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# Characterization of a diversity of tetraphyllidean and rhinebothriidean cestode larval types, with comments on host associations and life-cycles ☆

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ABSTRACT

Life-cycles of marine tapeworms of the orders Tetraphyllidea and Rhinebothriidea are poorly known primarily because their larvae typically lack species level, taxonomically distinguishing adult characteristics and using morphology they can be identified to genus, family or order only. This large-scale study conducted in the northern Gulf of Mexico includes adult cestodes (25 species) collected from sharks and rays (Elasmobranchii, eight species) and larval cestodes (27 species) collected from teleosts (Neopterygii, 46 species), bivalves and gastropods (Mollusca, 24 species), and shrimps (Crustacea, five species), comprising a phylogenetically (75 species in three phyla, 14 orders and 46 families) and ecologically (e.g., benthic, epibenthic, pelagic, euryhaline, stenohaline) diverse array of hosts of larval cestode. Molecular biology and morphology informed larval identification and facilitated the circumscription of suites of morphological features representing distinct larval types (i.e., collective groups). A total of 198 specimens comprising adult and larval tetraphyllideans and rhinebothriideans assigned to 12 genera were characterized for the partial (D1–D3) lsrDNA gene and analyzed separately and in combination with data derived from species belonging to an additional 21 genera available from GenBank. Eight larval types were identified and matched to one or several genera of Tetraphyllidea or Rhinebothriidea; morphological variation within these larval types was also documented. In combination with published reports of unique larval morphologies, 15 larval types were established and a key to their larvae presented. Overall, teleosts figured prominently in the life-cycles of tetraphyllideans and rhinebothriideans. Intermediate host specificity at the level of cestode genus was euryxenous, but limited host records suggest that host specificity at the level of cestode species may be more strict. To our knowledge, this is the first published study that approaches the elucidation of marine tapeworm life-cycles by incorporating morphological, molecular biological and phylogenetic methods using specimens collected on a regional scale and from wild-caught hosts from four metazoan phyla.



## 1. Introduction

The life-cycles of marine cestodes, especially those maturing in sharks and rays (Elasmobranchii), are surprisingly poorly known (Fuhrmann, 1931; Caira and Reyda, 2005; Jensen, 2005). Data on marine cestode life-cycles were most recently summarized by Caira and Reyda (2005). One of the primary factors contributing to the dearth of information on marine cestode life-cycles is that the larval stages of species belonging to many of the cestode orders do not resemble their adult counterparts. As a consequence, such larvae are difficult to accurately identify using the morphological criteria upon which cestode taxonomy is based (e.g., Joyeux and Baer, 1961), with the notable exception of elasmobranch cestodes of the order Trypanorhyncha, whose larvae develop taxonomically-distinctive hooked tentacles that identify them to species as pre-adults (e.g., Campbell and Beveridge, 1994; Palm, 2004). In fact, although most cestode species descriptions are based on adult specimens, some trypanorhynch species descriptions are based solely on larval specimens extracted from bony fish or invertebrate intermediate hosts, leaving the morphological features of the adult and the identity of the definitive elasmobranch host indeterminate (e.g., Palm, 2004; Beveridge et al., 2007).

In contrast, identification of many tetraphyllidean and rhinebothriidean (former members of the Tetraphyllidea; see Healy et al., 2009) larvae is more problematic given that their scoleces remain less differentiated until they infect the definitive host. Although a substantial body of literature includes mention of descriptions of these marine cestode larvae (e.g., Linton, 1897; Curtis, 1911; Dollfus, 1923, 1929, 1964, 1974; Yamaguti, 1934; Anantaraman, 1963;

☆ *Note:* Nucleotide sequence data reported in this paper are available in GenBank™ under the Accession Nos. GQ470001–GQ470202.

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Regan, 1963; Friedl and Simon, 1970; Vivares, 1971; Armstrong, 1974; Reimer, 1975; Cake, 1976, 1977; Stunkard, 1977; Chambers et al., 2000; Brickle et al., 2001; Agustí et al., 2005; Aznar et al., 2007), attempts to assign them to specific, generic or even familial cestode taxa are equivocal because they lack morphological clues about their identity. Surrendering to this inability to reliably identify them, a common practice has been to name the larval form by applying a "collective group name" (an assemblage of species, or stages of organisms (e.g., larvae), that cannot be allocated with confidence to nominal genera (International Commission on Zoological Nomenclature, 1999)). For example, the terms "*Scolex pleuronectis*" and "*Scolex polymorphus*" refer to larvae bearing an apical sucker and four acetabula, divided or undivided (e.g., Linton, 1905; Dollfus 1929, 1964; Yamaguti, 1934; Anantaraman, 1963; Reimer, 1975; Stunkard, 1977; Overstreet, 1978). Although presumed to be members of the order Tetraphyllidea (e.g., Fuhrmann, 1931; Yamaguti, 1934; Dollfus, 1953; Euzet, L., 1959. Recherches sur les cestodes tétraphyllides des sélaciens des cotes de France. Thèse présentées à la Faculté des Sciences l'Université de Montpellier, Montpellier (1956); Anantaraman, 1963; Overstreet, 1978; Caira and Reyda, 2005), the identities of these larvae remain indeterminate at all levels.

In vitro cultivation could help identify some marine cestode larvae, allowing the worker to observe the transformation of the larvae into an adult; thereby establishing its identity. Hamilton and Byram (1974), Avdeeva and Avdeev (1980), Carvajal et al. (1982) and Chambers et al. (2000) cultivated larvae until they determined them to be identifiable to a genus. However, many of these generic assignments need verification. As a result of these in vitro studies and studies documenting morphological change from plerocercoid to adult (e.g., Caira and Ruhnke, 1991), an understanding of the ontogeny of some larval features and, thus, their implications for identification purposes, has begun to emerge. However, because reportedly no marine cestode has been cultivated to maturity in vitro, this method needs improvement and further development to be widely employed; such a method is certainly far from being considered routine in cestode taxonomy. Thus, even with in vitro cultivation as a potential tool, correct and accurate identification of larvae beyond the level of genus is at present rarely possible.

With the advent of molecular methods, new molecule-based strategies have emerged to inform larval identifications. To date, five studies have used molecular data that match larvae of elasmobranch cestodes and their potential adult counterpart. Brickle et al. (2001) generated 626 bp of 28S rDNA data for larval forms found in the Patagonian squid (*Loligo gahi*) in the southwestern Atlantic Ocean off the Falkland Islands. Agustí et al. (2005) generated 486 bp of 28S rDNA (D2) data for larvae found in the striped dolphin (*Stenella coeruleoalba*) in the western Mediterranean Sea. Aznar et al. (2007) generated 515 bp of 28S rDNA (D2) for larvae from three cetacean species also in the Mediterranean Sea. Finally, Holland et al. (2009) generated 725 bp of 28S rDNA data and 2,067 bp of 18S rDNA data for larvae from the Florida amphioxus (*Branchiostoma floridae*) in the Gulf of Mexico off Florida, and Holland and Wilson (2009) generated 720 bp of 28S rDNA (D2) for larvae from the bivalve *Ensis minor*. In all cases, generic or specific identifications were asserted, and with the exception of one trypanorhynch included among the larvae investigated by Brickle et al. (2001), all larvae belonged to the order Tetraphyllidea. However, the accuracy and potential impact of such work is constrained by the limited comparable molecular data available for adults across the spectrum of marine cestode taxa and across geographic localities. Thus, when sequences are not identical it may be unclear whether the differences indicate the presence of different species, sequence error or intraspecific variation. Similarly, given the limited suite of taxa that has been sequenced and for which sequence data are available, even in instances of 100% sequence identity, conspecificity should not necessarily be assumed. This is clearly a promising avenue of exploration not only for larval identification to species, but also for informing the morphological identification of larvae into types (i.e., collective groups) based on shared morphological characters corroborated by phylogenetic groupings derived from molecular sequence data, once data for a sufficiently diverse suite of larval forms and their likely adults can be produced.

The present study aimed to construct a molecular sequence library for larval cestodes infecting invertebrates and teleosts, and adult cestodes infecting elasmobranchs ranging in the northern Gulf of Mexico. The Gulf of Mexico was suitable for such a targeted study because it is one of only two regions (the second being Heron Island, Australia; see Chambers et al., 2000) for which preliminary morphological data and a significant number of host records reportedly have been compiled for tetraphyllidean and rhinebothriidean larvae (e.g., Cake, 1976, 1977) as well as for their potential adult counterparts (e.g., Linton, 1909; Shuler, 1938; Chandler, 1954; Goldstein, 1964; Henson, 1975; Overstreet, 1978; Caira and Pritchard, 1986; Ruhnke, 1994; Caira et al., 1996, 2005b; Healy, 2003; Ruhnke and Thompson, 2006; Jensen, 2009). The Gulf of Mexico was suitable regarding logistics of host and parasite collections because (i) it supports active commercial and recreational fisheries for a variety of both fishes and shellfishes, (ii) collection sites were relatively easily accessible throughout all seasons of the year and (iii) access to the Parasitology Laboratory (Gulf Coast Research Laboratory, University of Southern Mississippi, Ocean Springs, Mississippi, USA), where the majority of dissections were conducted, allowed the expeditious extraction and fixation of live cestode larvae and adults. Existing data on the Gulf of Mexico cestode fauna consist of records of a total of 12 species in seven genera of Rhinebothriidea and 29 species in 13 genera of Tetraphyllidea (see Jensen, 2009). Regarding these Gulf of Mexico records, the region was also ideal because the 20 potential target cestode genera represent disparate scolex morphologies. Because marine fish cestodes are assumed to be transmitted trophically between hosts, every attempt was made to collect intermediate and definitive hosts simultaneously from the same sites; in some instances fish stomach contents informed a potential intermediate host(s). Thus, at least some conspecificity between larvae and adults collected over the course of the study was expected.

A region of the 28S rDNA gene (D1–D3) was used because (i) it varies among congeners in a diversity of cestode groups (e.g., Zehnder and Mariaux, 1999; Olson et al., 2001; de Chambrier et al., 2004; Caira et al., 2005a; Waeschenbach et al., 2007), (ii) sequence data are already available for many adult rhinebothriideans (e.g., Healy et al., 2009) and tetraphyllideans (Olson et al., 2001; Waeschenbach et al., 2007) and (iii) it is commonly used to identify cestode larvae (e.g., Brickle et al., 2001; Agustí et al., 2005; Aznar et al., 2007; Holland et al., 2009; Holland and Wilson, 2009).

The goals of this study were to (i) use molecular data to elucidate suites of morphological features facilitating tetraphyllidean and rhinebothriidean larval identification, (ii) investigate species level morphological variation among congeneric larvae, (iii) document which intermediate hosts are exploited by which cestode larvae and (iv) explore potential life-cycle strategies for tetraphyllidean and rhinebothriidean taxa in the context of these newly reported host associations.

## 2. Materials and methods

### 2.1. Collection strategy

The ubiquitous presence of adult tetraphyllideans in sharks and rays, as well as that of rhinebothriideans mainly in rays, has been widely recognized (e.g., Caira and Healy, 2004; Caira and Reyda, 2005). Thus, rather than aiming collections at specific elasmobranch taxa for the purposes of cestode species discovery, the goal herein

was to capture, necropsy and survey a broad ecological and phylogenetic diversity of elasmobranchs including estuarine, coastal and offshore species. Existing intermediate host records for tetraphyllideans and rhinebothriideans from the Gulf of Mexico (e.g., Linton, 1909; Chandler, 1935; Regan, 1963; Hutton, 1964; Harry, 1969; Hamilton and Byram, 1974; Wardle, W.J., 1974. A survey of the occurrence, distribution and incidence of infection of helminth parasites of marine and estuarine Mollusca from Galveston, Texas. Ph.D. Disssertation, Texas A & M University, College Station, Texas, USA; Cake, 1976, 1977) informed a strategy for collections of intermediate hosts, e.g., teleosts, bivalves and gastropods were prioritized as collection targets over, for example, decapods. Cephalopods, which host tetraphyllidean larvae (e.g., Dollfus, 1923, 1929, 1964, 1974; Gestal et al., 1998; Brickle et al., 2001), were not encountered and were not specifically targeted for collection. The Gulf of Mexico reportedly holds 1,409 species of actinopterygians (McEachran, 2009), 1,742 gastropods (Rosenberg et al., 2009) and 528 bivalves (Turgeon et al., 2009). Many gear types and collection methods were used in an attempt to maximize the diversity of those hosts that were sampled (e.g., pelagic versus benthic, etc.).

### 2.2. Collection of hosts

All collections were conducted in the northern Gulf of Mexico during March through October of 2005–2008. Elasmobranchs were captured off Florida in St. Joe Bay (29°46′3.27″N, 85°21′4.06″W), near Panama City (30° 8′4.69″N, 85°41′36.93″W), off Crooked Island Sound (30°0′14.42″N, 85°31′26.77″W), in Indian Pass (29°40′8.00″N, 85°13′30.00″W); off Mississippi in the vicinity of Horn Island (30°14′37.70″N, 88°46′37.62″W), Round Island (30°17′42.45″N, 88°35′11.55″W), Ship Island (30°14′24.54″N, 88°52′25.25″W), in Mississippi Sound (30°14′16.90″N, 89°13′35.13″W); and off Louisiana approximately 100–185 km offshore (29°58′58.20″N, 88°36′16.80″W), in Brenton Sound and around Chandeleur Island (29°57′9.54″N, 88°50′38.98″W). Elasmobranchs were collected by using a spear gun, Hawaiian sling, hand nets, cast nets, seines, otter trawl, fast drag treble hook, baited hook-and-line or gill nets. Intermediate hosts were collected off Florida in St. Andrews Bay and near Panama City (30°8′4.69″N, 85°41′36.93″W) and off Pensacola Beach (30°20′6.11″N, 87°8′18.41″W); off Mississippi near Horn Island (30°14′9.10″N, 88°46′2.00″W), in Davis Bayou (30°22′54.73″N, 88°46′47.23″W), in Mississippi Sound (30°14′16.90″N, 89°13′35.13″W), off Ship Island (30°14′24.54″N, 88°52′25.25″W); 50–100 km off Ocean Springs (29°59′N, 88°31′W), off Louisiana approximately 100–185 km offshore (29°58′58.20″N, 88°36′16.80″W) and in Brenton Sound and off Chandeleur Island (29°57′9.54″N, 88°50′38.98″W). Methods of intermediate host collection were as follows: teleosts were collected by otter trawl, commercial trawl, variously-sized seines, variously-sized hand nets, cast net, yabbie pump or hook-and-line. Molluscs (gastropods and bivalves) were collected with hand nets, yabbie pump and floating sieve and a modified A-frame dredge with variously-sized meshes towed by boat or by hand. Crustaceans were collected using an otter trawl or yabbie pump. Hosts were transported on ice or alive in coolers and buckets to either the Gulf Coast Research Laboratory or to the Panama City Laboratory of the National Marine Fisheries Service (NMFS, Panama City, Florida, USA) for necropsy. Common names of teleosts follow Nelson et al. (2004). Fishes were identified according to Carpenter (2002).

### 2.3. Dissection of hosts

Elasmobranchs were opened with a mid-ventral incision and the spiral intestine was removed. Spiral intestines were subsequently examined for adult cestodes under a dissecting microscope. A selection of cestodes encountered was removed and the spiral intestine preserved in 10% seawater buffered formalin for morphological study. Necropsies of the fish, mollusc and crustacean hosts were more extensive given the greater diversity of tissues exploited by cestode larvae. Stomach, intestine, pyloric caeca, body cavity, mesentery, liver and body wall musculature of fishes were examined, the latter via a series of diagonal slices. Gastropods and bivalves were shelled using a scalpel, vice-grips or forceps and organs of the digestive system, reproductive system, as well as the musculature were teased apart and examined under a dissecting microscope. In crustaceans, the dorsal carapace was removed and the thoracic haemocoel, organs of the digestive system, the digestive gland in particular and musculature were examined under a dissecting microscope.

### 2.4. Handling of specimens

Cestodes were fixed immediately in the field, or observed alive in a dish under high magnification with the aid of a dissecting microscope or as wet-mounted specimens with the aid of a compound microscope equipped with differential interference contrast (DIC) optical components. Some living specimens were observed, especially for the functionality of the scolex, in physiological saline comprising filtered seawater before being preserved. A subsample of each adult cestode taxon observed was preserved in 95% ethanol for molecular sequencing; remaining cestodes were preserved in 10% seawater buffered formalin for morphological study. Most larvae were heat-killed in hot tapwater. In some cases, a subsample of each larval form observed in vivo was preserved in 95% ethanol for molecular sequencing and the remaining sample of each form was preserved in 10% buffered formalin for morphological study. In other cases, larvae found were bisected such that the posterior half was preserved in 95% ethanol and the anterior, scolex-bearing half was preserved in 10% seawater buffered formalin. Adult and larval specimens were fixed in 10% seawater buffered formalin for at least 24 h before being transferred to 70% ethanol. Subsamples of each adult taxon and of each larval form from each site from each host, and the morphological vouchers of adults and larvae (i.e., the anterior, scolex-bearing half) sequenced were prepared as whole mounts as follows: specimens were hydrated in distilled water, stained in Delafield's hematoxylin, transferred to tapwater, followed by 70% acid ethanol, 70% basic ethanol, dehydrated in a graded ethanol series, cleared in methyl salicylate and mounted on glass slides in Canada balsam. A subsample of each adult taxon and larval form from each site from each host was prepared for examination with scanning electron microscopy (SEM). These were hydrated in distilled water, transferred to a solution of 1% osmium tetroxide overnight, dehydrated in a graded ethanol series, transferred to hexamethyldisilazane (Ted Pella Inc., Redding, CA, USA) allowed to air dry and mounted on aluminum stubs on carbon tabs, sputter coated with 35 nm of gold and examined in a LEO/Zeiss 1550 field emission scanning electron microscope.

