

Research Paper

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Evidence that host ecology drives first intermediate host use in the Didymozoidae (Trematoda: Hemiuroidea): an asexual infection in a vermetid (Gastropoda)

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Abstract

The Didymozoidae (Trematoda: Hemiuroidea) is among the most speciose trematode families, known from a wide range of marine teleost fishes. Despite their richness, however, didymozoid life cycles are unusually poorly known; only two first intermediate hosts are known, a marine bivalve (*Anadara trapezia*) and a pelagic gastropod (*Firoloida desmarestia*). This study uses multi-locus molecular sequence data to identify a novel first intermediate host for the family, a sessile gastropod of the genus *Thylacodes* Guettard (Vermetidae). The didymozoid infection is not identified to species but, based on molecular phylogenetic analyses, it is close to *Saccularina magnacetabula* Louvard *et al.*, 2022, which uses a bivalve as a first intermediate host. The distribution of known first intermediate hosts of didymozoids (a bivalve, a holoplanktonic gastropod and a sessile gastropod that feeds with the use of mucus nets) suggests that first intermediate host use within the Didymozoidae has been opportunistically driven by the trophic ecology of potential mollusc hosts and has involved significant host-switching events.

Introduction

The superfamily Hemiuroidea is a highly studied and rich group of trematodes. The 16 currently recognized hemiuroid families (Sokolov *et al.*, 2019; World Register of Marine Species, 2022b) infect a wide range of marine and freshwater fishes, amphibians and aquatic reptiles (e.g. Thomas, 1939; Vercammen-Grandjean & Heyneman, 1964; Blair, 1984; Hafeezullah, 1990; Shimazu *et al.*, 2014). Although hemiuroids are highly diverse morphologically, all the families of this group with known life cycles but one (the Ptychogonimidae Dollfus, 1937) have cystophorous cercariae (Dollfus, 1950). Cystophorous cercariae are unique to the Hemiuroidea and are characterized by a retractable cercarial body that is ejected from the caudal chamber through a delivery tube into the body cavity of the second intermediate host upon consumption of the cercaria (e.g. Køie, 1995); the apparent secondary loss of the caudal appendage (and possibly of the delivery tube) in *Ptychogonimus megastomum* (Rudolphi, 1819) may be due to modifications in the life cycle of that trematode, wherein sporocysts containing the cercariae are trophically transmitted to the second-intermediate host (Palombi, 1941, 1942).

Hemiuroid life cycles are thought to typically include four hosts (Nikolaeva, 1965; Kechemir, 1978; Køie & Lester, 1985). Known first intermediate hosts are molluscs (see review by Louvard *et al.* (2022)), principally gastropods and, much less frequently, bivalves (Wardle, 1975; Mateo *et al.*, 1985; Castro, 2019; Louvard *et al.*, 2022) and scaphopods (Palombi, 1941, 1942; Køie *et al.*, 2002). Most hemiuroid asexual infections are found in benthic environments, with exceptions from holoplanktonic molluscs (Bonnie, 1916; Vande Vusse, 1980; Hochberg & Seapy, 1985; Lester & Newman, 1986; Morales-Ávila *et al.*, 2018). Fewer than half of the families have elucidated life cycles, however, and first intermediate hosts are unknown for most species. Especially glaring is the lack of knowledge about life cycles of the Didymozoidae Monticelli, 1888. Only two first intermediate hosts are known. *Saccularina magnacetabula* Louvard *et al.*, 2022 infects the bivalve *Anadara trapezia* (Deshayes) (Arcoidea: Arcidae) (Louvard *et al.*, 2022), and two closely related but unidentified didymozoids infect the holoplanktonic gastropod *Firoloida desmarestia* Lesueur (Pterotracheoidea: Pterotracheidae) (Lozano-Cobo *et al.*, 2022).

Opposite to host–parasite coevolution, speciation by host-switching is the main driver of host–parasite relationships (Araujo *et al.*, 2015). Host-switching refers to the opportunistic establishment of a relationship between a parasite species and a previously unexploited host lineage (often not closely related to the usual hosts of that parasite) enabled by mutual

encounter and compatibility (Agosta & Klemens, 2008; Araujo *et al.*, 2015). The exploitation of the new host is followed by the co-adaptation of both parasite and host to each other (Araujo *et al.*, 2015). Host-switching often results in parasite adaptive radiation (Brooks *et al.*, 1985). Although trematode speciation by host-switching is widespread at both definitive (e.g. Shoop, 1989; Jousson *et al.*, 2000; Cribb *et al.*, 2001) and second intermediate host levels (e.g. Brooks *et al.*, 1985; Cribb *et al.*, 2001; Martin *et al.*, 2018b, 2020), remarkably few digenean families infect more than one class of first intermediate hosts.

