are no longer available to study. Some museum micrographs were obtained and studied - i.e., Meguro Parasitological Museum Collection No. 19551, (*Pristonchocotyle papuensis*, holotype). Morphological characters for more recently described genera were obtained from the literature. Sclerite robustness (see Boeger & Kritsky, 1989), a product of measurements, was not able to be obtained from the literature, thus was excluded from analysis due to lack of data, however these traits are discussed.

Characters are organized into four general categories and include characters 1 - 29 (see Boeger & Kritsky, 1989):

Egg filaments

1. Number of polar egg filaments - single 0; multiple 1; none 2
2. Length of egg filament - long 0; reduced 1

Male Reproductive Organs

The male reproductive system of Hexabothriidae consists of a cirrus, or male copulatory organ (MCO), vas deferens (aka seminal vesicle), vasa efferentia (aka sperm ducts) and testes.

3. Distal portion of cirrus - present 0; absent 1
4. Cirral spines - armed 0; unarmed 1
5. Shape of distal MCO - ovate to subpherical 0; elongate 1
6. Shape of proximal MCO - full dilation 0; nondilated 1; proximal dilation 2
7. Prostatic region - absent 0; present 1
8. Wall of proximal MCO - delicate 0; thick 1
9. Distal vas deferens - thin wall 0; thick wall 1

Female Reproductive Organs

The female reproductive system of Hexabothriidae consists of the ovary (germarium), vitellaria, vaginae, oviduct, ootype, seminal receptacle (SR),

10. Shape of vaginae - parallel 0; Y shaped 1; X shaped 2
11. Differentiation of vaginal duct - differentiated 0; undifferentiated 1
12. Distribution of glands of distal portion of vaginae - cells surrounding entire duct 0; cells only surrounding base 1
13. Musculature of distal portion of vaginae - muscular 0; non-muscular 1
14. Thickness of muscle layer of distal vaginae - delicate 0; thick 1
15. Shape of distal portion of vagina - expanded 0; tubular 1
16. Ootype - smooth 0; presence of longitudinal rows of large cells 1

17. Proximal end of germarium - lobate 0; 1 branched
18. Descending branch of germarium - sinuous/coiled 0; straight 1
19. Ascending branch of germarium - present 0; absent 1
20. Shape of ascending branch - straight 0; sinuous 1
21. Seminal receptacle - present 0; absent 1
22. Oviduct - nondilated 0; dilated 1
23. Modification of distal germarium for sperm storage - unmodified 0; modified 1

Body/haptor morphology

24. Origin of haptoral appendix - marginal 0; dorsal 1
25. Body midline vs longitudinal axis haptor - congruent axes 0; axes form </= 45 angle 1; axes form > 45 angle 2
26. Position of haptoral appendix - body midline 0; lateral to midline 1
27. Anchors - present 0; absent 1
28. Distribution of sucker complexes - symmetrical 0; asymmetrical type a 1; asymmetrical type b 2
29. Position of sucker complex 2 (symmetrical haptors only) - adjacent to remaining complex pairs 0; lateral displacement 1

4.2.3 Molecular Protocol

Extraction, amplification, and sequencing of DNA

Portions of DNA from the nuclear-encoded 18S and 28S ribosomal RNA genes (18S rDNA and 28S rDNA, respectively) were amplified and sequenced for phylogenetic analysis. Primers LSU5 (5'-TAGGTCGACCCGCTGAAYTTAAGCA-3'; Jensen & Bullard, 2010) and 28S_ECD2 (5'-CTTGGTCCGTGTTTCAAGACGGG-3'; Tkach *et*

al., 2003) were used to amplify an 1100 base pair portion of the 28S gene: a 25-µl total volume reaction contained 1X PCR Buffer (Promega, Madison, Wisconsin, USA), 3 mM MgCl2, 0.4 mM dNTPs (Promega), 0.4X Rediload gel loading buffer (Invitrogen, Waltham, Massachusetts, USA), 0.6 µM of each primer, 1 U GoTaq® Flexi DNA Polymerase (Promega), and 3 µl template DNA. Cycling was as follows: 5 min initial denaturation at 94 °C, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s, extension at 72 °C for 45 s, then a final extension at 72 °C for 5 min. DNA was extracted from parasite tissue using a DNeasy (Qiagen, Valencia, California, USA) Blood and Tissue kit following manufacturer's protocol.

DNA from almost the entire portion of the nuclear-encoded 18S ribosomal RNA gene (18S; ~1,800) was amplified and sequenced for analysis of a molecular phylogeny using primers WormA (5'-GCGAATGGCTCATTAAATCAG- 3'; Littlewood & Olsen, 2001) and WormB (5'-CTTGTTACGACTTTTACTTCC- 3'; Littlewood & Olsen, 2001): a 25-µl total volume reaction contained 2.5 µl 10X PCR Buffer (Invitrogen), 1.5 µl MgCl2 (50 mM), 0.4 µl dNTPs (25 mM) (Invitrogen, Waltham, Massachusetts, USA), 0.3 µl (50 pmol/µl) of each primer, 0.3 µl (5 U) Platinum Taq DNA-polymerase (Invitrogen), 2 µl of template DNA, and distilled water to complete volume. Cycling was as follows: 5 min initial denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 30 s, extension at 72 °C for 45 s, and then by a final extension at 72 °C for 5 min.

All products were electrophoresed in 1.5% agarose gels stained with GelRed ™ (Biotium, Fremont, California, USA) and visualized under a UV light. Products were purified using MiniElute purification kit (Qiagen, Valencia, CA) following the manufacturer's protocol, using ddH2O for the final elution step, and then sent to GoGenetic (UFPR) for direct, bi-directional Sanger sequencing using the same

primers as above. Complementary sequences were assembled, compared to their chromatograms, and edited accordingly using Geneious version (4.8.5) (http://www.geneious.com, Kearse *et al.,* 2012). Resulting sequences were compared with sequences available in the National Center for Biotechnology Information GenBank (NCBI GenBank) database using the Basic Local Alignment Search Tool (BLAST - Altschul *et al.,* 1990) and were deposited into GenBank (Table 1).

Table 1: Sequences of 18S and 28S rDNA used in this analysis and produced in the present study for phylogenetic analysis of family Hexabothriidae.

	GenBank No.		**Reference**
Hexabothriida	**18S**	**28S**	
Erpocotyle microstoma (Brooks, 1934)	9BNA-18S 3425-18S	x4_10/08/18, x5_10/8/18, x1_02/8/19, x11_02/08/19, x2_04/17/19, x7_04/17/19, x11_4/17/19, x12_4/17/19, x19_4/17/19, x20_4/17/19, x22_4/17/19, x32_4/17/19, x14_8/21/19	Present study
Erpocotyle sphyrnae (MacCallum, 1931)	-	x3_10/08/18, x9_02/08/19, x15_08/21/19	Present study
Hexabothrium sp. (Kuhn 1829)	-	AF131724, MT890136, MT890139, MT890140	Mollaret *et al.* (2000); Unpublished
Hypanocotyle bullardi Chero *et al.*, 2018	MG591249, MG591250	MG591251	Chero *et al.* (2018)
Narcinecotyle longifilamentus Torres-Carrera *et al.*, 2020	MN447332	MN367805, MN367803	Torres-Carrera *et al.* (2020)

Pseudohexabothrium taeniurae Agrawal, 1996	AJ228791	AF382035	Littlewood *et al.* (1998); Olson & Littlewood (2002)
Rhinobatonchocotyle pacifica Oliva & Luque, 1995	MH724313	MH714464	Chero *et al.* (2019)
Diclybothriidae (outgroup)			
Paradiclybothrium pacificum Bykhovskii & Gusev, 1950	KP796244	-	Rozhkovan & Shedko (2015)

4.2.4 Combined Data Analysis

Alignment and phylogenetic analysis

Sequences of 18S and 28S rDNA generated in this study and obtained from NCBI GenBank for species of variable genera of Hexabothriidae and from outgroup taxa were aligned using GUIDANCE 2 (Penn *et al.*, 2010a, 2010b) with our sequences using multiple sequence alignment (MSA) algorithm MAFFT (Katoh *et al.*, 2002) and set to 100 alternative guide-trees. Alignments of 28S sequences were used to infer a maximum likelihood molecular evolutionary history using the Hasegawa-Kishino-Yano model (HKY; Hasegawa *et al.,* 1985) and a discrete Gamma distribution (+G, parameter = 0.5253) for rate differences among sites, support values obtained by 1000 bootstrap replicates (Felsenstein, 1985), using MEGA11 (Tamura *et al.,* 2021) (Figure 3). According to the analysis, initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Likewise, an analysis of 18S sequences used the Kimura 2-parameter model (K2; Kimura, 1980) and discrete Gamma distribution (parameter = 0.0765), obtaining

support from a bootstrap of 1000 replicates (Figure 2). Consensus trees are presented with collapsed low support branches (less than 50%).

A concatenated matrix of molecular and updated morphological data (maximum evidence) was constructed using 29 transformation series and edited using Mesquite v3.61 (Maddison & Maddison, 2021) and includes 19 terminal taxa: 18 in-group taxa from Hexabothriidae and 1 out-group taxa as Diclybothriidae. Unknown states were coded as "?" and non-applicable characters or gaps were coded as "-"; if more than one state was present in the same genus, both were coded for (Figure 1).