### 2.5. DNA extraction, gene amplification and sequencing

Sequence data were generated for the D1–D3 portion of the nuclear 28S lsrDNA (~1300 nucleotides). In as many cases as possible, DNA was extracted from the posterior portion of an adult or larval specimen which had been preserved in 95% ethanol and a whole mount was prepared of the anterior portions of the same worm to serve as a hologenophore (sensu Pleijel et al., 2008) for the specimen sequenced. In the cases of minute larvae (i.e., <400 μm in total length) for which subdivision was not possible, photo vouchers were taken and the entire larva was sequenced. In only two cases was a representative voucher (i.e., paragenophore sensu Pleijel et al. (2008)) prepared. All hologenophores, paragenophores and photo vouchers have been deposited in the Lawrence R. Penner

Parasitology Collection (LRP), University of Connecticut, Storrs, Connecticut, USA.

Total genomic DNA was extracted from adults and large larvae (i.e., >400 μm) using a non-commercial guanidine thiocyanate protocol. Tissue was digested in 300 μl of cell lysis buffer (1 M NaCl, 0.1 M Tris–Cl (pH 8.0), 25 mM EDTA (pH 8.0), and 0.5% sodium dodecyl sulfate) and 4–8 μl of Proteinase K at 55 °C for 6–24 h. After digestion was complete, protein was precipitated out of solution by the addition of 4 M guanidine thiocyanate and 0.1 M Tris–Cl (pH 7.5) followed by vigorous mixing (10–15 s) and centrifuging (5 min at 15,871 g). The protein precipitate was discarded and DNA then precipitated from the supernatant by the addition of 300 μl of cold (−20 °C) 100% isopropanol. Samples were then gently mixed and centrifuged, as above. Isopropanol was discarded and the DNA pellet was washed in 70% ethyl alcohol. The ethanol was discarded and the samples were dried at room temperature for 10–24 h. DNA was then re-suspended in 30–40 μl of 10 μM Tris–Cl (pH 8.0) of $dH_2O$ and stored at −20 °C until subjected to PCR. In the case of smaller larvae (i.e., ⩽400 μm), DNA was extracted following Gloor et al. (1993). Entire specimens were placed in a 'squishing' buffer (10 mM Tris–Cl (pH 8.2), 1 mM EDTA, 25 mM NaCl and 200 μg/ml Proteinase K), incubated at 25–37 °C (or room temperature) for 20–30 min. The Proteinase K was inactivated by heating to 95 °C for 1–2 min and the solution was immediately subjected to PCR.

PCR was performed in 25 μl reactions using Ready-to-Go PCR beads (Amersham Pharmacia, Piscadoya, New Jersey, USA) (1 μl of 10 μM forward and reverse primers, 2–5 μl DNA template and 21–18 μl of DI water). PCR primers used were the forward primer LSU5 (5′-TAGGTCGACCCGCTGAAYTTAAGCA-3′) and the reverse primer 1200R (5′-GCATAGTTCACCATCTTTCGG-3′). Cycling conditions were as follows: initial denaturation for 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 55 °C, 2 min at 72 °C and completed by 5 min at 72 °C. PCR products were purified using ExoSap-IT™ (USB Corporation, Ohio, USA). Cleaned PCR products were cycle sequenced in 10 μl reactions using PCR primers LSU5 and 1200R, and internal primers 300F (5′-CAAGTACCGTGAGGGAAAGTTG-3′) and EDC2 (5′-CTTGGTCCGTGTTTCAAGACGGG-3′) with a Big Dye™ Terminator 3.1 kit (Perkin-Elmer, Boston, MA) following the manufacturer's instructions. Thermal cycles were as follows: initial denaturation for 2 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 20 s at 50 °C and 4 min at 54 °C. Cycle sequencing products were cleaned using Sephadex Medium (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Automated sequencing was completed on an ABI 3130*xl* Genetic Analyzer.

### 2.6. Phylogenetic analyses

Two analyses were conducted using different matrices. The initial analysis was conducted using a matrix comprised of the sequences of the larval and adult tetraphyllidean and rhinebothriidean specimens as ingroups and four lecanicephalidean specimens as outgroups, all generated as part of this study. Contiguous sequences for all specimens for which sequences were generated have been submitted to GenBank. These taxa, their GenBank accession numbers and voucher numbers (for hologenophores and photo vouchers) are provided in Tables 1 and 2. Also included in this matrix were six additional outgroup taxa taken from GenBank. These were the litobothriideans *Litobothrium amplifica* (AF286931) and *Litobothrium janovyi* (AF86930), and the four additional lecanicephalideans *Adelobothrium* cf. *aetobatidis* (EF095257), *Cephalobothrium aetobatidis* (AF286927), *Tylocephalum* sp. (AF286929) and *Eniochobothrium gracile* (AF286928). The second, more expanded analysis was designed to facilitate identification of larval taxa for which adult matches were not found in the initial analysis as well as to help confirm the identifications resulting from the initial analysis. Thus, it was based on the initial matrix expanded to include representation of all additional tetraphyllidean and rhinebothriidean genera for which 28S rDNA data were available in GenBank, as well as two members of the Proteocephalidea and one member of the Cathetocephalidea. This analysis included 70 additional species representing 12 tetraphyllidean and nine rhinebothriidean genera beyond those included in the initial analysis. These taxa and their GenBank accession numbers are provided in Table 3.

For generation of the initial matrix, individual sequences were edited and assembled using SEQUENCHER 4.5™ (GeneCodes Corporation, Ann Arbor, MI, USA) and compiled in Se-Al v2.0a11 (Rambaut, A., 1996. Se-Al: Sequence Alignment Editor (Se-Al v2.0a11). Available at http://tree.bio.ed.ac.uk/software/seal/). Sequences were aligned using MAFFT v6.240 (Kyoto University Bioinformatics Center, Japan) using the L-INS-I strategy with default parameters for Gap opening penalty and offset value, examined in Se-Al and cropped to a final aligned length of 1,321 bp. In the case of the expanded matrix, sequence data for the additional taxa were downloaded from GenBank and compiled together with the existing data in the Se-Al matrix. The expanded matrix was then realigned using MAFFT as above, and examined in Se-Al and cropped to a length of 1,441 bp. Using MacClade v.4.06 (Maddison, D., Maddison, W., 2003. MacClade 4: analysis of phylogeny and character evolution, version 4.06. Sinauer Associates, Inc.,Sunderland, Massachusetts, USA), regions that could not be unambiguously aligned were identified; 67 bp were subsequently excluded from the expanded matrix.

Maximum likelihood (ML) analyses were conducted on both the initial and expanded matrices. For both matrices, five analyses using GARLI v0.96 (Zwickl, D., 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. Dissertation, The University of Texas at Austin, Austin, Texas, USA) with default settings were run through the CIPRES Portal V2.0, and three analyses using GARLI v0.951 (Zwickl, 2006) with default settings were run on a desktop computer. In all cases, gaps and missing data were treated as missing data ("?"). The eight separate GARLI analyses for each matrix were run to ensure convergence on the same best tree, as evident from similar topology and lnL scores. Nodal support for the inferred ML clades was estimated by bootstrap analysis (100 bootstrap replicates) also conducted using GARLI v0.951. The 50% majority rule consensus tree was generated using PAUP* 4.0b10 (Swofford, D., 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods). Sinauer Associates, Inc., Sunderland).

### 2.7. Morphological characterization of larval types

Larvae were separated into types (i.e., collective groups) and named by Roman numeral according to shared morphological characters corroborated by phylogenetic groupings based on molecular sequence data. Groupings of larvae resulting from the ML analysis of the initial, more limited molecular matrix informed the preliminary circumscription and identification of larval types. A detailed morphological circumscription of these larval types was subsequently conducted using a combination of light microscopy (LM) and SEM data generated from the hologenophores as well as from the multitude of larval specimens preserved in formalin and prepared as whole mounts. Light micrographs were taken using a Leica DFC 480 digital camera mounted on a Zeiss Axioskop 2 compound microscope with DIC. Measurements were made of whole mounts of larvae using the above camera system and image analysis software OpenLab Demo 4.04 (Improvision, Massachusetts, USA). Measurements of photo vouchers and scanning electron micrographs were done using ImageJ (Rasband, W.S., 1997-2009. ImageJ, U. S. National Institutes of Health, Bethesda,

**Table 1**
Collection data for adult cestodes from elasmobranchs from the Gulf of Mexico by definitive host species.

| Host | No. of hosts necropsied | Collection date | Locality | Parasite order | Parasite species | Site in host | Stage, (sub)group | GenBank No. | LRP No. |
|---|---|---|---|---|---|---|---|---|---|
| Elasmobranchii (4 families; 8 spp.) | | | | | | | | | |
| Carcharhiniformes: Carcharhinidae | | | | | | | | | |
| *Carcharhinus brevipinna* | 9 | June–November 2005, | Florida, | Tetraphyllidea | *Paraorygmatobothrium* sp. 3A | Spiral intestine | Adult, E3 | GQ470011–2 | 7141–3 |
| | | May–October 2006 | Louisiana, | Tetraphyllidea | *Paraorygmatobothrium* sp. 5A | | Adult, E5 | GQ470031–2 | 7144–5 |
| | | | Mississippi | Tetraphyllidea | *Paraorygmatobothrium* sp. 6 | | Adult, E6 | GQ470001–4 | 7146–9 |
| | | | | Tetraphyllidea | *Phoreiobothrium* sp. 1A | | Adult, A1 | GQ470064–6, 82, 95 | 7150–5 |
| | | | | Tetraphyllidea | *Triloculatum bullardi* | | Adult, A6 | GQ470102 | 7155 |
| *Carcharhinus isodon* | 8 | June 2005, | Florida, | Tetraphyllidea | *Anthobothrium* sp. 1A | Spiral intestine | Adult, G1 | GQ470169 | 7156 |
| | | October 2006, | Mississippi | Tetraphyllidea | *Anthobothrium* sp. 2A | | Adult, G2 | GQ470160 | 7157 |
| | | May 2007 | | Tetraphyllidea | *Paraorygmatobothrium* sp. 5B | | Adult, E5 | GQ470037–44 | 7158–65 |
| | | | | Tetraphyllidea | *Phoreiobothrium* sp. 1B | | Adult, A1 | GQ470079–80, 83 | 7166–8 |
| *Carcharhinus limbatus* | 24 | June 2005, | Florida, | Tetraphyllidea | *Anthobothrium* sp. 1B | Spiral intestine | Adult, G1 | GQ470165–66 | 7169–70 |
| | | July–October 2006, | Mississippi | Tetraphyllidea | *Anthobothrium* sp. 2B | | Adult, G2 | GQ470159 | 7171 |
| | | May–June 2007 | | Tetraphyllidea | *Paraorygmatobothrium* sp. 3B | | Adult, E3 | GQ470005, 7 | 7172–3 |
| | | | | Tetraphyllidea | *Paraorygmatobothrium* sp. 5C | | Adult, E5 | GQ470034–6, 45, 47 | 7174–8 |
| | | | | Tetraphyllidea | *Phoreiobothrium* sp. 1C | | Adult, A1 | GQ470067–71, 74–76, 94 | 7179–87 |
| *Rhizoprionodon terraenovae* | 15 | June–October 2006, | Florida, | Tetraphyllidea | *Anthobothrium* sp. 1C | Spiral intestine | Adult, G1 | GQ470161 | 7188 |
| | | May–June 2007 | Mississippi | Tetraphyllidea | *Paraorygmatobothrium* sp. 2 | | Adult, E2 | GQ470020–21, 23–24 | 7189–92 |
| | | | | Tetraphyllidea | *Paraorygmatobothrium* sp. 3C | | Adult, E3 | GQ470006, 9, 13 | 7193–95 |
| | | | | Tetraphyllidea | *Paraorygmatobothrium* sp. 5D | | Adult, E5 | GQ470033, 46, 48 | 7196–98 |
| | | | | Tetraphyllidea | *Phoreiobothrium* sp. 1D | | Adult, A1 | GQ470072–3 | 7199–200 |
| Myliobatiformes: Myliobatidae | | | | | | | | | |
| *Mobula hypostoma* | 2 | October 2006 | Florida | Rhinebothriidea | *Rhabdotobothrium anterophallum* | Spiral intestine | Adult, H1 | GQ470179–80 | 7101–2 |
| Myliobatiformes: Rhinopteridae | | | | | | | | | |
| *Rhinoptera bonasus* | 13 | June–November 2005, | Florida | Lecanicephalidea | *Eniochobothrium* n. sp. (OG) | Spiral intestine | | GQ470201–2 | 7203–4 |
| | | March–October 2006, | | Rhinebothriidea | *Rhodobothrium paucitesticulare* | | Adult, H2 | GQ470172–74, 78 | 7205–8 |
| | | May 2007 | | Tetraphyllidea | *Dioecotaenia campbelli* | | Adult, D | GQ470156–7 | 7209–10 |
| | | | | Tetraphyllidea | *Duplicibothrium* n. sp. 1 | | Adult, C1 | GQ470125–32 | 7211–8 |
| | | | | Tetraphyllidea | *Duplicibothrium minutum* | | Adult, C2 | GQ470133, 36, 38, 40, 43 | 7219–23 |
| Rajiformes: Dasyatidae | | | | | | | | | |
| *Dasyatis sabina* | 15 | June 2005, | Florida, | Rhinebothriidea | *Spongiobothrium* sp. | Spiral intestine | Adult, H3 | GQ470184–9 | 7238–43 |
| | | February–October 2006, | Mississippi | | | | | | |
| *Dasyatis say* | 14 | June–July 2005, | Florida, | Lecanicephalidea | *Polypocephalus* sp. (OG) | Spiral intestine | | GQ470199–200 | 7224–5 |
| | | April–October 2006 | Mississippi | Rhinebothriidea | *Rhinebothrium* sp. 3 | | Adult, H6 | GQ470190–3 | 7226–9 |
| | | | | Rhinebothriidea | *Rhinebothrium* sp. 4 | | Adult, H7 | GQ470197–8 | 7230–1 |
| | | | | Tetraphyllidea | *Acanthobothrium* sp. 6A | | Adult, A6 | GQ470108–9, 11 | 7232–4 |
| | | | | Tetraphyllidea | *Acanthobothrium* sp. 6B | | Adult, A6 | GQ470110, 12 | 7235–6 |
| | | | | Tetraphyllidea | *Acanthobothrium* sp. 6C | | Adult, A6 | GQ470113 | 7237 |

LRP, Lawrence R. Penner Parasitology Collection, University of Connecticut, Storrs, Connecticut, USA.