This total maximum matrix was saved as a nexus file and analyzed using three methods: maximum parsimony, Bayesian, and maximum likelihood.

```
Hexabothrium            00000000010000000000000000000
Dasyonchocotvle         10001110000?00?0????0??000001
Rhinobatonchocotyle     100111??020?010???????00002?
Rajonchocotvle          1111?000011?10?10001000000000
Heteronchocotvle        100101{01}100??01?0001?11001102?
Paraheteronchocotyle    00011?????????0001?1?000012?
Erpocotyle              10010110100000001100000000000
Squalonchocotyle        10011110000110011000000000000
Epicotvle               10011110000100100000000101000
Protocotvle             10011210001?10?10001001000000
Callorhvnchocotyle      10010101000000100000000012101?
Neonchocotyle           10011110000100100000000012101?
Branchotenthes          10011001?0000??00000100000000
Hypanocotyle            10011?1100000110100?1??100000
Mobulicola              10?1100110000100000?100100000
Narcinecotyle           100110?1?0??0100000100?100000
Pristonchocotyle        10010????20?01000000100???0??
Pseudohexabothrium      00010????11?00?00000{01}00000001
Diclybothriidae         2-001000101???0000?010000000
```

Figure 1: Morphological character states shown as coded in the Nexus files for analysis. Question marks (?) are unknown states, numbers within brackets are multistate characters.

Maximum parsimony

Maximum parsimony was conducted in TNT v1.5 (Goloboff *et al.*, 2003) following a protocol of successive weighting (Farris, 1969) under parameters: max.trees = 9999, characters = nonadditive (unordered), wherein consecutive reweightings are run (REWT.RUN command) until character weights are stabilized (i.e. the program states no more changes). Weights are included in Table 2. An implicit enumeration analysis was then run, and a consensus tree was produced from the resulting best (most parsimonious) trees (Figure 4). Consistency and retention indices were used to compare trees. To avoid over- or underestimation of group support given different weights, symmetric resampling (P = 33) of differences in group frequencies (GC) was conducted for branch support (Goloboff *et al.,* 2003) for 1000 replicates of the strict consensus. For comparison, weights were left equal for a different run.

Maximum Likelihood

Maximum likelihood was executed in the raxmlGUI 2.0 (version 2.0.6) (Stamatakis, 2014; Silvestro, 2012) by uploading the sequence alignments and morphological matrix as separate files and running ML + rapid bootstrap under the GTR+G model for 1000 independent tree searches. RaxmlGUI 2.0 has no option for allowing "assumptions" such as character weights in the program, so only equally weighted characters were used (no "assumptions" coded into the Nexus file). As well, it does

not allow for coding of ambiguous character states, so for the two incidents recorded in our matrix, these were left as the states determined by Boeger & Kritsky (1989). The final consensus tree was edited in FigTree v1.4.4 with bootstrap (bipartition) values shown (Figure 5).

Bayesian analysis

Bayesian analysis was performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) with morphological characters reweighted using the values determined during the successive reweighting protocol in TNT. These values can be coded into a Nexus file under "assumptions". As with parsimony, weights were left equal in a separate run for comparison. When executed in the program, each dataset was partitioned into 3 parts: morphological (standard) and two parts DNA (18S and 28S, respectively). For DNA, the GTR+I model was used, and for morphological data a model with equal state frequencies and gamma-distributed rate variation across sites was used. Two runs of the Markov chain Monte Carlo (MCMC) were performed with 20 million generations. Convergence was determined by the stabilization of the log likelihood of the cold chain, and by the potential scale reduction factor (PSRF) stabilizing around 1.000 (Gelman & Rubin, 1992). By default, the first 25% of trees were discarded (burn-in). The resulting 50% consensus tree was edited via FigTree v1.4.4 with posterior probabilities shown as branch support (Figure 6).

Character Mapping & Tree Editing

Morphological characters were mapped onto each consensus tree using Mesquite v3.61 (Maddison & Maddison, 2019) using Parsimony Ancestral States in Ancestral

State Reconstruction. MS Powerpoint was used to mark each character state transition on the final edited trees (Figures 4, 5, 6).

Table 2: Character weights as assigned by successive reweighting (Farris, 1969) in TNT (Goloboff *et al.,* 2003). Weights are assigned on a scale 0 - 100, with 0 having the least amount of cladistic reliability (greater homoplasy/reversals) and 100 having the greatest amount of cladistic reliability (less homoplasy/reversals). Character numbers (1 - 29) are dark shaded, with respective weights in the row below.

1	2	3	4	5	6	7	8	9	10
67	1	1	33	20	50	25	50	33	67
11	12	13	14	15	16	17	18	19	20
33	50	100	100	50	100	33	1	100	50
21	22	23	24	25	26	27	28	29	
100	50	1	33	100	50	1	100	50	

4.3 RESULTS

4.3.1 Sequences generated

In total, two 18S and sixteen 28S sequences were generated: for *E sphyrnae* - 3 sequences of 28S; for *E. microstoma* - 13 sequences of 28S and two sequences of 18S. (accession numbers in Table 1; sequence compositions in Table 3).

Table 3: Sequence compositions, with no gaps, per *Erpocotyle* species collected from gills of *Sphyrna* spp.: ranges of lengths in base pairs (bp); variable sites (V); parsimony-informative sites (PI); percent composition of each nucleotide. Variable and PI sites of 28S sequences are compared between both species of *Erpocotyle*.

	Length (bp)	V	PI	A%	T%	C%	G%
18S							
E. microstoma	1700 - 1720	NA	NA	22.4	25.2	22.3	30.1
28S							
E. microstoma	748 - 881	18	17	25.1	19.9	33.5	21.6
E. sphyrnae	590 - 851			24.7	20.4	33.1	21.8

4.3.2 Phylogenetic analysis

Molecular analysis

Sequences of 18S and 28S showed a clear monophyly between *E. microstoma* and *E. sphyrnae* (Figures 2 & 3, respectively). Both molecular phylogenies of 18S and 28S show *Rhinobatonchocotyle* and *Hypanocotyle* forming a monophyletic clade. *Dasyonchocotyle* forms a monophyletic clade with *Pseudohexabothrium* in the 28S topology (Figure 3), also showing a close relationship in the 18S topology (Figure 2). Two *Hexabothrium* sequences form a monophyletic pair in the 28S sequences,

whereas the other two sequences of this genus are distinctly separated.

Figure 2: Consensus maximum likelihood molecular phylogenetic tree inferred using the K2+G model for Hexabothriidae 18S sequences from this study and from GenBank, performed in MEGA11 (Tamura *et al.,* 2021). Bootstrap support was obtained from 1000 replicates and is shown next to each branch, with branches that showed less than 50% support collapsed. The final dataset contained 9 nucleotide sequences representing six species from six genera with a total of 1988 positions.

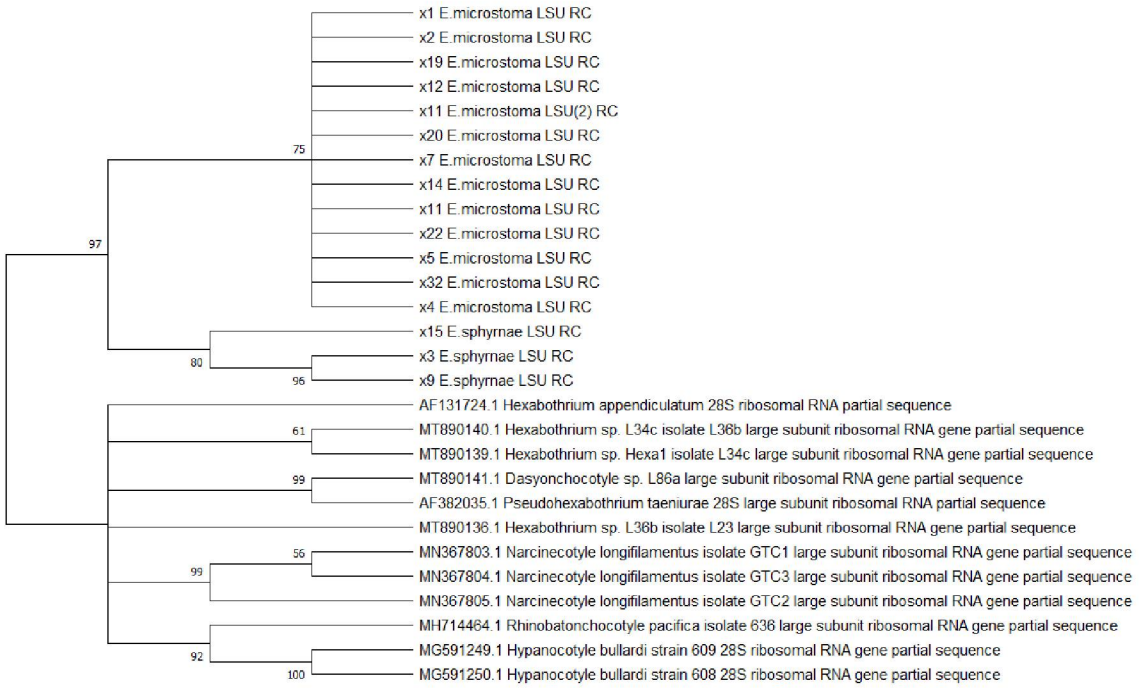

Figure 3: Consensus tree of maximum likelihood criterion inferred using the HKY+G model for 28S sequences of Hexabothriidae from this study and from GenBank, performed in MEGA11 (Tamura *et al.,* 2021). Bootstrap support was drawn from 1000 replicates and is shown next to each branch, with branches that showed less than 50% support collapsed. The final dataset contained 28 nucleotide sequences representing 10 species from seven genera with a total of 1409 positions.