**Table 2**
Crustaceans, molluscs and fishes from the Gulf of Mexico necropsied for larval tetraphyllideans and rhinebothriideans.

| Host | No. of hosts infected/ necropsied | Locality | Larval type, (sub)group | Larval identification | Site in host | GenBank No. | LRP No. |
|---|---|---|---|---|---|---|---|
| Crustacea (2 families; 5 spp.) | | | | | | | |
| Decapoda: Callianassidae | | | | | | | |
| *Callichirus islagrande* | 0/12 | Mississippi | | N/A | N/A | | |
| Decapoda: Penaeidae | | | | | | | |
| *Farfantepenaeus aztecus* | 0/2 | Mississippi | | N/A | N/A | | |
| *Farfantepenaeus duorarum* | 0/3 | Florida, Mississippi | | N/A | N/A | | |
| *Litopenaeus setiferus* | 0/1 | Mississippi | | N/A | N/A | | |
| *Trachypenaeus* sp. | 0/2 | Mississippi | | N/A | N/A | | |
| Bilvalvia (10 families; 12 spp.) | | | | | | | |
| Arcoida: Arcidae | | | | | | | |
| *Lunarca ovalis* | 0/1 | Mississippi | | N/A | N/A | | |
| Nuculoida: Nuculanidae | | | | | | | |
| *Nuculana concentrica* | 0/4 | Mississippi | | N/A | N/A | | |
| Osteroida: Pectinidae | | | | | | | |
| *Argopecten irradians concentricus* | 0/20 | Florida, Mississippi | | N/A | N/A | | |
| Veneroida: Cardiidae | | | | | | | |
| *Laevicardium mortoni* | 0/1 | Florida | | N/A | N/A | | |
| Veneroida: Donacidae | | | | | | | |
| *Donax variabilis* | 127/915 | Florida, Mississippi | Type III | *Duplicibothrium* n. sp. 1[a] | Digestive gland | | |
| | | | Type III, C2 | *Duplicibothrium minutum* | Digestive gland | GQ470134–5, 39[b], 42 | 7248–50 |
| | | | Type VIII, H2 | *Rhodothrium paucitesticulare* | Digestive gland | GQ470171, 75–77 | 7244–47 |
| Veneroida: Mactridae | | | | | | | |
| *Mulinia lateralis* | 0/10 | Mississippi | | N/A | N/A | | |
| Veneroida: Solecurtidae | | | | | | | |
| *Tagelus divisus* | 1/4 | Mississippi | | Unidentified tetraphyllidean or lecanicephalidean[a] | ? | | |
| Veneroida: Solenidae | | | | | | | |
| *Solen viridis* | 0/2 | Mississippi | | N/A | N/A | | |
| Veneroida: Tellinidae | | | | | | | |
| *Angulus versicolor* | 3/5 | Mississippi | Type III, C2 | *Duplicibothrium minutum* | Digestive gland | GQ470137, 41 | 7251–2 |
| | | | | Unidentified tetraphyllidean or lecanicephalidean[a] | Digestive gland | | |
| *Macoma mitchelli* | 2/8 | Mississippi | | Unidentified tetraphyllidean or lecanicephalidean[a] | ? | | |
| Veneroida: Veneridae | | | | | | | |
| *Chione elevata* | 0/10 | Florida | | N/A | N/A | | |
| *Dosinia discus* | 0/17 | Florida | | N/A | N/A | | |
| Gastropoda (10 families; 12 spp.) | | | | | | | |
| Neogastropoda: Buccinidae | | | | | | | |
| *Solenosteira cancellaria* | 3/9 | Florida, Mississippi | Type III, C3 | *Duplicibothrium* n. sp. 2 | Anterior digestive system | GQ470144–45, 49–50 | 7253–6 |
| Neogastropoda: Columbellidae | | | | | | | |
| *Costoanachis semiplicata* | 0/8 | Florida | | N/A | N/A | | |
| Neogastropoda: Fasciolariidae | | | | | | | |
| *Fasciolaria hunteria* | 0/6 | Florida | | N/A | N/A | | |
| Neogastropoda: Marginellidae | | | | | | | |
| *Prunum* sp. | 0/8 | Florida | | N/A | N/A | | |
| Neogastropoda: Melongenidae | | | | | | | |
| *Melongena corona* | 1/24 | Florida | Type III | *Duplicibothrium minutum*[a] | ? | | |
| Neogastropoda: Muricidae | | | | | | | |
| *Murex* sp. | 0/5 | Florida | | N/A | N/A | | |
| *Stramonita haemastoma* | 0/2 | Mississippi | | N/A | N/A | | |
| Neogastropoda: Nassariidae | | | | | | | |
| *Nassarius vibex* | 2/16 | Mississippi | Type III, C3 | *Duplicibothrium* n. sp. 2 | Anterior digestive system | GQ470152 | 7257 |
| Neogastropoda: Olividae | | | | | | | |
| *Oliva sayana* | 0/3 | Mississippi | | N/A | N/A | | |
| Neogastropoda: Cerithiidae | | | | | | | |
| *Cerithium* sp. | 0/24 | Florida | | N/A | N/A | | |
| *Cerithium* cf. *lutosum* | ?/? | Florida | Type III | *Duplicibothrium* n. sp. 2[a] | Anterior digestive system | | |
| Neogastropoda: Naticidae | | | | | | | |
| *Neverita duplicata* | 3/6 | Florida, Mississippi | Type III, C3 | *Duplicibothrium* n. sp. 2 | Anterior digestive system | GQ470146–8, 51 | 7258–61 |
| Neopterygii (24 families; 46 spp.) | | | | | | | |
| Batrachoidiformes: Batrachoididae | | | | | | | |
| *Opsanus beta* | 1/1 | Louisiana | Type I, A1 | *Phoreiobothrium* sp. 2 | Digestive system | GQ470062–3[c] | 7264–5 |

**Table 2** (*continued*)

| Host | No. of hosts infected/ necropsied | Locality | Larval type, (sub)group | Larval identification | Site in host | GenBank No. | LRP No. |
|---|---|---|---|---|---|---|---|
| | | | Type II | *Acanthobothrium* sp.[a] | Digestive system | | |
| | | | Type IV, E1 | *Paraorygmatobothrium* sp. 1 | Digestive system | GQ470030[c] | 7262 |
| | | | Type V, F1 | *Pedibothrium* sp. 1 | Digestive system | GQ470155[c] | 7263 |
| Beloniformes: Hemiramphidae | | | | | | | |
| *Hemiramphus brasiliensis* | 3/4 | Mississippi | Type I, A3 | *Phoreiobothrium* sp. 3 | Digestive system | GQ470103–4, 5–7[c] | 7266–70 |
| Clupeiformes: Clupeidae | | | | | | | |
| *Brevoortia patronus* | 0/3 | Mississippi | | N/A | N/A | | |
| Clupeiformes: Engraulidae | | | | | | | |
| *Anchoa hepsetus* | 1/1 | Mississippi | | Unidentified tetraphyllidean[a] | Digestive system | | |
| *Anchoa mitchilli* | 5/9 | Mississippi | Type I, A1 | *Phoreiobothrium* sp. 1 | Digestive system | GQ470081, 86–93, 96 | 7271–80 |
| Gadiformes: Gadidae | | | | | | | |
| *Urophycis floridana* | 5/8 | Mississippi | Type I | *Phoreiobothrium* sp. 1[a] | Digestive system | GQ470183 | 7281 |
| | | | Type I | *Phoreiobothrium* sp. 3[a] | Digestive system | | |
| | | | Type II | *Acanthobothrium* sp.[a] | Digestive system | | |
| | | | Type IV | *Paraorygmatobothrium* sp.[a] | Digestive system | | |
| | | | Type VI | *Anthobothrium* sp. 1[a] | Digestive system | | |
| | | | Type VII, H3 | *Spongiobothrium* sp. | Digestive system | | |
| Ophidiiformes: Ophidiidae | | | | | | | |
| *Lepophidium brevibarbe* | 0/1 | Mississippi | | N/A | N/A | | |
| Perciformes: Carangidae | | | | | | | |
| *Caranx crysos* | 0/2 | Mississippi | | N/A | N/A | | |
| *Caranx hippos* | 0/1 | Mississippi | | N/A | N/A | | |
| *Chloroscombrus chrysurus* | 0/1 | Mississippi | | N/A | N/A | | |
| *Selene vomer* | 0/1 | Mississippi | | N/A | N/A | | |
| Perciformes: Ephippidae | | | | | | | |
| *Chaetodipterus faber* | 0/1 | Mississippi | | N/A | N/A | | |
| Perciformes: Lobotidae | | | | | | | |
| *Lobotes surinamensis* | 2/3 | Mississippi | Type I, A5 | *Phoreiobothrium* sp. 5 | Digestive system | GQ470059–61[c] | 7288–90 |
| | | | Type IV, E3 | *Paraorygmatobothrium* sp. 3 | Digestive system | GQ470014–5[c], 18–19[c] | 7284–7 |
| | | | Type VII, H4 | *Rhinebothrium* sp. 1 | Digestive system | GQ470181–2 | 7182–3 |
| Perciformes: Lutjanidae | | | | | | | |
| *Lutjanus campechanus* | 3/7 | Mississippi | Type V, F2 | *Pedibothrium* sp. 2 | Digestive system | GQ470153–4[d] | 7291–2 |
| | | | | Unidentified tetraphyllidean[a] | Digestive system | | |
| *Lutjanus synagris* | 1/2 | Louisiana, Mississippi | Type I, A4 | *Phoreiobothrium* sp. 4 | Digestive system | GQ470056–8[c] | 7293–5 |
| *Rhomboplites aurorubens* | 0/1 | Mississippi | | N/A | N/A | | |
| Perciformes: Mugilidae | | | | | | | |
| *Mugil cephalus* | 0/2 | Mississippi | | N/A | N/A | | |
| *Mugil curema* | 0/1 | Florida | | N/A | N/A | | |
| Perciformes: Mullidae | | | | | | | |
| *Upeneus parvus* | 0/1 | Mississippi | | N/A | N/A | | |
| Perciformes: Nomeidae | | | | | | | |
| *Nomeus gronovii* | 1/2 | Mississippi | Type VII | Unidentified rhinebothriidean[a] | Digestive system | | |
| Perciformes: Sciaenidae | | | | | | | |
| *Bairdiella chrysoura* | 3/7 | Mississippi | Type II | *Acanthobothrium* sp.[a] | Digestive system | GQ470052[c], 4[c] | 7299–300 |
| | | | Type IV, E4 | *Paraorygmatobothrium* sp. 4 | Digestive system | | |
| *Cynoscion arenarius* | 5/8 | Mississippi | Type IV | *Paraorygmatobothrium* sp.[a] | Digestive system | | |
| *Cynoscion nebulosus* | 2/6 | Mississippi | Type II, B5 | *Acanthobothrium* sp. 5 | Digestive system | GQ470121 | 7296 |
| | | | Type IV, E4 | *Paraorygmatobothrium* sp. 4 | Digestive system | GQ470049–50[c] | 7297–8 |
| *Cynoscion nothus* | 0/1 | Mississippi | | N/A | N/A | | |
| *Leiostomus xanthurus* | 1/8 | Florida, Mississippi | Type IV | *Paraorygmatobothrium* sp.[a] | Digestive system | | |
| *Micropogonias undulatus* | 1/7 | Mississippi | Type IV | *Paraorygmatobothrium* sp.[a] | Digestive system | | |
| *Sciaenops ocellatus* | 1/2 | Mississippi | Type IV | *Paraorygmatobothrium* sp.[a] | Digestive system | | |
| *Stellifer lanceolatus* | 1/1 | Mississippi | | Unidentified tetraphyllidean[a] | Digestive system | | |
| Perciformes: Scombridae | | | | | | | |
| *Euthynnus alletteratus* | 0/3 | [Mississippi] | | N/A | N/A | | |
| *Scomberomorus cavalla* | 0/1 | Mississippi | | N/A | N/A | | |
| *Seriola dumerili* | 0/2 | [Mississippi] | | N/A | N/A | | |
| Perciformes: Serranidae | | | | | | | |
| *Centropristis philadelphica* | 0/1 | Mississippi | | N/A | N/A | | |
| *Diplectrum formosum* | 1/1 | Mississippi | Type II, B3 | *Acanthobothrium* sp. 3 | Digestive system | GQ470122–3 | 7303–4 |
| | | | Type II, B4 | *Acanthobothrium* sp. 4 | Digestive system | GQ470116–9 | 7305–8 |
| | | | Type II, B5 | *Acanthobothrium* sp. 5 | Digestive system | GQ470120 | 7309 |
| | | | Type VI, G1 | *Anthobothrium* sp. 1 | Digestive system | GQ470163 | 7310 |
| | | | Type VII, H5 | *Rhinebothrium* sp. 2 | Digestive system | GQ470195–6 | 7301–2 |
| *Mycteroperca microlepis* | 1/2 | Louisiana | Type VI | *Anthobothrium* sp.[a] | Digestive system | | |
| *Mycteroperca phenax* | 0/2 | [Mississippi] | | N/A | N/A | | |
| Perciformes: Sparidae | | | | | | | |
| *Lagodon rhomboides* | 1/4 | Florida, Mississippi | Type II, B1 | *Acanthobothrium* sp. 1 | ? | GQ470115 | 7312 |
| *Stenotomus caprinus* | 1/1 | Mississippi | Type VII, H5 | *Rhinebothrium* sp. 2 | Digestive system | GQ470194 | 7311 |

(*continued on next page*)

**Table 2** (*continued*)

| Host | No. of hosts infected/ necropsied | Locality | Larval type, (sub)group | Larval identification | Site in host | GenBank No. | LRP No. |
|---|---|---|---|---|---|---|---|
| Perciformes: Sphyraenidae | | | | | | | |
| *Sphyraena barracuda* | 0/1 | Mississippi | | N/A | N/A | | |
| Perciformes: Stromateidae | | | | | | | |
| *Peprilus burti* | 1/2 | Mississippi | Type VI | *Anthobothrium* sp.[a] | Digestive system | | |
| Perciformes: Trichiuridae | | | | | | | |
| *Trichiurus lepturus* | 2/2 | Mississippi | Type I, A1 | *Phoreiobothrium* sp. 1 | Digestive system | GQ470077–8, 84–5, | 7324–9 |
| | | | Type I, A6 | *Triloculatum* sp. | Digestive system | 97–8 | |
| | | | Type II | *Acanthobothrium* sp.[a] | Digestive system | GQ470099 | 7330 |
| | | | Type IV, E2 | *Paraorygmatobothrium* sp. 2 | Digestive system | GQ470022[c], 5–9[c] | 7318–23 |
| | | | Type VI, G1 | *Anthobothrium* sp. 1 | Digestive system | GQ470162, 67–8, | 7313–16 |
| | | | Type VI, G2 | *Anthobothrium* sp. 2 | Digestive system | 70GQ470158 | 7317 |
| Pleuronectiformes: Achiridae | | | | | | | |
| *Trinectes maculatus* | 0/2 | Mississippi | | N/A | N/A | | |
| Pleuronectiformes: Cynoglossidae | | | | | | | |
| *Symphurus pusillus* | 1/2 | Mississippi | | Unidentified tetraphyllidean[a] | Digestive system | | |
| Pleuronectiformes: Paralichthyidae | | | | | | | |
| *Paralichthys* cf. *lethostigma* | 0/1 | Mississippi | | N/A | N/A | | |
| *Paralichthys lethostigma* | 2/3 | Mississippi | Type II, B2 | *Acanthobothrium* sp. 2 | Digestive system | GQ470114[c], 24[c] | 7331–2 |
| | | | Type II, E3 | *Paraorygmatobothrium* sp. 3 | Digestive system | GQ470008[c], 10[c], 16–7[c] | 7333–6 |
| | | | Type VI | *Phoreiobothrium* sp.[a] | Digestive system | | |
| | | | Type VII | *Spongiobothrium* sp.[a] | Digestive system | | |
| Siluriformes: Ariidae | | | | | | | |
| *Ariopsis felis* | 4/7 | Mississippi | Type I, A6 | *Triloculatum* sp. | Digestive system | GQ470100–1 | 7341–2 |
| | | | Type IV, E4 | *Paraorygmatobothrium* sp. 4 | Digestive system | GQ470051[c], 3[c], 5[c] | 7338–40 |
| | | | Type VI, G1 | *Anthobothrium* sp. 1 | Digestive system | GQ470164 | 7337 |
| *Bagre marinus* | 1/1 | Mississippi | | Unidentified tetraphyllidean[a] | Digestive system | | |

[a]Identification based on morphology only; unidentified larvae assigned to larval type only. [b]No voucher. [c]Photo voucher only. [d]Representative voucher. LRP, Lawrence R. Penner Parasitology Collection, University of Connecticut, Storrs, Connecticut, USA.

Maryland, USA. Available at http://rsb.info.nih.gov/ij/). Measurements were taken of 195 larvae and consisted of total length, acetabular length and width, and apical organ width. Based on the terminology of Chervy (2002), all larvae are considered to be plerocercoids because each exhibits a scolex proper that is everted and lacks a primary lacuna. Attachment structure terminology follows Caira et al. (1999, 2001)

## 3. Results

### 3.1. Host infections

Eight species of elasmobranchs yielded the adult cestodes sequenced de novo for this study. Adult cestodes for which sequence data were generated were found to represent at least 25 species in seven genera of tetraphyllideans (*Acanthobothrium*, *Anthobothrium*, *Dioecotaenia*, *Duplicibothrium*, *Paraorygmatobothrium*, *Phoreiobothrium* and *Triloculatum*) and five species in four genera of rhinebothriideans (*Rhinebothrium*, *Rhabdotobothrium*, *Rhodobothrium* and *Spongiobothrium*) as well as two species in two genera of lecanicephalideans (*Eniochobothrium* and *Polypocephalus*) included as outgroups. Collection data for the adult cestodes, and GenBank accession numbers for the 98 adult ingroup specimens and four adult outgroup specimens for which 28S rDNA data were generated de novo are summarized by host species in Table 1.

A total of 75 species of potential intermediate hosts was examined; in combination these hosted over 600 specimens of cestode larvae: (i) neopterygians: 24 of 46 species with tetraphyllideans and six of 46 with rhinebothriideans, (ii) gastropods: five of 12 species with tetraphyllideans and none with rhinebothriideans, (iii) bivalves: two of 12 species with tetraphyllideans, one of 12 species with rhinebothriideans, and three of 12 species with larvae identifiable only as either tetraphyllidean or lecanicephalidean, and (iv) decapods: none of five species. In total, these larvae consisted of a minimum of 24 species in seven genera of tetraphyllideans and four species in three genera of rhinebothriideans. The collection data, including prevalence of infection, are summarized by species in the major intermediate host groups in Table 2. GenBank accession numbers for the 100 larval specimens for which 28S rDNA data were generated de novo are also provided in Table 2.

### 3.2. Initial maximum likelihood analysis

With respect to the initial matrix, the aligned 28S rDNA dataset for 208 taxa (i.e., 98 adult and 100 larval ingroup specimens and 10 adult outgroup taxa) consisted of 1,321 characters. The number of variable positions was 665, of which 646 were parsimony informative. Each of the eight independent ML analyses resulted in similar optimal topologies and similar lnL scores (i.e., ranging between −13,099.6494 and −13,104.4889). The topology of the tree resulting from the analysis with the lowest likelihood score is shown in Fig. 1.

The molecular analysis resulted in eight major groups, seven of them containing larvae, designated with the letters A to H. These consisted of six groups containing larvae and adults, one containing adults only and one containing larvae only. Seven of the eight groups (B to H), were strongly supported receiving bootstrap values of $\geqslant$99% (see Hillis and Bull, 1993). Group A was supported with a relatively lower bootstrap value of 58%. Four of the groups containing both larval and adult specimens each contained adults of only a single genus of cestode. These were Group B (*Acanthobothrium*), Group C (*Duplicibothrium*), Group E (*Paraorygmatobothrium*), and Group G (*Anthobothrium*). The remaining two groups each consisted of suites of related genera. These were Group A (*Phoreiobothrium* and *Triloculatum*) and Group H (*Rhabdotobothrium*, *Rhodobothrium*, *Spongiobothrium* and *Rhinebothrium*, i.e., the rhinebothriidean genera). Group D was comprised only of adult forms of *Dioecotaenia*. It was hoped that the identity of Group F, comprised of larvae only, would be informed by the expanded analysis.

In the cases of all but one of the eight groups (Group D), the molecular analysis revealed two or more subgroups. Within the eight groups, conservatively, a total of 32 subgroups was recog-

**Table 3**
Adult and larval cestode species included in the maximum likelihood analyses for wich sequence data was taken from GenBank.

| Host | Parasite order | Parasite species | Stage, (sub)group | GenBank No. |
|---|---|---|---|---|
| Elasmobranchii: Carcharhiniformes: Carcharhinidae | | | | |
| *Galeocerdo cuvier* | Tetraphyllidea | *Thysanocephalum* sp. | Adult | AF286963 |
| *Lamiopsis temmincki* | Cathetocephalidea | *Sanguilevator yearsleyi* | Adult | AY961397 |
| *Prionace glauca* | Tetraphyllidea | *Platybothrium auriculatum* | Adult | AF286955 |
| | Tetraphyllidea | *Prosobothrium armigerum* | Adult | AF286956 |
| Elasmobranchii: Carcharhiniformes: Scyliorhinidae | | | | |
| *Scyliorhinus canicula* | Tetraphyllidea | *Crossobothrium longicolle* | Adult | AF286958 |
| Elasmobranchii: Carcharhiniformes: Sphyrnidae | | | | |
| *Sphyrna mokarran* | Tetraphyllidea | *Phoreiobothrium* sp. | Adult, A5 | AF286954 |
| Elasmobranchii: Carcharhiniformes: Triakidae | | | | |
| *Mustelus asterias* | Tetraphyllidea | *Orygmatobothrium musteli* | Adult | AF382088 |
| | Tetraphyllidea | *Phyllobothrium lactuca* | Adult | AF286960 |
| Elasmobranchii: Lamniformes: Lamnidae | | | | |
| *Alopias pelagicus* | Litobothriidea (OG) | *Litobothrium amplifica* | Adult | AF286931 |
| | Tetraphyllidea | *Marsupiobothrium* sp. | Adult, E | AF286959 |
| *Alopias superciliosus* | Litobothriidea (OG) | *Litobothrium janovyi* | Adult | AF286930 |
| *Isurus oxyrinchus* | Tetraphyllidea | *Ceratobothrium xanthocephalum* | Adult | AF382089 |
| | Tetraphyllidea | *Clistobothrium montaukensis* | Adult | AF286957 |
| | Tetraphyllidea | *Clistobothrium montaukensis* | Adult | EF095259 |
| Elasmobranchii: Myliobatiformes: Myliobatidae | | | | |
| *Aetobatus* cf. *narinari* | Lecanicephalidea (OG) | *Adelobothrium* cf. *aetobatidis* | Adult | EF095257 |
| | Lecanicephalidea (OG) | *Cephalobothrium aetobatidis* | Adult | AF286927 |
| *Mobula hypostoma* | Rhinebothriidea | *Rhabdotobothrium anterophallum* | Adult, H1 | AF286961 |
| *Myliobatis californicus* | Tetraphyllidea | *Caulobothrium* n. sp. 1 | Adult | FJ177101 |
| | Tetraphyllidea | *Caulobothrium opisthorchis* | Adult | FJ177106 |
| *Pteromylaeus bovinus* | Tetraphyllidea | *Caulobothrium* n. sp. 2 | Adult | FJ177102 |
| Elasmobranchii: Myliobatiformes: Rhinopteridae | | | | |
| *Rhinoptera bonasus* | Rhinebothriidea | *Rhodobothrium paucitesticulare* | Adult, H2 | FJ177100 |
| | Rhinebothriidea | *Rhodobothrium* sp. | Adult, H2 | EF095258 |
| | Tetraphyllidea | *Duplicibothrium* cf. *minutum* | Adult, C | FJ177136 |
| *Rhinoptera* sp. | Lecanicephalidea (OG) | *Eniochobothrium gracilis* | Adult | AF286928 |
| | Tetraphyllidea | *Duplicibothrium* n. sp. | Adult, C | FJ177135 |
| Elasmobranchii: Orectolobiformes: Ginglymostomatidae | | | | |
| *Nebrius ferrugineus* | Tetraphyllidea | *Pachybothrium hutsoni* | Adult, F | EF095260 |
| Elasmobranchii: Pristiformes: Pristidae | | | | |
| *Pristis clavata* | Rhinebothriidea | Rhinebothriinae n. sp. | Adult, H | FJ177119 |
| Elasmobranchii: Rajiformes: Dasyatidae | | | | |
| *Dasyatis akajei* | Rhinebothriidea | *Rhinebothrium* sp. 4 | Adult, H | FJ177126 |
| *Dasyatis americana* | Rhinebothriidea | *Rhinebothrium maccallumi* | Adult, H | AF286962 |
| *Dasyatis brevis* | Rhinebothriidea | *Rhinebothrium* sp. 5 | Adult, H | FJ177127 |
| | Rhinebothriidea | *Spongiobothrium* sp. | Adult, H | AF382085 |
| *Dasyatis centroura* | Rhinebothriidea | *Anthocephalum* cf. *centrurum* | Adult, H | FJ177099 |
| *Dasyatis longus* | Rhinebothriidea | *Scalithrium* sp. | Adult, H | FJ177133 |
| | Tetraphyllidea | *Acanthobothrium* sp. 1 | Adult, B | AF286953 |
| *Dasyatis* sp. | Lecanicephalidea (OG) | *Tylocephalum* sp. | Adult | AF286929 |
| *Himantura* cf. *gerrardi* | Rhinebothriidea | n. gen. 1 n. sp. | Adult, H | FJ177107 |
| *Himantura lobistoma* | Tetraphyllidea | *Acanthobothrium* sp. | Adult, B | FJ843592 |
| *Himantura pastinacoides* | Rhinebothriidea | *Rhinebothrium* sp. 1 | Adult, H | FJ177121 |
| *Himantura polylepis* | Rhinebothriidea | n. gen. 4 *kinabatanganensis* | Adult, H | FJ177118 |
| | Rhinebothriidea | *Rhinebothrium megacanthophallus* | Adult, H | FJ177120 |
| | Tetraphyllidea | *Acanthobothrium masnihae* | Adult, A | FJ843605 |
| *Himantura* sp. | Tetraphyllidea | *Acanthobothrium oceanharvestae* | Adult, A | FJ843594 |
| | Tetraphyllidea | *Acanthobothrium popi* | Adult, A | FJ843600 |
| | Tetraphyllidea | *Acanthobothrium rodmani* | Adult, A | FJ843596 |
| | Tetraphyllidea | *Acanthobothrium romanowi* | Adult, A | FJ843598 |
| | Tetraphyllidea | *Acanthobothrium zimmeri* | Adult, A | FJ843602 |
| *Himantura astra* | Rhinebothriidea | n. gen. 3 sp. 3 | Adult, H | FJ177113 |
| | Rhinebothriidea | n. gen. 3 sp. 4 | Adult, H | FJ177114 |
| *Himantura uarnacoides* | Rhinebothriidea | *Rhinebothrium* sp. 2 | Adult, H | FJ177124 |
| *Himantura leoparda* | Rhinebothriidea | n. gen. 3 sp. 5 | Adult, H | FJ177115 |
| *Neotrygon kuhlii* | Rhinebothriidea | n. gen. 2 *shipleyi* | Adult, H | FJ177109 |
| *Pastinachus solocirostris* | Tetraphyllidea | *Caulobothrium* n. sp. 4 | Adult | FJ177104 |
| Stingray | Tetraphyllidea | *Acanthobothrium brevissime* | Adult, A | EU170363 |
| *Taeniura lymma* | Rhinebothriidea | n. gen. 2 *sexorchidum* | Adult, H | FJ177108 |
| | Rhinebothriidea | *Rhinebothrium* sp. 9 | Adult, H | FJ177131 |
| Elasmobranchii: Rajiformes: Potamotrygonidae | | | | |
| *Paratrygon* cf. *aiereba* | Rhinebothriidea | *Rhinebothrium* sp. | Adult, H | AY193880 |
| | Rhinebothriidea | *Rhinebothrium* sp. 8 | Adult, H | FJ177130 |
| *Potamotrygon* cf. *castexi* | Rhinebothriidea | *Rhinebothroides* cf. *freitasi* | Adult, H | FJ177132 |
| Elasmobranchii: Rajiformes: Rajidae | | | | |
| *Raja montagui* | Rhinebothriidea | *Echeneibothrium maculatum* | Adult, H | AF382086 |
| *Raja velezi* | Rhinebothriidea | *Echeneibothrium* sp. | Adult, H | FJ177098 |

(*continued on next page*)

**Table 3** (*continued*)

| Host | Parasite order | Parasite species | Stage, (sub)group | GenBank No. |
|---|---|---|---|---|
| Elasmobranchii: Rajiformes: Urolophidae | | | | |
| *Urobatis maculatus* | Tetraphyllidea | *Acanthobothrium parviuncinatum* | Adult, A | EF095264 |
| Elasmobranchii: Rhinobatiformes: Rhinobatidae | | | | |
| *Rhynchobatus* cf. *australiae* | Rhinebothriidea | *Spongiobothrium* sp. | Adult, H | FJ177134 |
| Cephalopoda: Teuthida: Loliginidae | | | | |
| *Loligo gahi* | Tetraphyllidea | *Clistobothrium* cf. *montaukensis* | Larva | AF382071 |
| | Tetraphyllidea | *Clistobothrium* cf. *montaukensis* | Larva | AF382072 |
| | Tetraphyllidea | *Clistobothrium* cf. *montaukensis* | Larva | AF382082 |
| | Tetraphyllidea | Tetraphyllidean plerocercoid | Larva | AF382083 |
| Neopterygii: Anguilliformes: Anguillidae | | | | |
| *Anguilla anguilla* | Proteocephalidea | *Proteocephalus macrocephalus* | Adult | EF095261 |
| Neopterygii: Amiiformes: Amiidae | | | | |
| *Amia calva* | Proteocephalidea | *Proteocephalus perplexus* | Adult | AF286940 |
| Mammalia: Cetacea: Delphinidae | | | | |
| *Grampus griseus* | Tetraphyllidea | *Monorygma grimaldii* | Larva | DQ839585 |
| | Tetraphyllidea | *Monorygma grimaldii* | Larva | DQ839586 |
| | Tetraphyllidea | Phyllobothriid sp. 2 | Larva | DQ839587 |
| *Stenella coeruleoalba* | Tetraphyllidea | *Monorygma grimaldii* | Larva | AY741594 |
| | Tetraphyllidea | Phyllobothriid sp. 1 | Larva | DQ839568 |
| | Tetraphyllidea | Phyllobothriid sp. 1 | Larva | DQ839576 |
| | Tetraphyllidea | Phyllobothriid sp. 2 | Larva | DQ839588 |
| | Tetraphyllidea | *Phyllobothrium delphini* | Larva | AY741606 |
| *Tursiops truncatus* | Tetraphyllidea | Phyllobothriid sp. 1 | Larva | DQ839580 |
| | Tetraphyllidea | *Phyllobothrium delphini* | Larva | DQ839590 |
| | Tetraphyllidea | *Phyllobothrium delphini* | Larva | DQ839592 |

nized based on number of pair-wise differences of specimens between subgroups and observed morphological differences of larvae between subgroups. Subgroups were designated with the letter of the group and a corresponding number. Group A consisted of six subgroups (A1–A6) that included one or more larvae, Group B of five (B1–B5), Groups E and H of four (E1–E4 and H2–H5, respectively) and Groups C, F and G of two (C2–C3, F1–F2 and G1–G2, respectively). The 32 subgroups consisted of nine subgroups containing larvae and adults, seven containing adults only and 16 containing larvae only. Specimens in 29 of 32 subgroups had identical sequences. Sequence variation among specimens in the remaining three subgroups (A6, G1 and H3) was 2, 5 and 2 bp, respectively. The lowest pair-wise difference between subgroups was 10 bp (0.76%) between subgroups E4 and E5, 15 bp (1.14%) between subgroups B4 and B5 and 22 bp (1.67%) between subgroups E1 and E2. All subgroups were supported with bootstrap values of >80%.

Molecular sequence data recovered only a subset of the 30 species of adult cestodes recognized based on morphology. In four of the 10 genera of tetraphyllideans and rhinebothriideans represented by greater than two adults (i.e., *Acanthobothrium*, *Phoreiobothrium*, *Paraorygmatobothrium* and *Anthobothrium*), adults specimens considered morphologically to represent different species were found to have 100% sequence identity. Conservatively, larvae with 100% sequence identity were considered conspecific, resulting in the recognition of 27 larval species (i.e., larvae in the 25 subgroups containing larvae and adults, or larvae only, with subgroups A6 and G1 considered to contain two species each differing in 2 and 5 bp, respectively).

### 3.3. Morphological circumscription of larval types and assignment of generic identities

Examination of the morphological features of larval specimens in each of the seven larva-containing groups revealed a number of morphological consistencies within each group and a number of morphological differences among groups, allowing for the recognition of distinct larval types. In each case, larval morphologies that were found either to correspond to a group, or in the case of Group G, to subgroups, have been given formal Roman numeral designations. Diagnoses of larval types are presented in telegraphic style based on morphology by group below. In most cases, morphological differences within groups were consistent with the molecular subgroups. Most variation within larval types (i.e., subgroups) could be quantified based on a combination of overall larval length, acetabular length and width and apical sucker width. Measurements of these features for 24 subgroups are presented in Table 4. Light and/or SEM images of specimens of each larval type and their subgroups are provided in Fig. 2. Integration of molecular data with morphological observations of distinct larval types was used to assign generic identities to larvae representing these types. Light and/or SEM images of specimens of each larval type and the corresponding adult scolex morphologies are provided in Fig. 3. While integration of molecular data with morphological observations on the subgroups did not allow universal assignment of specific identities to these subgroups, these subgroups may be useful to distinguish among groups of species within genera.

#### 3.3.1. Group A (*Phoreiobothrium* and *Triloculatum*): Larval Type I

Larvae of all six subgroups (A1–A6) had the following features: larvae elongate, <1,100 μm in total length, specimens representing A6 probably longer, tapering posteriorly. Scolex with apical sucker and four acetabula; acetabula in form of bothridia. Apical sucker large, 54–110 μm wide. Bothridia round to oval, 56–238 μm long by 43–157 wide, sessile or slightly free anteriorly and posteriorly, divided into anterior and posterior loculi, division between anterior and posterior loculi inconspicuous (see arrows in Fig. 1A1b and A3b); lateral margins of bothridia intact; anterior loculus semi-lunar, with straight posterior margin, of equal width or slightly narrower than posterior loculus; bothridida non-retractable. Hooks not seen. Larval body undivided.