Parsimony vs Bayesian vs Likelihood

In general, characters left with equal weights (EQ) produced trees with less resolution than reweighted characters (RW), thus only the topology from RW trees will be used for further discussion of parsimony and Bayesian results. For maximum parsimony (MP), reweighted characters produced 9 equally parsimonious trees (EPTs) with length 26,284. The strict consensus tree is shown in Figure 4. For the Bayesian inference (BI) analysis, convergence occurred after 10 thousand

generations, and PSRF was min/max: 1.000; average minESS: 1434. Lastly, final optimization likelihood for maximum likelihood (ML): -8170.95

Maximum parsimony and maximum likelihood produced the most resolved trees, while Bayesian inference left some clades unresolved (Figures 4, 5, and 6, respectively). Overall, several clades were consistently supported by the different criteria and are summarized in Table 4, along with their supporting character transformations.

Clade A (*Dasyonchocotyle* & *Pseudohexabothrium*; Figures 4 - 6) is supported by all three maximum evidence phylogenies, with significant statistical support (summarized in Table 4). However, the only character state shared by these two genera is a lateral displacement of sucker complex 2 (character 29), which given the score of 50, is neither considered homoplastic nor phylogenetically significant.

Clade B1 (*Rhinobatonchocotyle, Pristonchocotyle, Heteroncotyle,* & *Paraheteroncotyle*; Figures 4 - 6) is recovered in all three analyses, although with acceptable statistical support from likelihood only (Table 4), where it is nested within Clade C (Fig. 4) and remains fairly unresolved in BI (Fig. 6). Within the clade, *Heteronchocotyle* and *Paraheteronchocotyle* form a sister-pair, supported by absence of an ascending branch of the germarium (character 19) and the excluded sclerite robustness (sclerites 1, 1' and 2 more robust than 2', 3', and 3); however the sister-pairing of *Rhinopristonchotoyle* and *Pristonchocotyle* is only supported by parsimony, and morphologically an X-shaped vagina (character 10) is a shared character, however the exact relationship between these two (monophyletic or paraphyletic) and the others remains unclear.

Clade B2 (*Squalonchocotyle, Rajonchocotyle, & Protocotyle*; Figures 4-6) is presented in all three cladograms, although only with statistical support by MP and ML. Character state transitions for longitudinal rows of large cells in the ootype (character 16) - also known as "ootype côtelé" (Euzet & Maillard, 1974) - and a non-muscular distal portion of the vagina (character 13) show phylogenetic signal to support the monophyly of the clade composed by these three taxa. Both characters received high weights (Table 2) during successive reweighting, suggesting they present as synapomorphies with high cladistic reliability (Farris, 1969; DeSalle *et al.,* 2013). Within this clade, *Rajonchocotyle* and *Protocotyle* are further grouped by synapomorphies of an undifferentiated vaginal duct (character 11) and sinuous ascending germarium branch (character 20), and the pair is well supported statistically by likelihood and parsimony, however an undifferentiated vaginal duct appears to be homoplastic as it is also present in *Pseudohexabothrium* (Clade A), while a sinuous ascending germarium branch is homoplastic, present in *Narcinecotyle* (Clade C).

Clade B3 (*Epicotyle, Callorhynchocotyle, & Neonchocotyle*; Figures 4 - 6) is statistically supported in all three analyses, with several character series with somewhat cladistic reliability (scores inTable 2; summary Table 4). Within this group, *Callorhynchocotyle* and *Neonchocotyle* form a monophyletic sister-group, significantly supported statistically (GC = 78; BS = 63; PP = 97), while *Callorhynchocotyle* retains the plesiomorphic trait of cells surrounding the entire distal duct of the vaginae (character 12).

Clade C is supported in parsimony as a paraphyly between *Branchotenthes, Hypanocotyle*, *Narcinecotyle*, and *Mobulicola*, and these genera tend to cluster in each cladogram (Figures 4 - 6). These genera share a fully dilated proximal MCO

(character 6), a plesiomorphic trait per Boeger & Kritksy (1989), a thick muscle wall of proximal MCO (character 8) and thick layer of the distal vaginae (character 14), traits that are shared by Clade B1; as well, the absence of a seminal receptacle (character 21) is shared by all except *Narcinecotyle*. Finally, a dorsal origin of the haptoral appendix (character 24) is shared by *Narcinecotyle, Hypanocotyle*, and *Mobulicola*, but this trait is also present in Clade B3. If character weights are considered, characters 14 and 21 have high scores representing high cladistic reliability (Table 2). Thus, the phylogenetic positioning of a paraphyly formed by *Mobulicola, Branchotenthes, Hypanocotyle,* and *Narcinecotyle* within or near Clade B1, as shown by all three cladograms, would be the most supported by morphological character series.

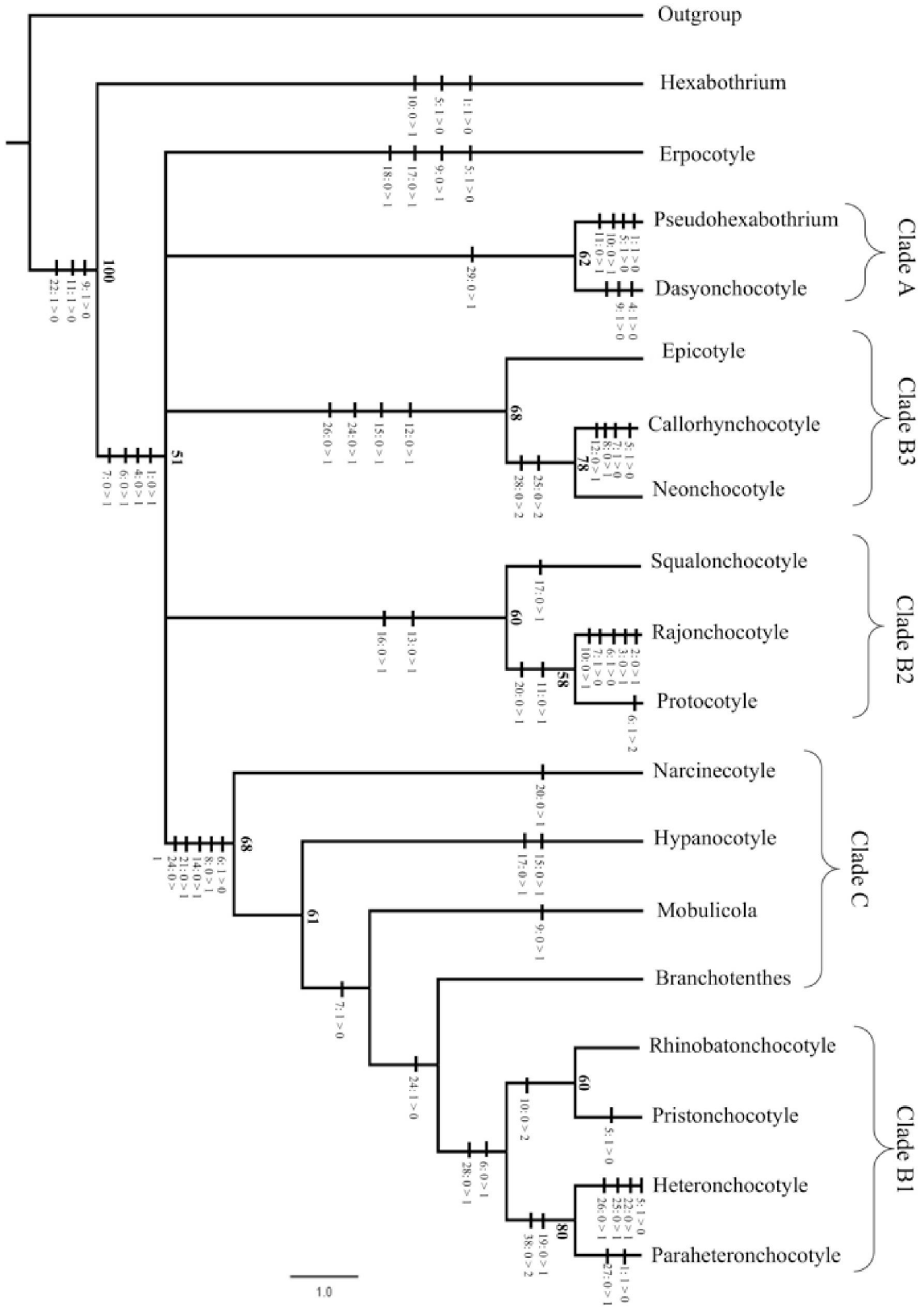

Figure 4: Consensus parsimony tree for genera of Hexabothriidae produced using successive reweighting (Farris, 1969) with the support shown as group frequency (GC)

values from symmetric resampling (P = 33) next to each node, using 1000 replicates, conducted in TNT v1.5. Consistency Index (CI) = 0.708, Retention index (RI) = 0.602. Main clades are labeled (A, B1, B2, B3, C) using brackets. Character state transitions are mapped onto the topology, with the character number followed by the state transition.