Group A consisted of a subgroup comprised of adults representing four species of *Phoreiobothrium* and larvae of Type I (A1), four subgroups comprised of larvae of Type I only (A2–A5), a subgroup comprised of an adult specimen of *Triloculatum bullardi* and larvae

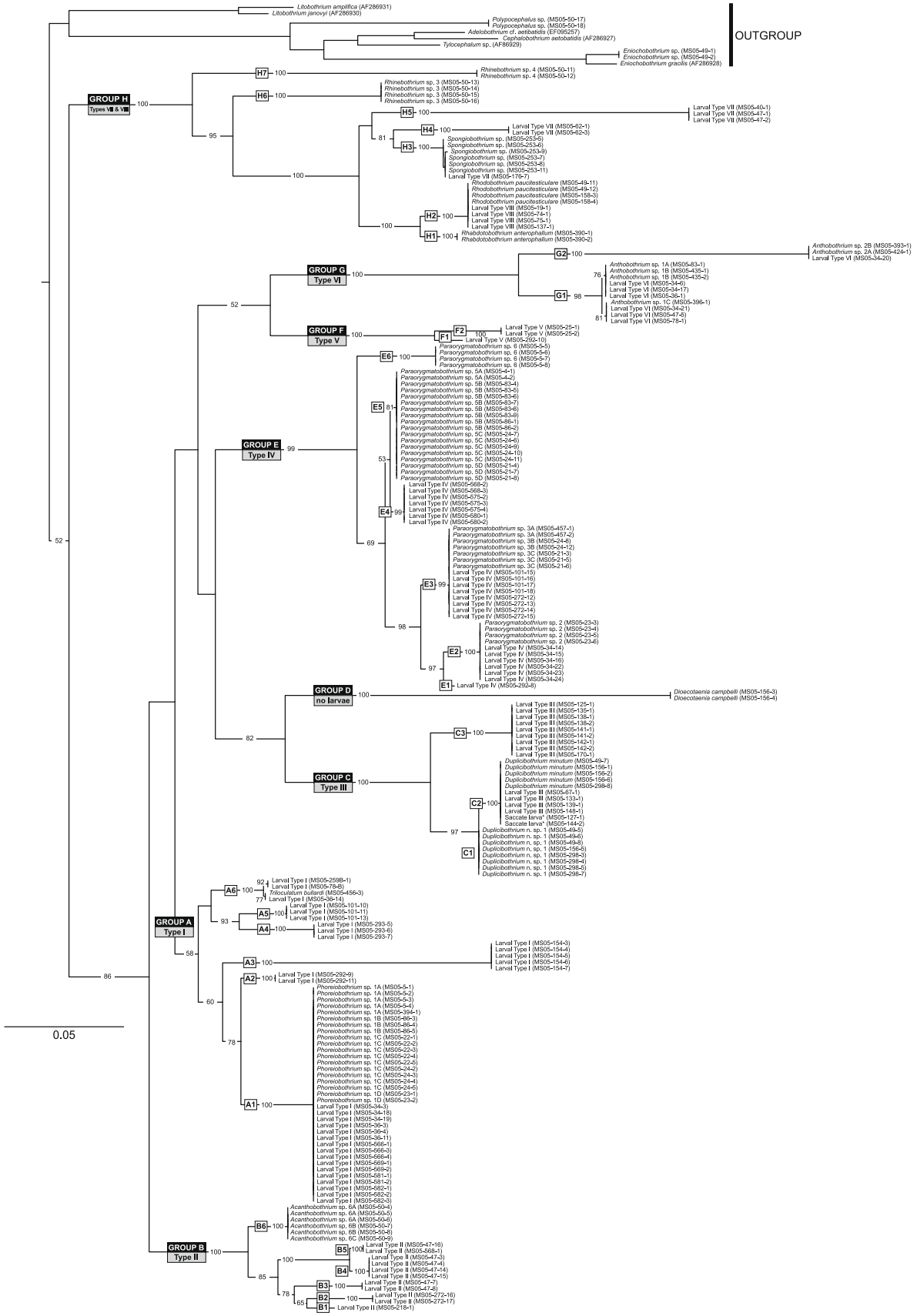


**Fig. 1.** Maximum likelihood (ML) tree (ln likelihood = −13,099.6494) of initial matrix including sequence data from 98 adult and 100 larval ingroup specimens of tetraphyllideans and rhinebothriideans from the Gulf of Mexico and 10 adult outgroup taxa. Nodal support on branches is only given if bootstrap values >50%. Major groups are indicated by black boxes above the line, with the corresponding larval type in grey boxes below the line; subgroups are indicated by white boxes; * indicates saccate larval forms of *Duplicibothrium* (see Discussion); scale: substitutions per site.

**Table 4**
Measurements[a] of morphological features of larvae from the Gulf of Mexico by larval type.

| | Total length | Acetabular length | Acetabular width | Apical organ width |
|---|---|---|---|---|
| Group A (*Phoreiobothrium* and *Triloculatum*): Larval Type I | | | | |
| A1 | 653–1,082 ($n$ = 12) | 104–144 ($n$ = 23; $N$ = 44) | 77–108 ($n$ = 23; $N$ = 45) | 54–71 ($n$ = 24) |
| A2 | 708 ($n$ = 1) | 96–113 ($n$ = 1; $N$ = 2) | 62–85 ($n$ = 2) | 70–92 ($n$ = 2) |
| A3 | 310–653 ($n$ = 10) | 58–95 ($n$ = 14; $N$ = 28) | 50–77 ($n$ = 14; $N$ = 25) | 49–76 ($n$ = 12) |
| A4 | 553–889 ($n$ = 7) | 72–94 ($n$ = 7; $N$ = 14) | 43–65 ($n$ = 7; $N$ = 11) | 84–107 ($n$ = 7) |
| A5 | 249–570 ($n$ = 15) | 56–79 ($n$ = 14; $N$ = 28) | 53–70 ($n$ = 14; $N$ = 27) | 90–110 ($n$ = 14) |
| A6 | [unknown] | 170–238 ($n$ = 6; $N$ = 3) | 119–157 ($n$ = 3; $N$ = 6) | 61–74 ($n$ = 3) |
| Group B (*Acanthobothrium*): Larval Type II | | | | |
| B1 | [unknown] | 276–303 ($n$ = 2; $N$ = 4) | 147–184 ($n$ = 2; $N$ = 4) | 110–111 ($n$ = 2) |
| B2 | 299–914 ($n$ = 5) | 62–154 ($n$ = 5; $N$ = 10) | 25–59 ($n$ = 5; $N$ = 10) | 29–45 ($n$ = 3) |
| B3 | 618–850 ($n$ = 7) | 129–179 ($n$ = 9; $N$ = 18) | 64–77 ($n$ = 6; $N$ = 12) | 73–86 ($n$ = 6) |
| B4 and B5 | 2.8–3.8 mm ($n$ = 4) | 133–168 ($n$ = 10; $N$ = 17) | 74–100 ($n$ = 10; $N$ = 18) | 74–99 ($n$ = 9) |
| Group C (*Duplicibothrium*): Larval Type III | | | | |
| C1 | [unknown] | 265–276 ($n$ = 1; $N$ = 2) | 173–197 ($n$ = 1; $N$ = 2) | 127 ($n$ = 1) |
| C2 | 1,025 ($n$ = 1) | 162–236 ($n$ = 5; $N$ = 10) | 89–116 ($n$ = 5; $N$ = 9) | 84–102 ($n$ = 5) |
| C3 | 693–2.8 mm ($n$ = 11) | 258–350 ($n$ = 9; $N$ = 15) | 79–102 ($n$ = 9; $N$ = 14) | 88–118 ($n$ = 11) |
| Group E (*Paraorymatobothrium*): Larval Type IV | | | | |
| E1 | 232 ($n$ = 1) | 36–37 ($n$ = 1; $N$ = 2) | 32–40 ($n$ = 1; $N$ = 2) | 49 ($n$ = 1) |
| E2 | 139–270 ($n$ = 16) | 26–49 ($n$ = 12; $N$ = 22) | 23–44 ($n$ = 11; $N$ = 20) | 42–54 ($n$ = 12) |
| E3 | 113–321 ($n$ = 12) | 30–43 ($n$ = 13; $N$ = 24) | 25–38 ($n$ = 13; $N$ = 20) | 39–57 ($n$ = 12) |
| E4 | 163–288 ($n$ = 8) | 22–42 ($n$ = 7; $N$ = 12) | 20–34 ($n$ = 6; $N$ = 8) | 33–43 ($n$ = 7) |
| Group F (no adults sequenced): Larval Type V | | | | |
| F1 | 395 ($n$ = 1) | 100–111 ($n$ = 1; $N$ = 2) | 80–85 ($n$ = 1; $N$ = 2) | [not visible] |
| F2 | 246–293 ($n$ = 3) | 90–115 ($n$ = 3; $N$ = 6) | 54–67 ($n$ = 3; $N$ = 6) | [not visible] |
| Group G (*Anthobothrium*): Larval Type VI | | | | |
| G1 | 881–1,592 ($n$ = 10) | 86–156 ($n$ = 15; $N$ = 15) | 79–134 ($n$ = 15; $N$ = 29) | 69–114 ($n$ = 15) |
| G2 | 439–1,023 ($n$ = 5) | 48–83 ($n$ = 6; $N$ = 12) | 55–75 ($n$ = 6; $N$ = 12) | 46–90 ($n$ = 5) |
| Group H (*Rhinebothrium* and *Spongiobothrium*): Larval Type VII | | | | |
| H3 | 734–787 ($n$ = 2) | 214–242 ($n$ = 5; $N$ = 10) | 75–134 ($n$ = 5; $N$ = 8) | 111–158 ($n$ = 5) |
| H4 | 978 ($n$ = 1) | 458–587 ($n$ = 4; $N$ = 6) | 148–190 ($n$ = 2; $N$ = 3) | 190–217 ($n$ = 4) |
| H5 | 12.6 mm ($n$ = 1) | [unknown] | [unknown] | 181–281 ($n$ = 6) |

[a] Measurements (in μm unless otherwise stated) are given as ranges followed in parentheses by the number of larvae measured ($n$) and the total number of measurements taken ($N$).

of Type I (A6). Larvae of Type I were identified as either *Phoreiobothrium* or *Triloculatum*.

### 3.3.2. Group B (Acanthobothrium): Larval Type II

Larvae of all five subgroups (B1–B5) had the following features: larvae elongate, 299 μm to up to 3.8 mm in total length, tapering posteriorly. Scolex with apical sucker and four acetabula; acetabula in form of bothridia. Apical sucker large, 29–111 μm wide. Bothridia elongate, 62–303 μm long by 25–185 μm wide, mainly sessile anteriorly, free posteriorly, divided into anterior pad and posterior loculus; lateral margins of bothridia conspicuously indented at junction of anterior pad and posterior loculus (see upper arrow in Fig. 1B3a); anterior pad triangular, with straight posterior margin, narrower than posterior loculus; one horizontal septum subdividing posterior loculus visible in many specimens (see lower arrow in Fig. 1B3a); two horizontal septa subdividing posterior loculus visible in some specimens; bothridia non-retractable. Hooks not seen. Larval body in some with horizontal divisions.

Group B consisted of a subgroup comprised of adults representing three species of *Acanthobothrium* (B6) and five subgroups of larvae of Type II (B1–B5). Larvae of Type II were identified as *Acanthobothrium*.

### 3.3.3. Group C (Duplicibothrium): Larval Type III

Larvae of subgroups C2 and C3 had the following features: larvae elongate, 693 μm–2.8 mm in total length, tapering posteriorly. Scolex with apical sucker and four acetabula; acetabula in form of bothridia. Apical sucker large, 84–127 μm wide. Bothridia oblong, 162–350 μm long by 79–197 μm wide, fused anteriorly into dorsal–ventral pairs, free posteriorly, slightly expanded posteriorly, facially loculated, occasionally crumpled; facial loculation consisting of 1–3 columns of rectangular loculi, with or without posterior row of rectangular loculi; lateral margins of bothridia intact; bothridia non-retractable. Hooks absent. Larval body with conspicuous anterior constriction posterior to scolex.

Group C consisted of a subgroup comprised of larvae of Type III (C3) and a subgroup comprised of adults of *Duplicibothrium minutum* and larvae of type III (C2) to the exclusion of adults representing a new species of *Duplicibothrium* designated as C1. Larvae of Type III were identified as *Duplicibothrium*.

### 3.3.4. Group E (Paraorygmatobothrium): Larval Type IV

Larvae of all four subgroups (E1–E4) had the following features: larvae elongate to subspherical, tiny, <350 μm in total length, tapering posteriorly. Scolex with apical sucker and four acetabula; acetabula in form of suckers. Apical sucker small, 33–57 μm wide, protruded in some. Suckers round to oblong, 22–49 μm long by 20–44 μm wide, sessile, undivided, non-retractable. Hooks absent. Larval body not divided.

Group E consisted of two subgroups comprised of adults representing one species (E6) and four species (E5) of *Paraorygmatobothrium*, respectively, two subgroups comprised of adults representing 1–3 species of *Paraorygmatobothrium* and larvae of Type IV (E2 and E3), and two subgroups comprised of larvae of Type IV only (E1 and E4). Larvae of Type IV were identified as *Paraorygmatobothrium*.

### 3.3.5. Group F (no adults sequenced): Larval Type V

Larvae of both subgroups had the following features: larvae subspherical, tiny, <400 μm in total length, tapering posteriorly. Scolex with four acetabula; acetabula in form of bothridia; apical

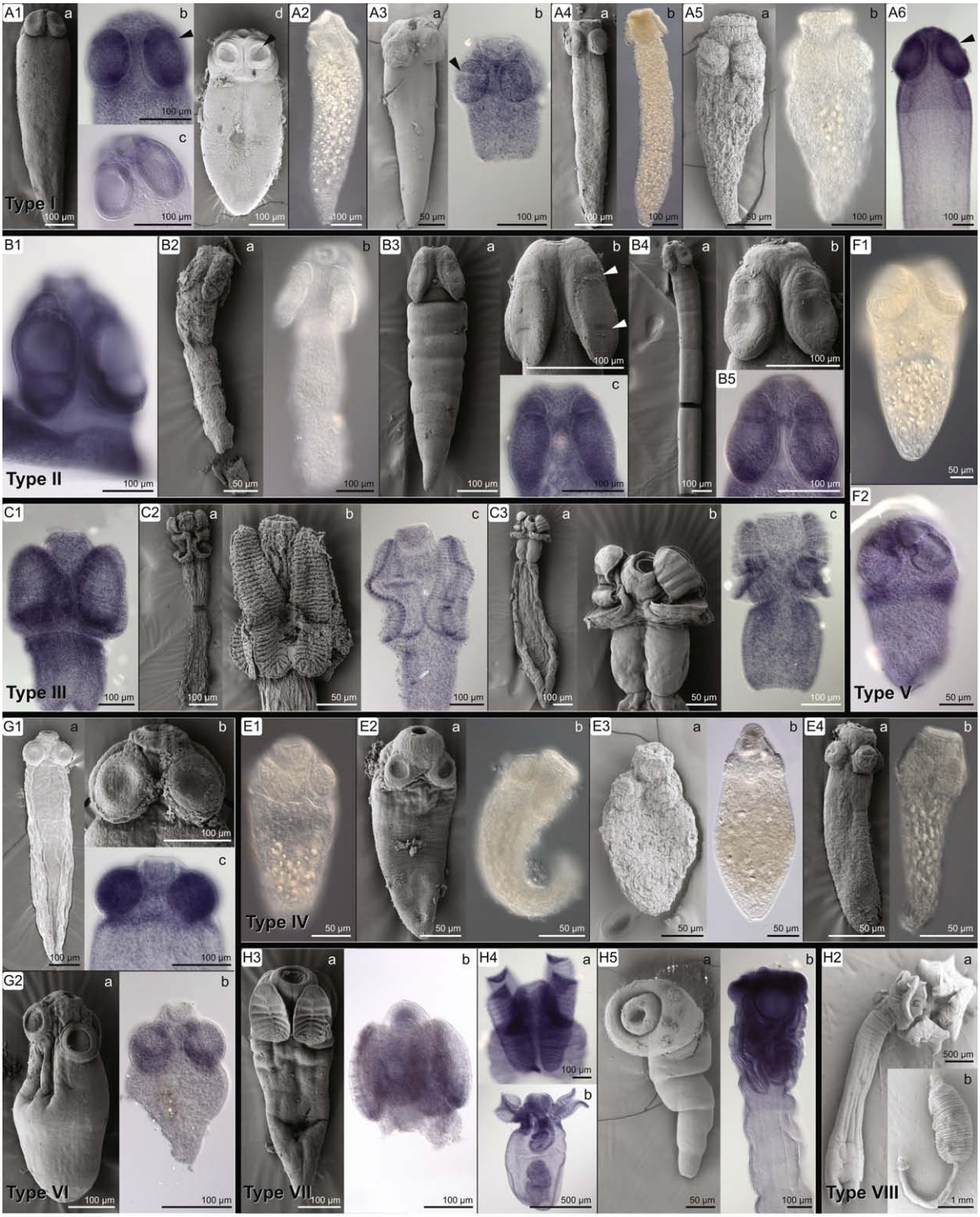


**Fig. 2.** Tetraphyllidean and rhinebothriidean Larval Types I–VIII. Note, for hologenophores or photo vouchers of specimens sequenced, specimen numbers are given. Type I. A1: a, ex *Trichiurus lepturus*; b, ex *Trichiurus lepturus* (MS05–36-3V); c, ex *Anchoa mitchelii* (MS05–566-3V); d, ex *Trichiurus lepturus*; A2: ex *Opsanus beta* (MS050–11V); A3: a, ex *Hemiramphus brasiliensis*; b, ex *Hemiramphus brasiliensis* (MS05–154-3V); A4: a, ex *Lutjanus synagris*; b, ex *Lutjanus synagris* (MS05–293-7V); A5: a, ex *Lobotes surinamensis*; b, ex *Lobotes surinamensis* (MS05–101-14V); A6: ex *Ariopsis felis* (MS05–259B-1V). Type II. B1: ex *Bairdiella chrysoura* (MS05–307-1V); B2: a, ex *Paralichthys lethostigma*; b, ex *Paralichthys lethostigma* (MS05–16V); B3: a, ex *Diplectrum formosum*; b, ex *Diplectrum formosum*; c, ex *Diplectrum formosum* (MS05–47-8V); B4: a, ex *Diplectrum formosum*; b, ex *Diplectrum formosum*; B5, ex *Diplectrum formosum* (MS05–47-16V). Type III. C1: ex *Donax variabilis*; C2: a, ex *Neverita duplicata*; b, ex *Neverita duplicata*; c, ex *Neverita duplicata*; C3: a, ex *Donax variabilis*; b, ex *Donax variabilis*; c, ex *Donax variabilis* (MS05–139-1V). Type IV. E1: ex *Opsanus beta* (MS05–292-8V); E2: a, ex *Trichiurus lepturus*; b, ex *Trichiurus lepturus* (MS05–34-24V); E3: a, ex *Lobotes surinamensis*; b, ex *Paralichthys lethostigma*; E4: a, ex *Bairdiella chrysoura*; b, ex *Cynoscion nebulosus* (MS05–568-2V). Type V. F1: ex *Opsanus beta* (MS05–292-10V); F2: ex *Lutjanus campechanus* (paragenophore). Type VI. G1: a, ex *Trichiurus lepturus*; b, ex *Trichiurus lepturus*; c, ex *Diplectrum formosum* (MS05–47-5V); G2: a, ex *Trichiurus lepturus*; b, ex *Trichiurus lepturus* (MS05–34-20V). Type VII. H3: a, ex *Urophycis floridana*; b, ex *Urophycis floridana* (MS05–176-6V); H4: a, ex *Lobotes surinamensis* (MS05–62-2V); b, ex *Lobotes surinamensis*; H6: a, ex *Diplectrum formosum*; b, ex *Diplectrum formosum* (MS05–47-2V). Type VIII. H2: a, ex *Donax variabilis*; b, ex *Donax variabilis*. Note, arrowheads in A1b, A1d, A3b, A6, and B3b indicate septa.

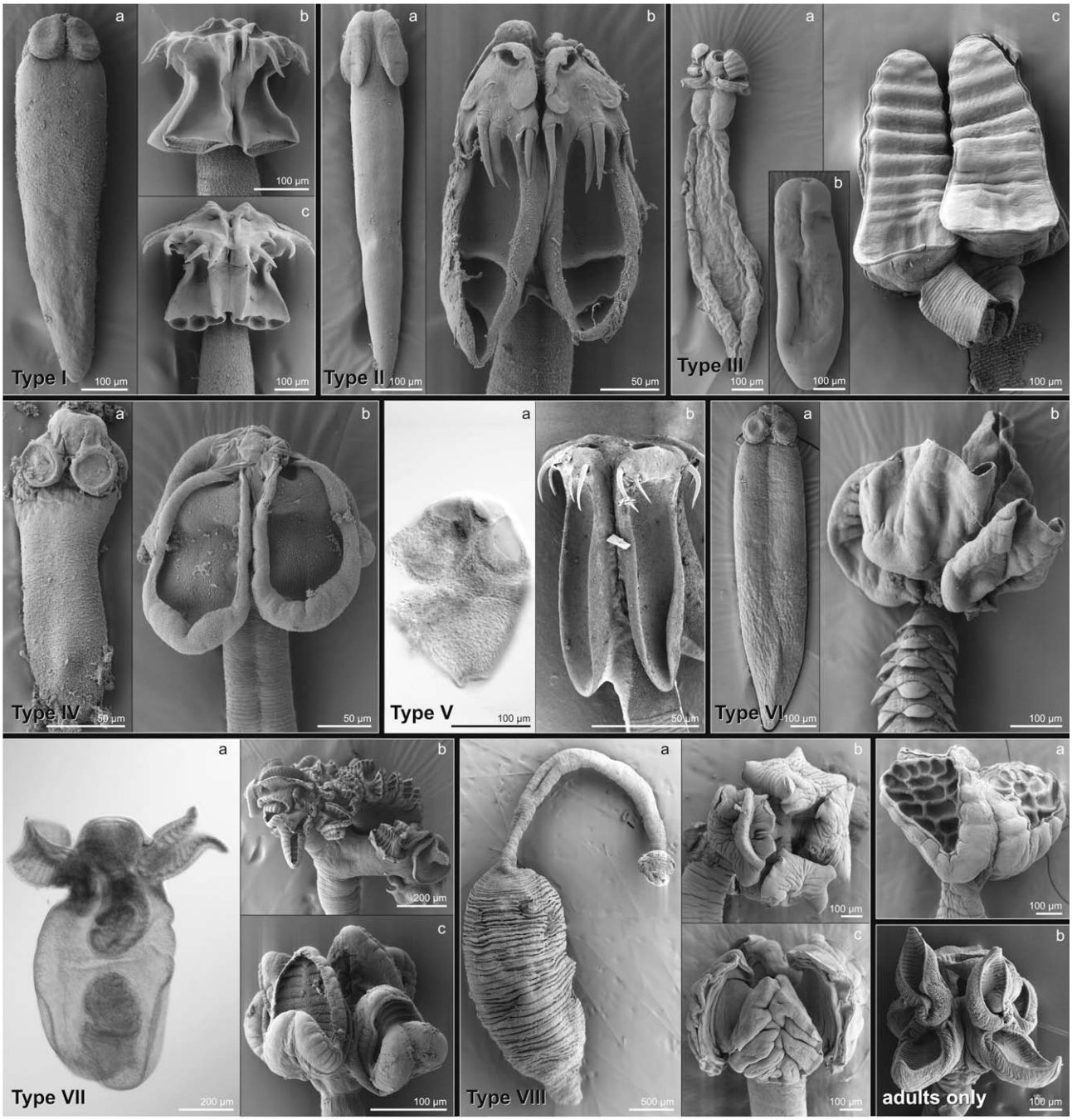


**Fig. 3.** Tetraphyllidean and rhinebothriidean larva Types I–VIII, with corresponding adult forms. Type I: a, Larval Type I ex *Trichiurus lepturus*; b, *Phoreiobothrium* sp. 1C ex *Carcharhinus limbatus*; c, *Triloculatum geeceearelensis* ex *Carcharhinus isodon*. Type II: a, Larval Type II ex *Diplectrum formosum*; b, *Acanthobothrium* sp. 6A ex *Dasyatis say*. Type III: a, Larval Type III ex *Donax variabilis*; b, saccate larva ex *D. variabilis*; c, *Duplicibothrium minutum* ex *Rhinoptera bonasus*. Type IV: a, Larval Type IV ex *Trichiurus lepturus*; b, *Paraorygmatobothrium* sp. ex *Carcharhinus brevipinna*. Type V: a, Larval Type V ex *Lutjanus campechanus*; b, *Pedibothrium longispine* ex *Ginglymostoma cirratum*. Type VI: a, Larval Type VI ex *Trichiurus lepturus*; b, *Anthobothrium* sp. ex *Carcharhinus limbatus*. Type VII: a, Larval Type VII ex *Lobotes surinamensis*; b, *Spongiobothrium* sp. ex *Dasyatis sabina*; c, *Rhinebothrium* sp. 3 ex *Dasyatis say*. Type VIII: a, Larval Type VIII ex *Donax variabilis*; b, Larval Type VIII ex *Donax variabilis*; c, *Rhodobothrium paucitesticulare* ex *Rhinoptera bonasus*. Adults only: a, *Dioecotaenia campbelli* ex *Rhinoptera bonasus*; b, *Rhabdotobothrium anterophallum* ex *Mobula hypostoma*.

sucker absent. Bothridia oval, 90–115 μm long by 54–85 μm wide, slightly free anteriorly and posteriorly, thick rimmed, divided into anterior and posterior loculi; lateral margins of bothridia conspicuously indented at junction of anterior and posterior loculi; anterior loculus semi-lunar, narrower than posterior loculus, with straight posterior margin; bothridia non-retractable. Hooks not seen. Larval body undivided.

Group F consisted of two subgroups comprised of larvae of Type V only (F1 and F2). The absence of adults from these groups in the initial analysis did not allow identification of this type.

*3.3.6. Group G (Anthobothrium): Larval Type VI*

Larvae of both subgroups had the following features: larvae elongate, 439 μm–1.6 mm in total length, tapering posteriorly. Scolex with apical sucker and four acetabula; acetabula in form of suckers. Apical sucker 46–114 μm wide. Acetabula round, 48–156 μm long by 55–134 μm wide, sessile, undivided, not retractable. Hooks absent. Larval body not divided.

Group G consisted of a subgroup comprised of adults representing three species of *Anthobothrium* and larvae of Type VI (G1) and a subgroup comprised of adults representing two species of *Antho-*

*bothrium* and larvae of Type VI (G2). Larvae of Type VI were identified as *Anthobothrium*.

*3.3.7. Group H (rhinebothriideans): Larval Types VII and VIII*

Two distinct larval morphologies were found in Group H. Group H consisted of a subgroup comprised of adults of *Rhabdotobothrium anterophallum* (H1), a subgroup comprised of adults of *Rhodobothrium paucitesticulare* and larvae of Type VIII (H2), a subgroup comprised of adults of *Spongiobothrium* and larvae of Type VII (H3), two subgroups of larvae only of Type VII (H4 and H5) and two subgroups comprised of adults representing two species of *Rhinebothrium* (H6 and H7).

*3.3.8. Larval Type VII (Rhinebothrium and Spongiobothrium)*

Larvae elongate, 734 μm–12.6 mm in total length, tapering posteriorly. Scolex with apical sucker and four acetabula; acetabula in form of bothridia. Apical sucker large, 111–281 μm wide. Bothridia elongate, 214–587 μm long by 75–190 μm wide (may be much longer in larvae in subgroup H5), stalked, facially loculated, fully retractable into scolex proper; facial loculation consisting of 1–2 columns of rectangular to subrectangular loculi. Bothridia retract at point of attachment to stalk, with anterior and posterior bothridial margins retracting last. Hooks absent. Larval body with horizontal divisions in some.

Larvae of Type VII were identified as *Rhinebothrium or Spongiobothrium*

*3.3.9. Larval Type VIII (Rhodobothrium)*

Larvae ~7 mm in total length, consisting of large, fluid-filled bladder connected to slender peduncle terminating in distinct swelling. Scolex within bladder, folded, consisting of four acetabula; acetabula in form of bothridia; apical sucker absent. Bothridia stalked, with crenulated margins.

Larvae of Type VIII were identified as *Rhodobothrium*.

*3.4. Expanded maximum likelihood analysis*

With respect to the expanded matrix, the aligned 28S rDNA dataset for 281 taxa (98 adult and 100 larval ingroup specimens, and 10 adult outgroup taxa for the initial analysis to which were added 58 adult and 15 larval ingroup taxa from GenBank) consisted of 1,374 characters. The number of variable positions was 779, of which 713 were parsimony informative. Each of the eight independent ML analyses resulted in similar optimal topologies and similar lnL scores (i.e., ranging between −24,438.5439 and −24,444.8872). The topology of the tree resulting from the analysis with the lowest likelihood score is shown in Fig. 4.

Six of the eight groups resulting from the initial analysis (i.e., Groups C to H), remained strongly supported, receiving bootstrap values of ⩾99%; support for Groups A and B was substantially reduced, with both groups receiving bootstrap values of <50% and 67%, respectively. Inclusion of the 70 additional species, representing 12 tetraphyllidean and nine rhinebothriidean genera beyond those included in the initial analysis, supported the assignments of generic identities for larval types in Group A (Type I as *Phoreiobothrium* or *Triloculatum*), Group B (Type II as *Acanthobothrium*), Group C (Type III as *Duplicibothrium*), subgroup H2 (Type VIII as *Rhodobothrium*) and subgroup H3 (Type VII [in part] as *Spongiobothrium*). The two larvae belonging to Group F, for which no equivalents were found among the adults sequenced in the more restricted analysis, grouped with a specimen of the Indo-Pacific genus *Pachybothrium*, suggesting that this, or one of its Atlantic close relatives (i.e., *Pedibothrium*; see Caira and Pritchard, 1986), might be represented by this larval form. Also interesting was the fact that *Marsupiobothrium* sp. was the only additional species that grouped among species in Group E, which in the initial analysis had consisted only of adults of *Paraorymatobothrium* and larvae of Type IV. Given that this genus failed to group with any of the larval containing subgroups, the identity of these larvae should stand as *Paraorygmatobothrium*. The addition of species of rhinebothriidean genera not previously represented, i.e., a number of new genera, *Anthocephalum*, *Scalithrium* and *Rhinebothroides*, as well as species of *Rhinebothrium* not previously included, did not unambiguously suggest generic identities for the remaining larvae of Type VII (H4 and H5).

## 4. Discussion

One of our primary goals herein was to identify suites of morphological features, confirmed with molecular data, which together would facilitate or make possible morphology-based identification of tetraphyllidean and rhinebothriidean larval forms thereby allowing the creation of a dichotomous key to larval types (see below). This goal has been achieved in that eight larval types, corresponding to specific genera, or at least sets of related genera, have been identified and morphologically characterized. In combination, we believe that the suites of characters articulated for each larval type are sufficient to allow identification of at least a subset of the more common tetraphyllideans and two rhinebothriideans to the generic level. Formalized morphological criteria to distinguish between the larvae of tetraphyllideans and those of the newly erected closely related order Rhinebothriidea are also now available.

Based on our results, in general, larvae with a scolex bearing an apical sucker and four acetabula that can be completely retracted into the scolex proper belong to the Rhinebothriidea. These larvae, at least the ones studied herein, also possess acetabula that are facially loculated. Among rhinebothriideans, the exception is the larval form of *Rhodobothrium*, which is confirmed herein to consist of a bladder containing the scolex and a distal swelling supported on a peduncle, as has been reported previously on multiple occasions (e.g., Gallien, 1949; Bahamonde and Lopez, 1962; Dollfus, 1964, 1974; Cake, E.W., 1973. Larval cestode infections several edible bivalve mollusks from the vicinity of St. Teresa. Florida. Proceeding of the National Shellfisheries Association 63, 1. (Abstract); Campbell and Carvajal, 1979; Carvajal et al., 1982; Carvajal and Mellado, 2007). Larvae bearing acetabula that do not retract into the scolex proper probably belong to members of the Tetraphyllidea. This generalization has some special bearing on larvae identified previously as belonging to *Caulobothrium*, a genus with facially loculated, stalked bothridia, that was until recently considered to belong to the Rhinebothriinae (see Healy et al., 2009). This genus has been considered among other facially loculated genera (e.g., Chambers et al., 2000) for larvae exhibiting loculated bothridia that have been shown to retract into the scolex proper. Our results would suggest this is an unlikely generic identity for these larvae.