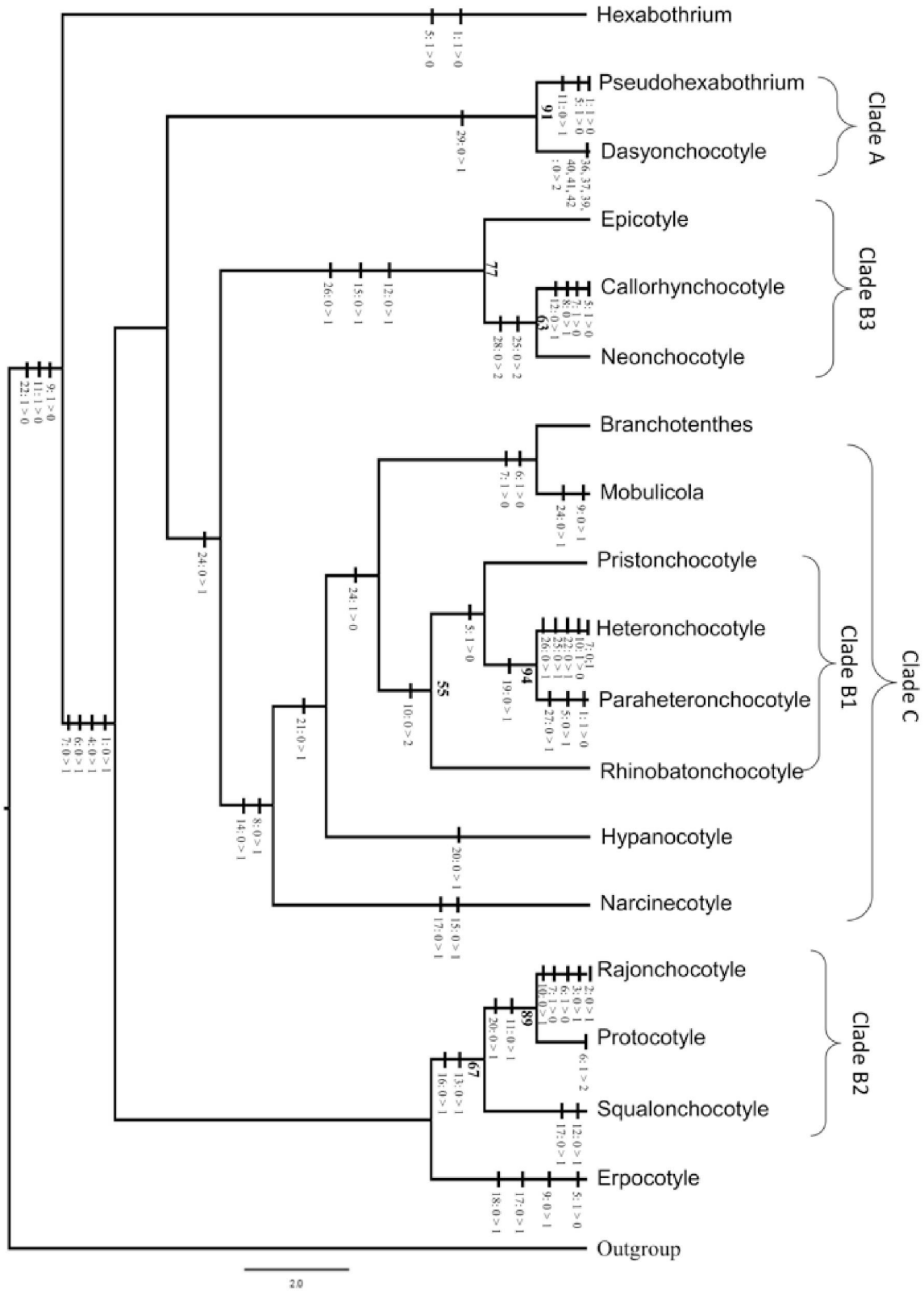
Hexabothrium
Pseudohexabothrium
Dasyonchocotyle
Clade A
Epicotyle
Callorhynchocotyle
Neonchocotyle
Clade B3
Branchotenthes
Mobulicola
Pristonchocotyle
Heteronchocotyle
Paraheteronchocotyle
Rhinobatonchocotyle
Clade B1
Clade C
Hypanocotyle
Narcinecotyle
Rajonchocotyle
Protocotyle
Squalonchocotyle
Erpocotyle
Clade B2
Outgroup
2.0

Figure 5: Maximum likelihood tree topology for family Hexabothriidae with support shown as bootstrap (BS) values next to each node, produced in raxmlGUI 2.0 (version 2.0.6) (Stamatakis, 2014; Silvestro, 2012) using the GTR+G model for 1000 independent tree searches for a concatenated dataset of equally weighted morphological characters and 18S and 28S sequence alignments. Main clades are labeled (A, B1, B2, B3, C) using brackets. Character state transitions are mapped onto the topology, with the character number followed by the state transition.

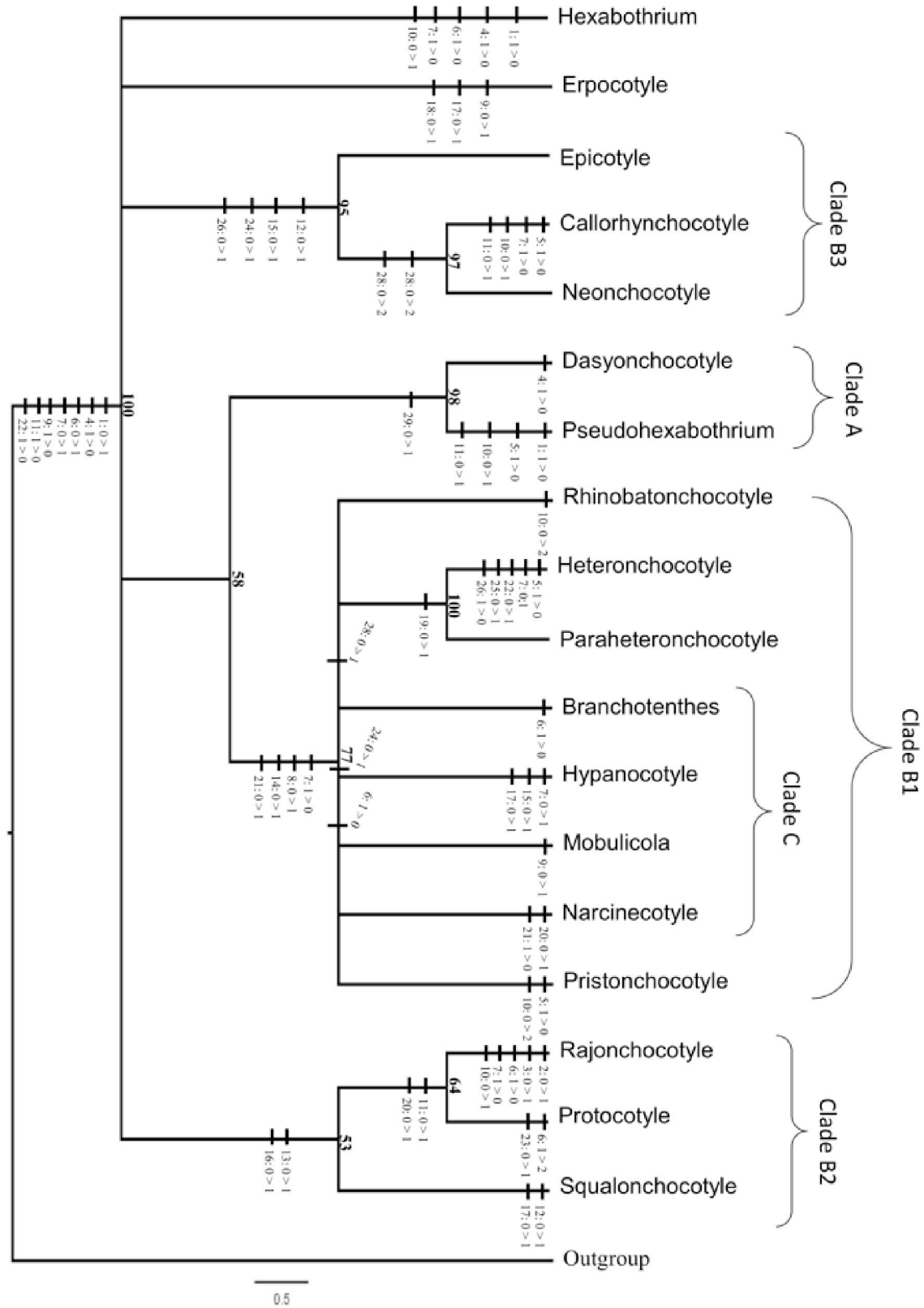
Hexabothrium
Erpocotyle
Epicotyle
Callorhynchocotyle
Neonchocotyle
Dasyonchocotyle
Pseudohexabothrium
Rhinobatonchocotyle
Heteronchocotyle
Paraheteronchocotyle
Branchotenthes
Hypanocotyle
Mobulicola
Narcinecotyle
Pristonchocotyle
Rajonchocotyle
Protocotyle
Squalonchocotyle
Outgroup
Clade B3
Clade A
Clade B1
Clade C
Clade B2
0.5

Figure 6: Bayesian inference 50% consensus tree for family Hexabothriidae; posterior probabilities as support next to each node, produced using a concatenated dataset of reweighted morphological characters (Table 2), 18S and 28S sequence alignments performed in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). The GTR+G model was used for molecular data and equal state frequencies and gamma-distributed rate variation across sites for morphological data, for two runs of the Markov chain Monte Carlo (MCMC) with 20 million generations.

Table 4: Summary of taxa in the main clades presented by the various analyses in the present study. Statistical support (GC = group frequencies, BS = bootstrap, PP = posterior probability) is provided under each type of analysis (MP = maximum parsimony, ML = maximum likelihood, BI = Bayesian analysis); dashes (-) mean the clade was not present in the tree generated by the respective analysis. Apomorphies of each clade are included with the character number in parentheses.

Clade	Taxa	MP (GC)	ML (BS)	BI (PP)	Characters
A	*Dasyonchocotyle* *Pseudohexabothrium*	62	91	98	• Lateral displacement of sucker complex 2 (29)
B1	*Rhinobatonchocotyle* *Pristonchocotyle* *Heteroncotyle* *Paraheteroncotyle*	-	55	77	• Absence of an ascending branch (germarium) (19) • Asymmetrical type a distribution of sucker complexes (28)
B2	*Squalonchocotyle* *Rajonchocotyle* *Protocotyle*	60	67	53	• Non-muscular distal portion of vaginae (13) • Longitudinal rows of cells in ootype (16)
B3	*Epicotyle* *Callorhynchocotyle* *Neonchocotyle*	68	77	95	• Distal portion of vaginae tubular (15) • Dorsal origin of haptoral appendix (24) • Lateral to midline position of haptoral appendix (26)
C	*Branchotenthes* *Narcinecotyle Mobulicola* *Hypanocotyle*	68	-	77	• Full dilation of proximal MCO (6) • Thick wall of proximal portion of MCO (8) • Thick muscular layer of distal vaginae (14) • Absence of seminal receptacle (21) • Dorsal origin of haptoral appendix (24)

4.4 DISCUSSION

Three clades presented in this analysis are also present in Boeger & Kritsky's (1989) hypothesis: Clade B1, B2, and B3. In our analysis, *Pristonchocotyle* was included in Clade B1, which was not included in the analysis of Boeger & Kritsky's (1989). An asymmetrical type a distribution of sucker complexes (character 28) appears to be a synapomorphy for the entire clade B1, however *P. papuensis* Ogawa 1991 is described as having "five arranged in a row along ophistohaptoral margin…[with the] remaining hook…situated inside and posterior to the anteriormost hook". In the description of the type species and generic diagnosis provided by Watson & Thorson's (1976), the symmetry of the haptor is not described, and it is noted that the holotype is an "abnormal specimen" due to a damaged haptor. Museum specimens did not clarify the type of asymmetry, although it is apparent that the haptor is asymmetrical in *Pristonchocotyle*. It is most likely the same as *Rajonchocotyle* (type b), which would support the grouping of these two.

Hexabothrium formed a basal branch in all three cladograms, consistent with conclusions based on morphology by Boeger & Kritsky (1989) and the present study. It does, however, share several character series of plesiomorphic traits (according to Boeger & Kristky 1989) of a single egg filament (character 1) and ovate to subspherical distal MCO (character 5) with *Pseudohexabothrium*, as well as a Y-shaped vagina (character 10), which appears to be homoplastic in *Rajonchocotyle.* However, these genera appear relatively distant, given the evidence of the present study, and that *Pseudohexabothrium* is more closely related to *Dasyonchocotyle,* although morphologically these two have little in common. Euzet & Maillard (1974)

originally suggested greater phylogenetic proximity of *Pseudohexabothrium* to *Rajonchocotyle* and *Rhinobatonchocotyle* than to *Hexabothrium,* based on the morphology of the vaginae - at the time only two states were recognized (V-shape or parallel). This character was revised by Boeger & Kritsky (1989) to have three states (Y- or X- shaped vaginae, or parallel). Also, it was stated by Boeger & Kritsky (1989) that *Hexabothrium* "clearly show the vaginal duct in a Y-shaped configuration", and the only accepted confirmed species is *H. appendiculatum*. Euzet & Maillard (1974) included in their study species of *Hexabothrium* which were later designated to other genera, with parallel vaginae. *Pseudohexabothrium* was not included in the Boeger & Kritsky (1989) tree because of the poor quality of available specimens for determining character states. However, the genus was subsequently amended by Agrawal *et al.* (1996) and sequenced by Littlewood *et al*. (1998). The sequence and amended polymorphic state (presence or absence) of the seminal receptacle (character 21) were included in the maximum evidence analyses. This same character also scored a weight of high cladistic reliability, although it appears to display some plasticity. The other *Hexabothrium* sp. sequences used only in the molecular analysis are most likely of another genera, or even possibly a different family, given that the hosts listed for these specimens in GenBank are a member of Osteichthyes (*Macrourus holotrachys* Günther, 1878), a member of Squaliformes (*Etmopterus granulosus* (Günther, 1880)), and a member of Rajiformes (*Bathyraja peruana* McEachran & Miyake, 1984). The type species *H. appendiculatum* has only been identified in host species of *Scyliorhinus* Blainville, 1816 (Carcharhiniformes).

Glennon *et al.* (2005) amended the generic diagnosis of *Branchothenthes* to have a thick or thin walled (polymorphic) distal vaginae (character 14) in their description of *Branchotenthes octohamatus* Glennon *et al.*, 2005. Given the

suggested proximity of the genus to Clade B1, in which a thick layer of the distal vagina is a synapomorphy, polymorphy is accepted as a retention of the primitive state of this clade in *Branchotenthes.*They also stated that the proximal wall of the MCO (character 8) was much thinner in their species than in the type species, although this trait demonstrates some homoplasy.

In Boeger and Kritsky's (1989) analysis and in the present analysis, the grouping of *Callorhynchocotyle* and *Neonchocotyle* was supported by two synapomorphies: a greater than 45-degree angle formed by the body midline vs longitudinal axis of the haptor (character 25) and asymmetrical type a distribution of sucker complexes (character 28). At the time of Boeger & Kritsky's (1989) publication, there existed only one paratype specimen, and it was stated that the dorsal origin of haptoral appendix (character 24) needed to be verified as the existing specimen was not helpful for the morphological characterization of this trait. Subsequently, *Neonchocotyle* was revised by Quiterio-Rendon, et al. (2018) when they described a new species, which confirmed the dorsal originating appendix. However, while these authors stated that the angle of the haptoral axis was approximately a 45-degree angle, this could not be confirmed in their illustrations.

Boeger & Kritsky (1989) originally considered the absence of a seminal receptacle as plesiomorphic but determined through *a posteriori* optimization that the presence of the SR is actually plesiomorphic, and absence is considered a secondary loss of the structure. This character appears polymorphic in *Pseudohexabothrium* and is likely a novel secondary loss of the structure, however absence of the seminal receptacle is a state of Clade B1 and Clade C, except for *Narcinecotyle,* which is suggested as a basal member of Clade C per likelihood and parsimony, thus the SR could have been lost in a common ancestor between the rest

of Clade C and Clade B1. A close common ancestor between Clades B1 and C is further supported by previous molecular phylogenies (Chero et al., 2018; Torres-Carrera et al., 2020), and by the 28S molecular phylogeny presented here.

Similar to *Psuedohexabothrium,* the state of the SR appears polymorphic in *Rhionopristonchocotyle*; when Boeger & Kritsky (1989) reviewed the type specimen for *Rhinobatonchocotyle cyclovaginatus* Doran, 1953, they noted the absence of a seminal receptacle (character 21) and the presence of a smooth ootype (character 16). Chero et al. (2019) confirmed in their specimens and in the type of the later described *R. pacifica* Oliva & Luque 1995 the smooth ootype, however noted a presence of the seminal receptacle. Unlike *Psuedohexabothrium*, *Rhinobatonchocotyle* is a member of Clade B1, which as discussed are largely supported by the secondary loss of the organ. Given the nestedness of this genus far into the clade, the SR may likely be a reappearance of an ancestral state.

Torres-Carrera et al. (2020) claimed the origin of the haptoral appendix was not phylogenetically informative because *Rhinobatonchocotyle* and *Hypanocotyle* did not group with *Narcinecotyle* in their analysis, however they mistakenly stated that species of *Rhinobatonchocotyle* have a dorsal-originating haptoral appendix, but it has been confirmed several times that these depict a marginally originating haptoral appendix (Boeger & Kritsky 1989; Chero et al. 2019). Chero et al. (2018) proposed *Hypanocotyle* for species having an appendix "originating from dorsal surface of haptor" but later in the same article compare it to *Erpocotyle* as "both genera have…an appendix that originates from the haptoral margin between the posterior-most pair of haptoral suckers". These types of morphological discrepancies need to be clarified through study of museum specimens, of which we did not have access during this study.

Hexabothriids are found to infect species of almost all orders of Chondrichthyes, and it is probable that they in fact infect all orders. According to the literature, *Erpocotyle* is considered to infect the species from most orders (7 total), however Boeger & Kritsky (1989) determined most of those species to be unconfirmed and stated that many of these described species likely belong to other genera. Given this conclusion, Hexabothriidae do not appear to have extreme host-specificity and, instead, utilize opportunity to expand their host repertoires; this would explain incidences of polymorphism and retained character states, and the overall diversity found within the family (Araujo et al., 2015; Brooks et al., 2019). In conclusion, members of Hexabothriidae comprise at least 4 to 5 monophyletic clades, with *Hexabothrium* being a basal member.