With respect to other tetraphyllideans, the presence of an apical sucker and four non-retractable acetabula of various forms is common among the larvae of the genera examined herein with the possible exception of *Pedibothrium*. This is generally consistent with larvae assigned to Tetraphyllidea by previous authors (e.g., Yamaguti, 1934; Anantaraman, 1963; Dollfus, 1964; Vivares, 1971; Reimer, 1975; Cake, 1976, 1977; Stunkard, 1977; Chambers et al., 2000). However, more specifically, the following generalizations can now be made with some confidence. The larvae of *Paraorygmatobothrium* are substantially smaller than those of *Anthobothrium* (<350 μm versus 439 μm–1.6 mm) and are subspherical rather than elongate. However, larvae of both *Paraorygmatobothrium* and *Anthobothrium* bear sucker-like (i.e., completely sessile) acetabula; whereas the acetabula of *Acanthobothrium*, *Phoreiobothrium*, *Triloculatum* and *Duplicibothrium* are bothridiate (i.e., possess a

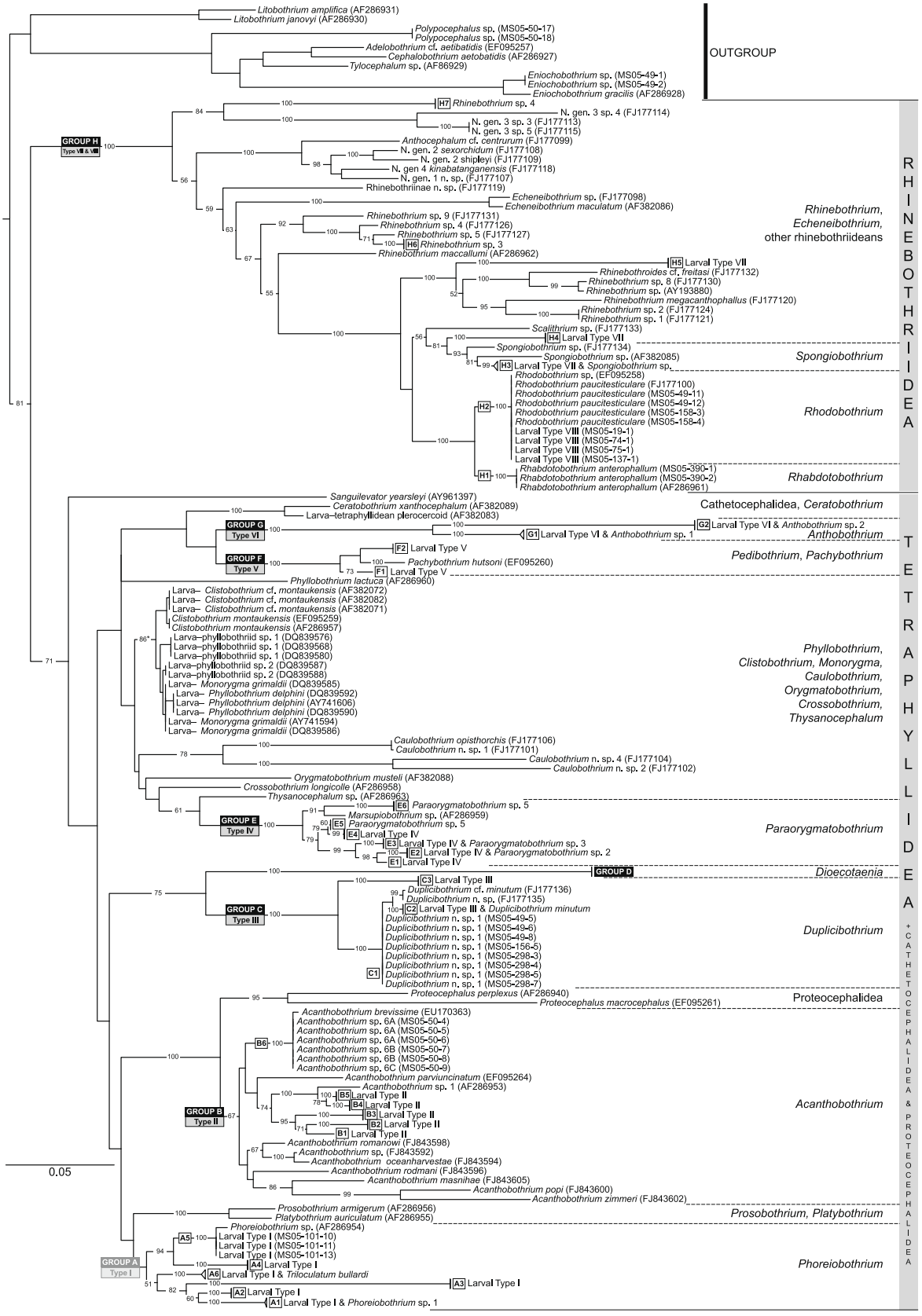


**Fig. 4.** Maximum likelihood (ML) tree (ln likelihood = −24,438.5439) of expanded matrix including sequence data from 98 adult and 100 larval ingroup specimens of tetraphyllideans and rhinebothriideans from the Gulf of Mexico and 10 adult outgroup taxa from the initial matrix to which were added sequence data of 58 adult and 15 larval tetraphyllidean and rhinebothriidean ingroup taxa from GenBank. Nodal support on branches is only given if bootstrap values >50%. Major groups are indicated by black boxes above the line, with the corresponding larval type in grey boxes below the line (a lighter major group box and larval type box indicates a group with bootstrap value <50%); subgroups are indicated by white boxes; if subgroup (or group) constituency did not change with inclusion of additional GenBank specimens, individual specimen labels were substitutes for subgroup (group) boxes with more general labels; * indicates clade within which support values are not shown; scale: substitutions per site.

distinct proximal surface). Criteria for distinguishing larvae of *Triloculatum*, *Phoreiobothrium* and *Acanthobothrium* come also from features of the bothridia. Although the boundary between the anterior pad and the remainder of the bothridium is marked by distinct lateral constrictions in larvae of *Acanthobothrium*, this is not the case in larvae of *Triloculatum* or *Phoreiobothrium*, which both have straight bothridial margins. In addition, the bothridia of larvae of *Acanthobothrium* are elongate rather than rounded to ovoid in larvae of *Phoreiobothrium* and *Triloculatum*. With respect to the distinction between larvae of the latter two genera, it appears that the larvae of *Triloculatum* are longer than those of *Phoreiobothrium*, but this requires confirmation. The larvae of *Duplicibothrium* exhibit bothridia that are arranged in dorso-ventral pairs, with each bothridium bearing one or more columns of facial loculi, with or without a posterior row of loculi. While the bothridia of *Duplicibothrium* may be contracted, they apparently cannot be retracted within the scolex; this is probably true for the larvae of species of *Dioecotaenia*, a close relative, but a genus having no larvae represented herein. Among the genera treated above, the bothridia of larvae of species of *Duplicibothrium* most closely resemble those of its adult form. This is also probably true of *Dioecotaenia* as was depicted by Cake (1977).

The phylogenetic analysis conducted herein allows us to make predictions about the larval forms described herein relative to the additional genera of rhinebothriideans and tetraphyllideans that have been reported to occur in the Gulf of Mexico. All six genera of rhinebothriideans that have been reported from the Gulf of Mexico (Jensen, 2009) were included among the adults for which molecular data were either generated de novo or obtained from GenBank. Among these, we have identified larvae for *Rhinebothrium* and *Spongiobothrium* (both Type VII) and, although it has not been reported from the Gulf of Mexico as an adult, *Rhodobothrium* (Type VIII). Genera reported from the Gulf of Mexico for which larvae were not identified are *Echeneibothrium*, *Anthocephalum*, *Scalithrium* and *Rhabdotobothrium*.

Based on the tree resulting from our expanded analysis (Fig. 4), we would predict that the former three genera are likely to exhibit larvae of Type VII. Based on its sister-taxon status relationship to *Rhodobothrium*, we would predict that *Rhabdotobothrium* perhaps exhibits larvae of Type VIII. All 11 genera of tetraphyllideans reported from the Gulf of Mexico (see Jensen, 2009) were included among the adults for which molecular data were either generated de novo or obtained from GenBank. In this study, larvae have been identified of species of *Phoreiobothrium* and *Triloculatum* (Type I), *Acanthobothrium* (Type II), *Duplicibothrium* (Type III), *Paraorygmatobothrium* (Type IV), *Anthobothrium* (Type VI) and, by inference, *Pedibothrium* (Type V). Predictions of larval types for the four genera for which larvae were not recovered (i.e., *Crossobothrium*, *Dioecotaenia*, *Platybothrium* and *Thysanocephalum*) are more uncertain because none of these genera occurred within clades for which larvae had been identified. Moreover, in all cases, the nodes associated with clades grouping them as sister taxa to those containing larval specimens are poorly supported. However, based on these tentative sister–taxon relationships, we predict that *Platybothrium* may exhibit larvae of Type I, *Dioecotaenia* larvae similar to those of Type III and *Crossobothrium* and *Thysanocephalum* possibly larvae of Type IV, or perhaps more likely larvae that are of some other form. Although the identities of larvae of three of these genera have, in our view, never been identified convincingly, our prediction for *Dioecotaenia* is consistent with the larva of *Dioecotaenia cancellata* described by Cake (1976, 1977).

It is seemingly not possible to reconcile the eight larval types articulated herein with much of the previous 200 years of literature aimed at identifying tetraphyllidean and rhinebothriidean larval forms, but nevertheless that literature certainly continues to prove invaluable as a foundation of data for investigating cestode life history. Previous work has involved a wide array of larval morphologies and a plethora of associated names for these forms (e.g., Linton, 1897; Dollfus, 1929, 1964; Yamaguti, 1934; Vivares, 1971; Cake, 1976, 1977; Stunkard, 1977; Chambers et al., 2000). Unfortunately, many of these names have been applied inconsistently among authors. For example, historically, the collective group names "*Scolex polymorphus*" (e.g., Monticelli, 1888; Linton, 1905; Curtis, 1911; Dollfus, 1974) and "*Scolex pleuronectis*" (e.g., Dollfus, 1964; Reimer, 1975; Cake, 1976) have been commonly used in reference to larvae putatively indentified as tetraphyllideans. However, it was not uncommon to apply the same name to different larval forms, e.g., Reimer (1975) used the collective group name "*Scolex pleuronectis*" for both uniloculate and triloculate larvae. Similarly, it was not uncommon for the same form to have different names, e.g., Curtis (1911) used "*Scolex polymorphus*" for a triloculate larva while Reimer (1975) used "*Scolex pleuronectis*" for that same larval form. In some instances, group names, appearing as sub-specific designations, have been modified to accommodate variation in larval forms. For example, Dollfus (1974) used "*Scolex polymorphus unilocularis*," "*Scolex polymorphus bilocularis*," and "*Scolex polymorphus trilocularis*" for larvae that possessed bothridia with one, two and three loculi, respectively. More recently, some authors have tried to develop a more detailed nomenclature for marine cestode larvae. Cake (1977), for example, developed a suite of terms (e.g., bothridio-postplerocercoid, uniacetabulo-postplerocercoid, etc.) based on the overall morphological features of larvae. In other cases, no attempt was made to assign genera to specific forms (e.g., Dollfus, 1974) or the assignment of suites of genera to specific larval forms was a primary goal (Cake, 1977). However, authors have not necessarily agreed upon such generic assignments. For example, larvae bearing four bothridia each subdivided into four loculi were attributed to *Calliobothrium* by Linton (1905) but to *Acanthobothrium* by Cake (1977). Friedl and Simon (1970) suggested that such larvae could belong to either genus but also to *Onchobothrium*. Caira (1987) added *Acanthobothroides* to the list of potential genera for this larval form.

In a more recent effort to categorize tetraphyllidean larval diversity more broadly, Chambers et al. (2000) recognized 11 types of larvae found parasitizing teleosts on Heron Island in Australia to provide putative generic identities for each. Owing to the morphological detail provided for each larval type they recognized, our results can be directly compared to theirs as follows. We did not encounter their larval Types 1, 2, 7 or 9, perhaps because the genera to which Chambers et al. (2000) attributed their Types 1, 2 and 7 (i.e., *Uncibilocularis*, *Megalonchos* and *Carpobothrium*) do not reportedly occur in the Gulf of Mexico. This may also be true of their Type 9, which they putatively assigned to *Phyllobothrium* and *Clydonobothrium*. However, the generic assignments of Chambers et al. (2000) need additional confirmation, as do ours because, for example, Healy et al. (2009) considered the latter genus to be a rhinebothriidean and thus it is likely to exhibit bothridia that retract into the scolex. Our results lead us to combine their Types 3 and 11, recognized herein as Type II. Our results support recognition of their Types 4, 5, 6, 8 and 10, referred to herein as our Types VI, VII, V, I and VII, respectively. We also found several larval types (IV, III and VIII) not recognized by Chambers et al. (2000). The absence of at least the latter two types is likely accounted for by the fact that these larvae were found in bivalves while Chambers et al. (2000) restricted their work to teleosts only. The absence of larvae of Type IV, which we attributed to *Paraorygmatobothrium*, is puzzling given that this genus is well known from the Indo-Pacific region (e.g., Ruhnke and Thompson, 2006; Ruhnke et al., 2006; Cutmore et al., 2009). Our results can be used to further inform some of the larval identifications encountered by Chambers et al. (2000). Our data support the following: Type 3 as *Acanthobothrium*, Type 4 as *Anthobothrium*, Type 5 as *Rhinebothrium* or *Rhabdotobothrium* but, based on its position outside of the Rhinebothriidea (see

Healy et al., 2009), not *Caulobothrium*. Furthermore, we suggest that unidentified larval Type 6 of Chambers et al. (2000) represents a species of *Pedibothrium* or *Pachybothrium*, their unidentified larval Type 7 represents that of *Carpobothrium* and their unidentified larval Type 8 represents that of *Phoreiobothrium*. Noteworthy is that our sampling methods yielded mostly tetraphyllidean larvae such that rhinebothriidean larvae have not been treated in comparable detail. It is likely that more comprehensive sampling of rhinebothriidean hosts will reveal additional larval types and allow a more complete characterization of larval morphology within that order.

It seems appropriate now to begin to standardize the names and criteria for recognizing marine cestode larvae. Based on their position in the phylogenetic analysis performed herein, we propose that the eight types encountered in the Gulf of Mexico be formally recognized as Types I–VIII. To this end, we have provided telegraphic descriptions for each of these types above. Furthermore, we propose that the additional larval types encountered by Chambers et al. (2000) be formally referred to as follows: Type IX (for their Type 9: scolex with apical sucker and non-retractable bothridia that are facially undivided) and Type X (for their Type 7: scolex with apical sucker and non-retractable bothridia that are pouch-like and muscular bands on the anterior and posterior margins of the aperture). The morphological criteria employed herein to recognize Types I–VIII lead us to doubt that Types 1 and 2 of Chambers et al. (2000) represent distinct larvae; in fact, no criteria were articulated by Chambers et al. (2000) to distinguish between these two forms. Thus, we propose that larvae of this form, having an apical sucker and four bothridia each subdivided into three loculi, be referred to as Type XI. We propose that the following additional larval types should be recognized based on descriptions from other literature sources. Larvae bearing an apical sucker and four non-retractable non-fused bothridia each with one or two columns of facial loculi should be referred to as Type XII (e.g., Wardle, W.J., 1974. A survey of the occurrence, distribution and incidence of infection of helminth parasites of marine and estuarine Mollusca from Galveston, Texas. Ph.D. Disssertation, Texas A & M University, College Station, Texas, USA). Larvae bearing an apical sucker and four non-retractable non-fused bothridia bearing three columns of facial loculi should be referred to as Type XIII (e.g., Wardle, W.J., 1974. A survey of the occurrence, distribution and incidence of infection of helminth parasites of marine and estuarine Mollusca from Galveston, Texas. Ph.D. Disssertation, Texas A & M University, College Station, Texas, USA). Larvae lacking an apical sucker and bearing four non-retractable bothridia each with an anterior accessory sucker and one or more pairs of lateral lappets should be referred to as Type XIV (e.g., Dollfus, 1964). Finally, larvae with an apical sucker and four non-retractable bothridia each with an anterior accessory sucker, but lacking lateral lappets should be referred to as Type XV (e.g., Dollfus, 1964). With respect to examples of cestode genera exhibiting larvae of these additional four types, larvae of Type XII have been attributed to *Caulobothrium* (e.g., Carvajal, 1977), larvae of Type XIII to *Dioecotaenia* (e.g., Wardle, W.J., 1974. A survey of the occurrence, distribution and incidence of infection of helminth parasites of marine and estuarine Mollusca from Galveston, Texas. Ph.D. Disssertation, Texas A & M University, College Station, Texas, USA; Cake, 1976) and larvae of Type XIV to *Dinobothrium* (e.g., Dollfus, 1964). Larvae of Type XV have historically often been referred to as *Phyllobothrium delphini* and/or *Monorygma grimaldii* (e.g., see Baer, 1932; Testa and Dailey, 1977; Siquier and Le Bas, 2003). However, recent comparisons of molecular sequence data derived from adults and juveniles of these taxa have suggested that these larvae may actually belong to species of *Clistobothrium* (e.g., see Brickle et al., 2001; Agustí et al., 2005). The larvae of *Pelichnobothrium* are also likely to exhibit larvae of Type XV (e.g., Scholz et al., 1998). A key to the 15 larval types recognized herein is provided below.

The method employed herein using molecular data for adults and larvae differs conceptually from that employed by most previous authors working with morphology in that, rather than attempting to assign a larvae to a genus, we have attempted to identify larval forms associated with specific genera. This was also the strategy followed by Brickle et al. (2001) and Agustí et al. (2005). This has allowed a more detailed circumscription of the morphological features associated with the larvae of particular genera and has confirmed that different genera may exhibit essentially the same larval Type (e.g., Type I of *Phoreiobothrium* and *Triloculatum*). Hence, from a practical standpoint, caution should be exercised when assigning larval types to a cestode genus, particularly given that larval forms of tens of genera of tetraphyllideans and rhinebothriideans have yet to be described. Thus, the full spectrum of larval types found in these orders remains to be explored and the full complement of genera that exhibit specific larval types remains to be identified.