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5 FINAL CONSIDERATIONS & FUTURE RESEARCH: DISTRIBUTION AND HOST-RANGE OF *LOIMOSINA WILSONI*, THE STOCKHOLM PARADIGM AND MONOCOTYLIDAE

Short communication

Monogenoidea (Platyhelminthes), a class of mainly ectoparasitic (with some examples of endoparasitic) flatworms, can be found globally infecting all orders of fishes, some reptiles/amphibians, cephalopods, and one example of a mammal (Stunkard, 1924) in both marine and freshwater ecosystems. Dalrymple (Chapter 1) noted that the host range of *Loimosina wilsoni* is within *Sphyrna* spp. (Sphyrnidae; Carcharhiniformes), a genus of sharks currently known to be composed of 9 species.

Species of *Loimosina* were allocated in the family-group Loimoidae Price 1936 which was originally composed by *Loimos* MacCallum, 1917 (the type genus), *Loimopapillosum* Hargis, 1955, and *Loimosina* Manter, 1944. However, the family is presently considered polyphyletic due to the phylogenetic position of *L. wilsoni* (Boeger *et al.* 2014; Dalrymple, Chapter 1) and Loimopapillosum (Chero *et al.,* 2021). Both species are subordinate taxa of the Monocotylidae (Boeger *et al.*, 2014). *Loimosina wilsoni* is phylogenetically related to species of *Neoheterocotyle* Hargis, 1955 and *Troglocephalus* Young, 1967, while *Loimopapillosum pascuali* groups with *Heterocotyle capricornensis* Chisholm & Whittington, 1996 - *L. wilsoni* and *Loi. pascuali* are not closely related in the phylogeny of Monocotylidae. However, while other members of Monocotylidae have been found to infect Carcharhiniformes, the genera forming a clade with *Loimosina* are largely found infecting members of Rhinopristiformes and Myliobatiformes (Figures 2 & 3).

Monogenoids have a reputation for narrow host ranges, so this poses the question as to how these parasites colonized species of taxa phylogenetic distantly-related to their original host?

The hosts of *L. wilsoni, Sphyrna* spp., are members of Carcharhiniformes, an order to sharks with species distributed in all oceans. A modern phylogeny of the parphylum Chondrichthyes, provided by Kousteni *et al.* (2021) (Figure 1) shows the main classes (Elasmobranchii and Holocephali) and infraclasses (Selachii and Batoidea), and the orders contained in each. Elasmobranchii and Holocephali have

an estimated divergence of ~338.0 - 471.0 MYA (TimeTree, Kumar *et al.*, 2017; estimation derived from 7 studies), and Selachii and Batoidea have an estimated divergence time of about 249.5 - 343.0 MYA (TimeTree, Kumar *et al.,* 2017; based on 8 studies).

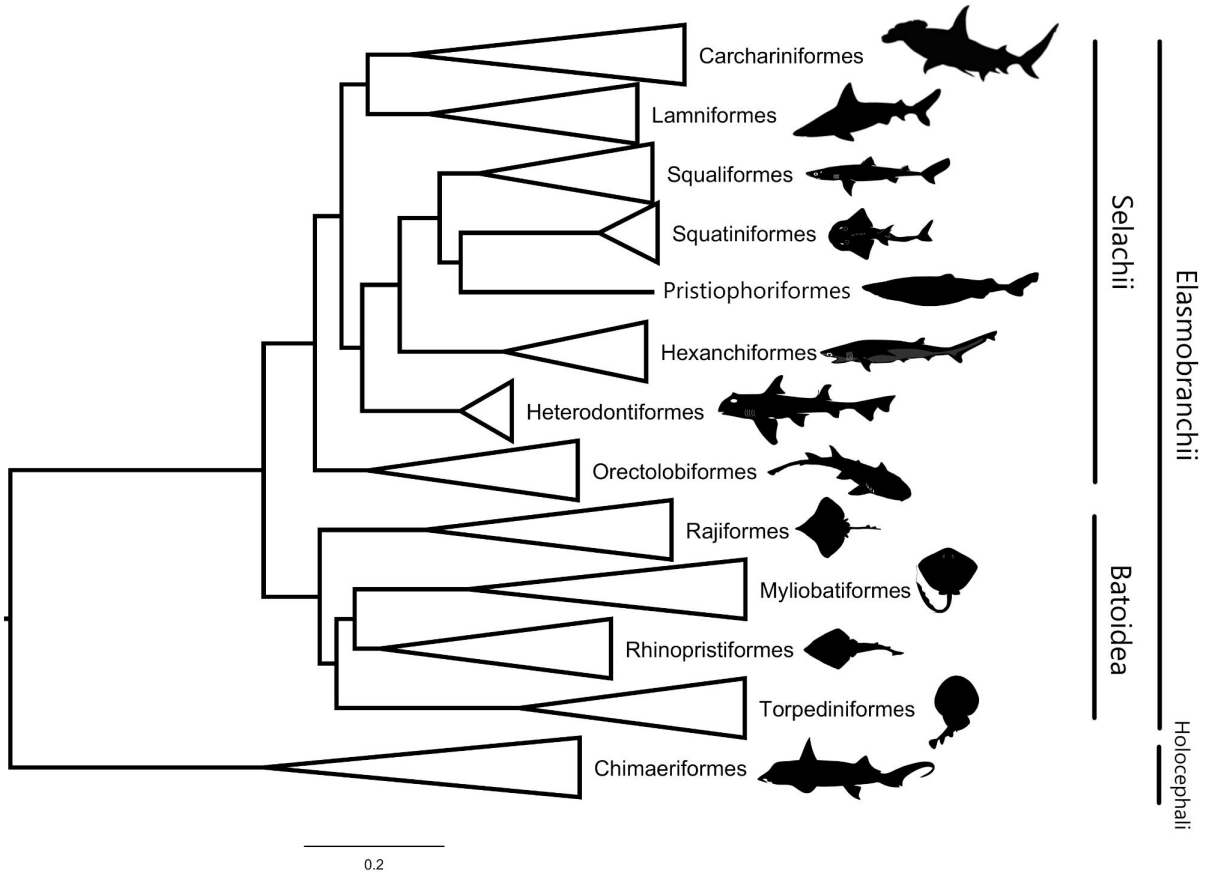

Figure 1: Maximum likelihood phylogenetic tree of Chondrichthyes based on a concatenated dataset of 13 individual protein coding genes, provided by Kousteni *et al.* (2021). Image credit for Rajiformes to Ignacio Contreras and for Chimaeriformes to Tambja (vectorized by T. Michael Keesey; https://creativecommons.org/licenses/by/3.0/legalcode).