Larvae attributed to all but two of the 10 cestode genera held species that infected teleosts, mainly in the intestine and pyloric caeca. The exceptions were the larvae of species of *Duplicibothrium* (Tetraphyllidea) collected herein from bivalves and gastropods, as well as larvae of a species of *Rhodobothrium* (Rhinebothriidea) collected herein from bivalves only. Broadly speaking, bivalves and gastropods had most cestodes in the digestive gland and lumen of the anterior portion of the digestive tract, respectively. On average, each of 26 teleost hosts harboured cestode larvae representing two cestode species; on average each of the eight species of molluscan hosts had larvae representing 1.6 cestode species. However, a subset of hosts had much greater larval diversity: both the epibenthic/benthic southern hake, *Urophycis floridana* (Gadidae), and the pelagic Atlantic cutlassfish, *Trichiurus lepturus* (Trichiuridae), each harboured larvae belonging to six species in five genera. In both cases, the definitive hosts of the majority of the species were sharks rather than rays.

Based on the criteria we used for assigning larvae to particular genera (see Key above), it was possible to include larvae from all of the hosts listed in Table 2 in analyses of generic level intermediate host specificity. In total, potential intermediate hosts sampled represented three classes, 14 orders and 46 families of animals. They consisted of five species of crustaceans, 12 species of bivalves, 12 species of gastropods and 46 species of teleosts (see Table 2) (75 species in total). Of note, given previous records of tetraphyllidean larvae (e.g., Dollfus, 1923, 1929, 1974; Brown and Threlfall, 1968; Stunkard, 1977; Pascual, 2001; Aznar et al., 2007), was the fact that no cephalopods were examined. However, it should be recognized that the generalizations made herein regarding intermediate host use come from a subset of the potential intermediate hosts in this system.

Host specificity index values (see Caira et al., 2003) generated for cestode larvae at the generic level ($HS_g$), suggest that the larvae of all genera encountered in this study, with the exception of those of *Rhodobothrium* larvae, exhibited euryxenous host specificity (i.e., infect more than a single host family). Larvae of *Rhodobothrium* exhibited oioxenous host specificity (i.e., infect a single host species) for the variable coquina, *Donax variabilis* (Donacidae). Least host-specific were larvae of *Duplicibothrium*, which infected host species of two classes, two orders, six families, six genera, and six species ($HS_g = 9.39$) and larvae of *Paraorygmatobothrium* which infected host species of one class, five orders, seven families, 11 genera and 12 species ($HS_g = 8.26$). However, such index values are heavily influenced by sampling bias or sampling effort focused on individual species. For example, the larva of *Rhodobothrium* herein infected *D. variabilis* only, but it reportedly also infects bivalves of the families Veneridae, Mactridae, Mesodesmatidae, Psammobiidae and Tellinidae, even members of other orders Arcoidea and Osteroidea (see Cake, 1976, 1977; Carvajal and Mellado, 2007).

**Key to larval types**

| | |
|---|---|
| 1a | Larva in form of large, fluid-filled bladder (containing folded scolex) connected to slender peduncle terminating in distinct swelling (Fig. 2H2b) ..... Type VIII (e.g., *Rhodobothrium*) |
| 1b | Larva in form of scolex and larval body ..... 2 |
| 2a | Scolex with four acetabula in form of suckers (Fig. 2G1–2, E1–4) ..... 3 |
| 2b | Scolex with four acetabula in form of bothridia; bothridia undivided or divided into 2 or more loculi (e.g., Fig. 2A1, B3, C3 and H3) ..... 4 |
| 3a | Larva small, <350 μm in total length; scolex apical sucker usually wider than acetabula (Fig. 2E1–4) ..... Type IV (e.g., *Paraorygmatobothrium*) |
| 3b | Larva large,>400 μm in total length; scolex apical sucker of equal width to or slightly wider than acetabula (Fig. 2G1–2) ..... Type VI (e.g., *Anthobothrium*) |
| 4a | Scolex apical sucker absent (Fig. 2F1–2) ..... 5 |
| 4b | Scolex apical sucker present (e.g., Fig. 2A1d, C3b and H3a) ..... 6 |
| 5a | Bothridia undivided, with one or more pairs of anterior lappets, and anterior accessory sucker ..... Type XIV (e.g., *Dinobothrium*) |
| 5b | Bothridia divided into two loculi (e.g., Fig. 2F2); anterior lappets and anterior accessory sucker absent ..... Type V (e.g., *Pedibothrium*, *Pachybothrium*) |
| 6a | Bothridia undivided ..... 7 |
| 6b | Bothridia divided into two or more loculi (e.g., Fig. 2A3b, B1 and H4) ..... 8 |
| 7a | Bothridia foliate, with prominent anterior accessory sucker; scolex apical sucker reduced ..... Type XV (e.g., *Phyllobothrium delphini*, *Pelichnibothrium*) |
| 7b | Bothridia foliate, without anterior accessory sucker; scolex apical sucker prominent ..... Type IX (e.g., Chambers et al. (2000), Fig. 1i) |
| 7c | Bothridia pouch-like; aperture of bothridia with anterior and posterior muscular bands; scolex apical sucker prominent ..... Type X (e.g., *Carpobothrium*) |
| 8a | Bothridia divided into two to four loculi (e.g., Fig. 2A3b and B1) ..... 9 |
| 8b | Bothridia divided into >5 loculi; loculi arranged in one or more columns (Fig. 2H3–4) ..... 10 |
| 9a | Bothridia divided into two loculi (e.g., Fig. 2A3b) ..... Type I (e.g., *Phoreiobothrium*, *Triloculatum*) |
| 9b | Bothridia divided into three loculi ..... Type XI (e.g., *Uncibilocularis*) |
| 9c | Bothridia divided into four loculi (e.g., Fig. 2B1) ..... Type II (e.g., *Acanthobothrium*, *Calliobothrium*) |
| 10a | Bothridia retractable into scolex proper (e.g., Fig. 2H4b) ..... Type VII (e.g., *Rhinebothrium*, *Spongiobothrium*) |
| 10b | Bothridia not retractable into scolex proper (e.g., Fig. 2C2) ..... 11 |
| 11a | Bothridia fused anteriorly into dorsal–ventral pairs (e.g., Fig. 2C3b) ..... Type III (e.g., *Duplicibothrium*) |
| 11b | Bothridia not fused anteriorly into dorsal–ventral pairs ..... 12 |
| 12a | Bothridia divided into single column of loculi ..... Type XII (e.g., *Caulobothrium*) |
| 12b | Bothridia divided into three columns of loculi ..... Type XIII (e.g., *Dioecotaenia*) |

Because specific identifications of larvae based on morphological criteria were not possible, only those larvae for which sequence data were generated were included in considerations of host specificity at the species level. As a consequence, only those hosts infected by these larvae (i.e., a subset of those in Table 2) were considered. With respect to cestode species, intermediate host specificity was more strict. While the species level data were insufficient to allow calculation of informative $HS_s$ values, the following observations can be made. Although potentially underestimating diversity, larvae exhibiting 100% sequence identity for the D1–D3 28S rDNA were treated as conspecific, and herein comprised a total of 27 species. Of these 27 species, 19 (70%) infected a single host species only (i.e., four species each of *Phoreiobothrium* and *Acanthobothrium*, two species each of *Triloculatum*, *Paraorygmatobothrium*, *Pedibothrium* and *Anthobothrium*, and one species each of *Rhinebothrium*, *Spongiobothrium* and *Rhodobothrium*). Larvae of five cestode species infected two host species (one species each of *Phoreiobothium*, *Paraorygmatobothrium*, *Acanthobothrium*, *Rhinebothrium* and *Duplicibothrium*) and their hosts represented one to two families in one to two host orders. Larvae of one species in each of *Paraorygmatobothrium*, *Anthobothrium* and *Duplicibothrium* infected three host species in one to three families and one to two orders. No cestode species infected more than three host species.

The present study focused on the presumptive terminal larval stage in the life-cycles of tetraphyllidean and rhinebothriidean cestodes. Except when larvae of different forms were found to be genetically identical (e.g., *Duplicibothrium*; see discussion below), we consider that the diversity of larval types characterized herein as representing different taxa and not merely morphologically different "developmental stages." Thus, in the context of discussing cestode life-cycles, the infected hosts we studied are considered as terminal intermediate hosts, i.e., the host consumed by an elasmobranch thereby transmitting the infective larval stage to the digestive tract of the predator. Obviously, we cannot know which hosts are paratenic hosts or which are so-called "dead-end hosts" nor the extent to which some of these cestodes utilize paratenic hosts in their life-cycle to increase the probability of horizontal dispersal in the pelagic marine environment. However, regarding our results and in the absence of experimental infections wherein we could make direct observations of larval development, it was not possible to distinguish between these types of hosts. And, in reality, such distinctions are inconsequential regarding the elucidation of which hosts are utilized as hosts in cestode life-cycles. Needless to say, one cannot predict which infected hosts will be eaten by the "correct" elasmobranch host and which will not; hence, one cannot make the distinction between hosts that serve to allow completion of the life-cycle and those that represent so-called "dead-end hosts," different from paratenic hosts, which serve to maintain the larvae as viable or infective until the larva's host is eaten.

Tetraphyllideans can be divided into two groups based on whether their definitive hosts are rays or sharks. Among the ray tetraphyllideans, substantial progress was made with the life-cycles of *Duplicibothrium* species that infect cownose rays (Rhinopteridae) (e.g., see Williams and Campbell, 1978; Ruhnke, 2000).

Larvae of Type III (*Duplicibothrium*) infected several species of gastropods and bivalves. The notion that the life-cycles of species of *Duplicibothrium* may routinely involve mollusc intermediate hosts is consistent with results of dietary studies involving the cownose ray, *Rhinoptera bonasus*, suggesting that this ray consumes shelled prey items such as bivalves (e.g., Smith and Merriner, 1985; Blaylock, 1993). In fact, valves and crushed shell belonging to species of *Donax* (probably either or both of the variable coquina, *D. variabilis* or the Texas coquina, *Donax texasiana*) were casually observed in the stomach of several cownose rays examined herein. In this respect, species of *Duplicibothrium* differ markedly from species of other tetrapyllidean genera investigated herein, all of which were found as larvae in teleosts. An interesting observation remains to be explored regarding larval development in *Duplicibothrium minutum*. A subset of larvae of Type III were determined to be identical in sequence to two specimen of a saccate larval form (*in Fig. 1) bearing an apical organ, but lacking external acetabula also found parasitizing the bivalve *D. variabilis*. The larvae of Type III were assigned to *D. minutum* based both on their sequence identity with, and morphological resemblance to, adults of this species. However, based on sequence identity, the saccate larval form should also be assigned to *D. minutum* despite its distinct morphology relative to larva of Type III. We suggest that the saccate larval form may actually represent an earlier stage in the life-cycle of *D. minutum*, particularly given its relatively undifferentiated form. This is supported by the fact that both the saccate larvae and larvae of Type III parasitized *D. variabilis* suggesting the transformation occurs within this host. Nonetheless, while the present study has revealed much about what we believe are the terminal larval stages (i.e., pleroceroids and meroceroids sensu Chervy (2002)) of tetraphyllidean and rhinebothriidean cestodes, much remains to be determined about the earlier elements of their life-cycles.

Generalizations about the life-cycles of species in the most speciose genus of tetraphyllideans, *Acanthobothrium*, which generally use rays as their definitive hosts, can be made with much less precision. Larvae of Type II, identified as a species of *Acanthobothrium*, infected species in six teleost families. Yet many previous records of larvae identified as *Acanthobothrium* come from a diversity of gastropods and bivalves (e.g., Regan, 1963; Harry, 1969; Cake, 1976; Holland and Wilson, 2009) as well as the lancelets (Holland et al., 2009). The single adult species examined herein was collected from the bluntnose stingray, *Dasyatis say* (Dasyatidae). However, additional records of adults of species of *Acanthobothrium* from the Gulf of Mexico include additional dasyatids as well as species in Rajidae, Gymnuridae, Narcinidae and Myliobatidae. The present study emphasized teleost intermediate hosts and is intriguing given that reports on the diet of the batoid families listed above are largely comprised of invertebrates (Froese, R., Pauly, D. (eds.), 2009. FishBase. World Wide Web electronic publication. www.fishbse.org, version (06/2009)).

Among the tetraphyllidean genera including species that mature in sharks, the case of *Pedibothrium* appears to be relatively straightforward. Larvae of this form (Type V) infected the Gulf toadfish, *Opsanus beta* (Batrachoididae) and the red snapper, *Lutjanus campechanus* (Lutjanidae). All six species of *Pedibothrium* reported from the Atlantic Ocean reportedly infect the nurse shark, *Ginglymostoma cirratum* (Ginglystomatidae) (see Caira, 1992). Nurse sharks eat toadfishes (*Opsanus* spp.) and snappers (Lutjanidae), with teleosts comprising ~90% of their diet (Castro, 2000), strongly indicating that teleosts are certainly plausible intermediate hosts for species of *Pedibothrium* that mature in the nurse shark.

Somewhat more complex is determining plausible life-cycles for species herein assigned to the four remaining tetraphyllidean genera (*Phoreiobothrium*, *Triloculatum*, *Paraorygmatobothrium* and *Anthobothrium*), all of which mature in whaler sharks (Carcharhiniformes). Larvae assigned to these four genera infected teleosts only. This result, combined with published intermediate host records (e.g., Chambers et al., 2000), suggests that teleosts likely figure prominently in the life-cycles of species in all four genera. Adults were found that exhibited genetic identity with one to three larval species in each genus. These adults infected the spinner shark (*Carcharhinus brevipinna*), the finetooth shark (*C. isodon*), the blacktip shark (*C. limbatus*) and the Atlantic sharpnose shark (*Rhizoprionodon terraenovae*). These sharks eat teleosts (e.g., Hoffmayer and Parsons, 2003; Bethea et al., 2004; Tavares, 2008) that host larvae of species belonging to all four tetraphyllidean genera. However, a diversity of other carcharhinid and sphyrnid sharks have been reported as hosts for species of these genera in the Gulf of Mexico (Jensen, 2009), indicating that the web of trophic connections among intermediate teleost hosts and elasmobranch definitive hosts involved in the life-cycles of species in these genera is likely much more complex than can be unraveled herein given the data at hand.

Rhinebothriideans reportedly infect rays as adults, but our results suggest that there is a novel dichotomy in play among cestode taxa with respect to the types of intermediate hosts involved in their life-cycles. In short, rhinebothriideans infect both teleosts and molluscs. Larvae of Type VII identified as belonging to species of *Spongiobothrium* were found in the southern hake (Gadidae) and the southern flounder, *Paralichthys lethostigma* (Paralichthyidae), indicating that teleosts are intermediate hosts for members of this genus. Because species of *Spongiobothrium* commonly mature in dasyatids and because these same dasyatids prey upon teleosts (see Hess, 1961; Funicelli, N., 1975. Taxonomy, feeding, limiting factors, and sex ratios of *Dasyatis sabina*, *Dasyatis americana*, *Dasyatis sayi*, and *Narcine brasiliensis*. Ph.D. Dissertation, University of Southern Mississippi, Hattisburg, Mississippi, USA), this seems like a plausible life-cycle scenario. Similarly, the remaining Type VII larvae belonging to two species of *Rhinebothrium* infected the sand perch, *Diplectrum formosum* (Serranidae), the longspine porgy, *Stenotomus caprinus* (Sparidae) and the Atlantic tripletail, *Lobotes surinamensis* (Lobotidae). Although the species identities of the larvae encountered herein were indeterminate, published records of adults of species of *Rhinebothrium* from the Gulf of Mexico include dasyatids, suggesting that the fish/batoid scenario herein again seems plausible, although perhaps not necessarily with these teleost species. This is consistent with the results of Chambers et al. (2000), who reported larvae of Type VII from hosts representing seven teleost families. In contrast, *D. variabilis* apparently is an intermediate host for the rhinebothriidean *Rhodobothrium paucitestiulare* and the life-cycle of this cestode appears similar to species of *Duplicibothrium* that exploit seasonal molluscivory of its cownose ray definitive host.

The methods employed herein have resulted in what we hope will be viewed as a foundational study to which future morphological investigations of cestode life-cycles in the ocean can be added. Based on morphology, 15 larval types have been characterized and a key presented to facilitate recognition of these larval types and to allow future assessment and refinement of what has been considered herein to represent variation within each type. When combined with morphology, sequence data for the nuclear gene 28S rDNA effectively differentiate larvae assigned to species of cestode genera reported herein as well as, in some cases, larvae of species within these genera. However, caution should be exercised in attempting to identify larvae or adults to species using data from this gene region alone because in several instances herein adult congeners had identical sequences. This was also true for sequence data for a ~550 bp region of the CO1 gene generated as part of this project for a subset of the specimens included in the initial matrix, but not presented herein. It now seems clear that teleosts figure relatively prominently in the life-cycles of a diversity of tetra-

phyllidean and rhinebothriidean genera. At the level of genera, cestodes apparently exhibit euryxenous specificity for their intermediate hosts; however, our data presented herein suggests that host specificity of cestodes to their intermediate hosts may be higher at the species level.

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