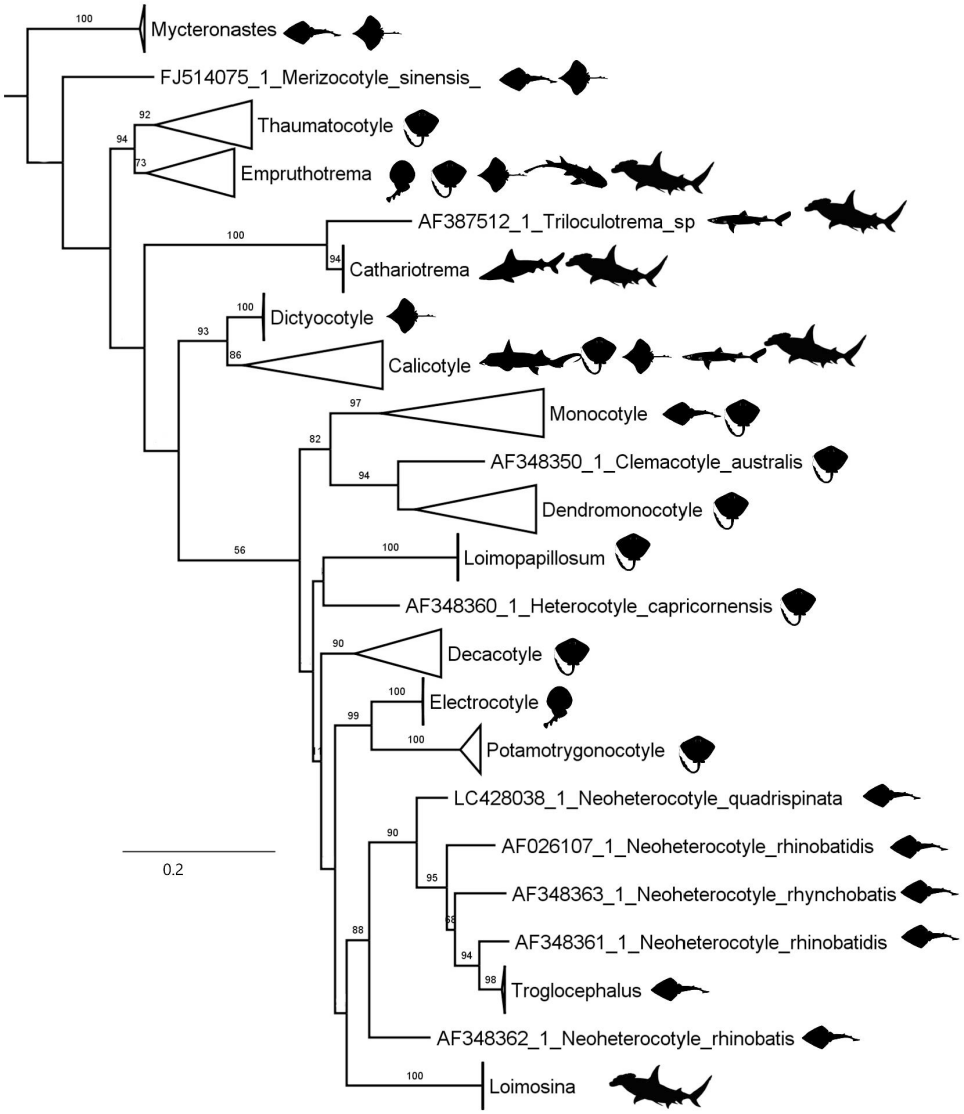

Figure 2: Maximum likelihood phylogenetic tree for Monocotylidae (GTR+G substitution model, 1000 bootstraps) constructed using 92 (28S) sequences for 54 species and model GTR+G in raxmlGUI 2.0 (version 2.0.6) (Stamatakis, 2014; Silvestro, 2012) with 1000 bootstraps. Final ML optimization = -20733. Bootstrap values shown as branch support, with support less than 50% removed Image credit for Rajiformes to Ignacio Contreras and for Chimaeriformes to Tambja (vectorized by T. Michael Keesey; https://creativecommons.org/licenses/by/3.0/legalcode).

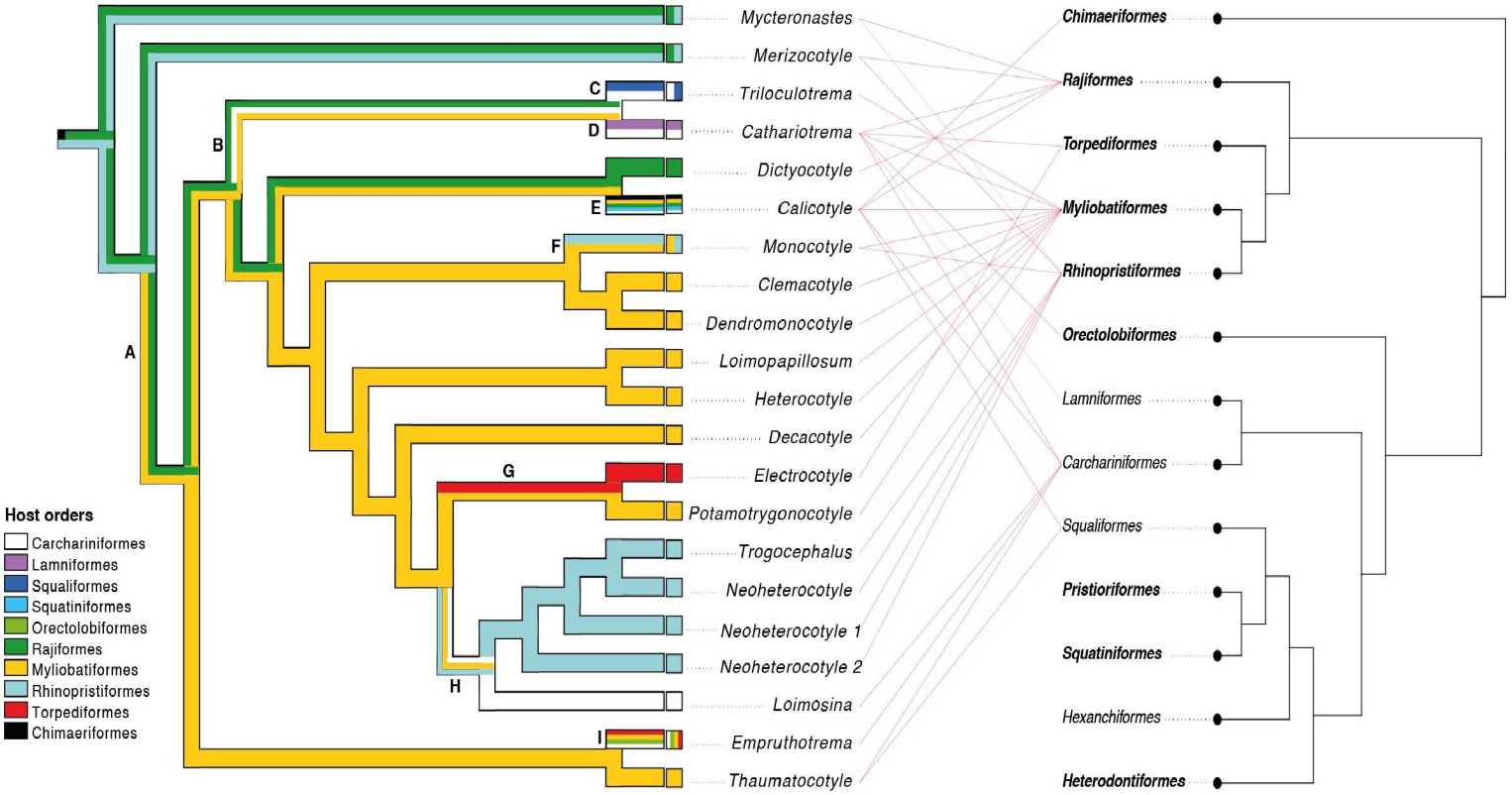

Figure 3: Tanglegram of Monocotylidae and hosts Chondrichthyes, produced using *cophylo* in R studio. The host Ancestral State Reconstruction (using parsimony in Mesquite v3.61 (Maddison & Maddison, 2019) modified to comply with the algorithm of Lieberman, 2000) is presented in the left cladogram (parasites). Capital letters indicate reconstructed events of host-range expansion (Table 1).

Table 1: Evolutionary distances (a proxy to the nature of the resource provided by host species) for the host expansion events recovered in Figure 3 (and indicated by capital letters – first column). The age of the last common ancestor of the donor (from) and recipient host (recipient) species ages are given in millions of years ago (MYA) and is followed by lower and upper limits of 95% confidence interval.

	From	to	Mean	Min	Max
A	Rajiformes	Myliobatiformes	201	187.8	291.0
B	Myliobatiformes	Carcharhiniformes	272	249.5	343.0
	Rajiformes	Carcharhiniformes	272	249.6	344.0
C	Carcharhiniformes	Squaliformes	225	163.6	350.0
D	Carcharhiniformes	Lamniformes	173	168.2	259.0
E	Rajiformes	Carcharhiniformes	272	249.5	343.0
	Rajiformes	Rhinopristiformes	201	187.8	291.0

	Rajiformes	Chimaeriformes	413	338.0	471.0
	Myliobatiformes	Carcharhiniformes	272	249.5	343.0
	Myliobatiformes	Rhinopristiformes	222	178.2	265.0
	Myliobatiformes	Chimaeriformes	413	338.0	471.0
F	Myliobatiformes	Rhinopristiformes	222	178.2	265.0
G	Myliobatiformes	Torpediniformes	226	177.4	274.0
H	Myliobatiformes	Rhinopristiformes	222	178.2	265.0
	Myliobatiformes	Carcharhiniformes	272	249.5	343.0
I	Myliobatiformes	Carcharhiniformes	272	249.5	343.0
	Myliobatiformes	Orectolobiformes	272	249.5	343.0
	Myliobatiformes	Torpediniformes	226	177.4	274.0

Sphyrnidae, comprising two genera, *Sphyrna* and *Eusphyra*, has an estimated divergence time within the order of about 40 - 50 MYA (see Kousteni *et al.*, 2021). These genera are then estimated to have diverged about 15 - 20 million years ago (MYA), with the diversification of *Sphyrna* spp. occurring within the last 10 MYA (Lim *et al.,* 2010). The divergence between *S. lewini* and *S. giberti* is estimated to be about 4.5 MYA (Pinhal *et al.*, 2012; Quattro *et al.*, 2013).

Currently, data for calibration times of parasites is extremely sparse. However, if we assume that *L. wilsoni* has only been found to infect *Sphyrna* spp., then the oldest this lineage could be is 15 - 20 MYA; this estimate becomes even lower (~10 MYA) if the assumption is that it can only be found in *S. lewini*, an assumption not so outrageous considering what we know about host-specificity and diversification.

Other genera of Monocotylidae have species that are known from species of Selachii, like the clade composed of *Cathariotrema*, another genus found to infect *Sphyrna* spp., and *Triloculotrema,* which infects members of Squalidae and Triakidae (Carcharhiniformes). However, these genera are distantly related to *Loimosina* (Fig. 2). Other monocotylids found to infect Carcharhiniformes are also genera with the widest host-range, i.e., *Empruthotrema* and *Calicotyle*.

Reconstruction of host onto the phylogeny of the Monocotylidae (using Lierberman algorithm – Lieberman, 2000) provides some insights on the coevolutionary dynamics of host-range and diversification of this group of parasites (Fig. 3). Origin of the Monocotylidae was reconstructed in Batoidea with a subsequent large number of events representing host-range expansion and isolation. Events of expansion are labeled by capital letters on the left cladogram (parasites) in

Figure 3. Table 3 presents the distance, in millions of years ago, the putative date of the last common ancestor (LCA) for the donor and recipient host order as a measure of compatibility of the parasite lineage/species and these hosts (see Charleston & Robertson, 2002; Engelstädter & Fortuna, 2019). The origin of *Loimosina* and closely related clade (*Neoheterocotyle* and *Troglocephalus*) is associated with an event of host-range expansion origination from a Myliobatiformes host into Rhinopristiformes and Sphyrnidae (Carcharhiniformes) (H in Figure 3). The LCA of the present host group of *Loimosina* and the donor host species was postulated to be around 272 MYA (Kumar *et al.,* 2017) and represents one of the highest distances recorded among the events reconstructed in this study (Fig. 3). This may provide a perspective in understanding the limitation of *L. wilsoni* to a single species of *Sphyrna*, despite maximum opportunity of encounter.

This phylogenetic pattern of range-change, oscillating between host-range expansion and contraction (i.e., isolation) are associated with two elements of the theoretical framework of the Stockholm Paradigm (Agosta & Brooks, 2020) – Oscillation and Taxon Pulse.

The Stockholm Paradigm is a fusion of three hypotheses: Ecological Fitting (EF), Oscillation Hypothesis (OH), and Taxon Pulse (TP) (Brooks *et al.,* 2019). Separately, they describe evolutionary events such as the ability for range-expansion without prior emergence of evolutionary novelty (EF), the evolutionary variation in the potential to EF (OH), and the effective pattern of sequential host-range expansion and isolation (TP) usually associated with cyclic or intermittent environmental disruptions (Hoberg & Brooks, 2008). The ability of EF is graphically modeled as the Fitness Space (FS), which is composed of the Fundamental FS (FFS) and the more limited Operational or Realized Fitness Space (RFS) (Figure 4). In a certain host, fitness is typically maximized within the RFS, but the capacity exists to expand – hence, the FS is called “sloppy”. A narrow host-range is thus a narrow-realized fitness-space, where fitness and reproduction is maximized within this range (Brooks *et al.,* 2019) – although the “sloppy” portion of the FFS represents the ability of the parasite to expand to new hosts by EF. However, host-range expansion is dependent on opportunity. Opportunity is the possibility of encounter between parasites and hosts – and if the parasite has capacity (represented by the FS), it will be able to colonize and incorporate new host species by EF (Brooks *et al.,* 2019).

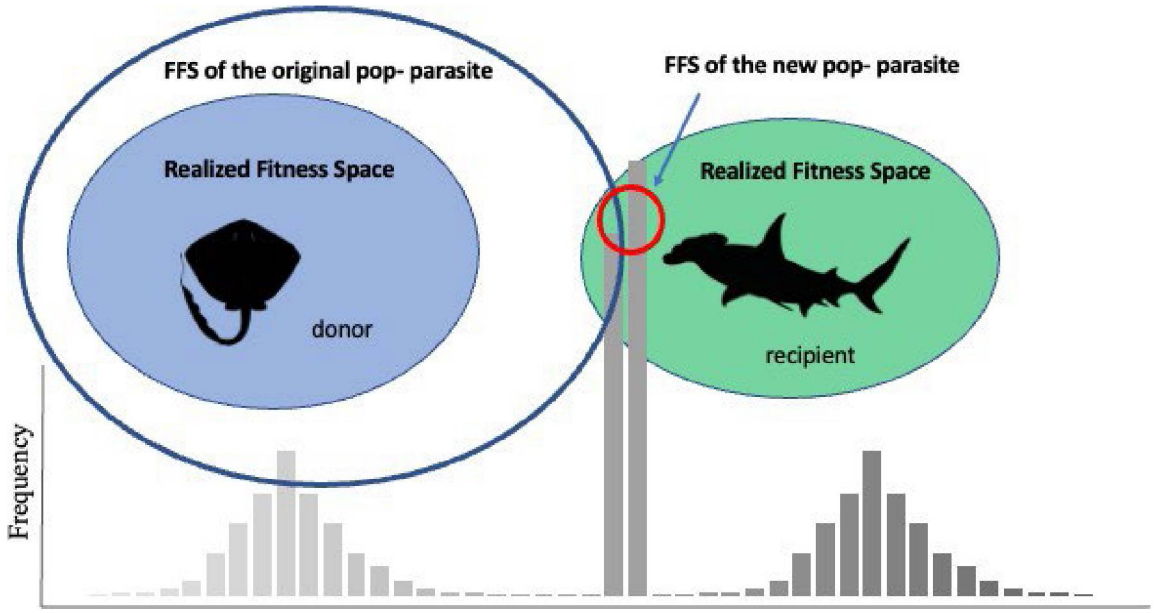

Figure 4: Depiction of phenotype frequencies changes within the fitness space (FS) (adapted from Brooks *et al.,* 2019). The current host is the realized fitness space (RFS), in which natural selection maintains high frequencies (bars) of optimal genes. At the edges of the RFS are low frequencies of below-optimal genes, which through exploration are able to colonize the tail end of a compatibility curve of a new host species. The tall bars represent the expected fitness of parasites on the respective hosts.

Whittington & Kearn (2011) summarized several factors in hatching requirements for the larvae of monogenoids that promote host-infection: quantity of light (day/night cycles, or even a shadow projected by a passing host), host-derived chemical cues (urea produced from the skin, for example), or mechanical disturbance (e.g., foraging/feeding activity of the host). If a different host species comes into contact with a monocotylid egg, the egg becomes stimulated into hatching, and the oncomiracidium are able to explore this new host, then the potential for colonization exists.

Keeping in mind that actual and potential phenotypic variability is both a requirement and an assumption for a healthy population, we can visualize a normal distribution of genotypic frequency to survivorship (Figure 4). Certain genotypes and phenotypes have been naturally selected for due to their ability to maximize the fitness under the curve, thus there is medium variability/medium frequency. However, the variants that exist in the tails of the curve exhibit lower fitness and may or may

not be expressed phenotypically. Individuals with these variants would have the greatest potential to explore and expand their range than highly fit individuals under the present host species (Brooks *et al.,* 2019). When only a small part of the sloppy FS of a parasite population overlaps with the putative RFS of a new host, the resulting population that colonizes this new resource (i.e., host) has usually low real and potential phenotypic variability (and, hence, also genetic), which depict high frequency in the new population. This can create situations analogous to a populational bottlenecks (Mayr, 1954, see also Bush, 2001).

Considering the distances associated with the donor (Myliobatiformes) and the recipient host (Carcharhiniformes) associated with the origin (following isolation) of *Loimosina*, we expect that the real and putative phenotypic variability of the initial population was immensely reduced, due to the postulated quali- and quantitative distance between the resources offered by the involved hosts. The isolation of the two host clades, through more than 272 MY, is assumed to have generated these differences – the greater the distance between the host resources, the greater the reduction in the FS of the colonizing population (see Brooks *et al.,* 2019; Araujo *et al.,* 2015). It is possible that the reduced FS of the ancestor of *L. wilsoni* precluded its colonization of *S. gilberti*.

While it is expected that the FS of a population/species increases with time – with the accumulation of evolutionary novelties through time – the right novelty to allow the use and colonization of the nature of resources provided by *S. gilberti* never emerged. Hence why there are currently only 2 known species (possibly only 1, see Chapter 1) for this genus that infects taxa with broad geographic distribution.

Of the 8 species known for *Sphyrna*, smaller sized species (*S. corona, S. media, S. tiburo,* and *S. tudes*) are observed as range-restricted, being found only in specific regions or oceans of the world (see Lim *et al.,* 2010, range-data in Compagno, 1984). The larger species (*S. lewini, S. mokarran, S. zygaena*) tend to be found globally. Unfortunately, complete range-data for *S. gilberti* is unknown, however neonates have been found in coastal nurseries along the southeastern Atlantic coast of the US, and a few adults were found in southeastern Brazil fisheries (Abercrombie *et al.,* 2005; Barker *et al.*, 2021; Duncan *et al.,* 2006; Quattro *et al.,* 2006; Pinhal *et al.,* 2012). Data on adult size is insufficient, although it is known that neonates of *S. gilberti* are typically smaller than those of *S. lewini* (Quattro *et al.,* 2013, see also Barker *et al.,* 2021). This could explain why *S. gilberti* has thus far

been observed with a more restricted range than *S. lewini*, although the extent of which remains uncertain. *Loimosina wilsoni* has been found infecting *S. lewini,* a host with a known global distribution, and its hybrids, in a habitat shared sympatrically with *S. gilberti*, and yet is demonstrating a specificity that prevents inclusion of *S. gilberti* in the current host repertoire. This could be a result of a 10 MY old genetic bottleneck.

Unfortunately, the answer to this question is not so simple to obtain. Ideally, many more sequences from species of *Loimosina*, especially mitochondrial sequences which are better for population studies, from many different hosts around the world, would shed light on genetic diversity within this genus. Life history strategies of the parasite, like those studied by Kearn (see Whittington & Kearn, 2011) could also aid in the identification of what causes (and prevents) host-range expansion by *Loimosina*.

In conclusion, we have observed examples of taxon-pulse cycles in Monocotylidae that have led to narrower host-ranges in many clades (Fig. 3). Hence, we hypothesize that the founder effect in the evolutionary history of *Loimosina* prevents it from expanding to *S. gilberti*, a species closely related to its current host species, due to a small FS or to the accumulation of a new FS that does not overlap with the required RFS. We propose that future studies should focus on genetic diversity between populations and life history strategies of these parasites to clarify how these monogenoids evolve their present host-parasite distribution.

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