Didymozoids are known from holoplanktonic gastropods and benthic bivalves. Here, a didymozoid infection is reported from a dramatically different group of gastropods, the Vermetidae, on the basis of molecular phylogenetic analyses. The presence of didymozoid infections in arcid bivalves, holoplanktonic gastropods and sessile vermetids suggests that first intermediate host use within that family is driven by host ecology.

Materials and methods

Sample collection

Vermetid gastropods were chiselled by hand from emerged rocks as part of the study on trematodes infecting six species of vermetids conducted by Corner *et al.* (2022) on Heron Island (HI), Lizard Island and Moreton Bay (MB) on the coast of Queensland, Australia. From all the locations and species sampled, a single hemiuroid infection was found in Polka Point, North Stradbroke Island (NSI), MB (27°29'46.3"S 153°23'54.6"E).

Adult hemiuroid trematodes were collected by the Marine Parasitology Laboratory (University of Queensland, Australia) from a wide range of fishes as part of a wider parasitological study. The search for didymozoids proceeded as per the protocol of Louvard *et al.* (2022).

All fishes and vermetids were dissected in saline. All digeneans, including larval stages, were killed in near-boiling saline and stored in 80% ethanol for morphological and molecular identifications. Anterior ends of infected vermetids were stored separately in 80% ethanol for morphological identification.

Morphological analyses

All procedures related to morphological analyses were performed on ethanol-preserved samples. For adult didymozoids, hologenophores *sensu* Pleijel *et al.* (2008) were produced by removing a small portion of tissue from the side of the worms with a scalpel. The tissue sample was sequenced and the remainder preserved for morphological analyses; cercariae and sporocysts were left whole. Larvae were rinsed in fresh water, stained with Mayer's haematoxylin, destained in 1% hydrochloric acid, neutralized in 1% ammonium hydroxide, dehydrated in increasing ethanol concentrations from 50% to 100%, cleared in methyl salicylate and mounted in Canada balsam. Specimens were measured in micrometres using cellSens standard imaging software on an Olympus BX-53 compound microscope fitted with an Olympus SC50 digital camera. Specimens were drawn using a camera lucida and digitized with Adobe Illustrator CC 2018. Voucher specimens of both sporocysts and adult didymozoids were lodged in the Queensland Museum (QM), Brisbane, Queensland, Australia.

Vermetids were identified with both morphological and unpublished molecular data by Professor Timothy Rawlings (Cape Breton University, Canada) using molecular sequences

produced by Corner *et al.* (2022). Morphological vouchers and molecular sequences of vermetid hosts were lodged by Corner *et al.* (2022) in the QM and GenBank, respectively.

Adult didymozoids were identified on the basis of the work of Yamaguti (1970) and Pozdnyakov & Gibson (2008), as well as previous host–trematode–site records. Hologenophore specimens were lodged in the QM.

Molecular sequencing of trematodes and hosts

Total genomic DNA was extracted from individual sporocysts, adult trematodes and sections of vermetid tissue using a method derived from Sambrook & Russell (2001). Briefly, specimens were dried, then incubated thrice: at 37°C for >7 h in Tris (Merck, Australia)-ethylenediaminetetraacetic acid (Sigma Aldrich, UK); at 55°C for 2 h with added proteinase K (Invitrogen™, Australia; 10 mg/ml); and at 65°C for 10 min with added sodium chloride (Selby Biolab, Australia; 5 M) and cetyltrimethylammonium bromide (Sigma Aldrich, Australia). DNA was purified with chloroform (Merck, Australia) and phenol–chloroform–isoamyl alcohol (Ambion, Australia), precipitated with cold isopropanol (Sigma Aldrich, Australia) at room temperature, washed in 70% molecular-grade ethanol (Supelco, Germany), dried, rehydrated in 25 µl of Invitrogen™ ultraPURE™ (USA) distilled water at 4°C for >7 h and stored at –20°C.

Complete internal transcribed spacer 2 (ITS2) rDNA and a partial D1–D3 fragment of 28S rDNA regions, and the partial 16S mtDNA region, were amplified for parasites and vermetids, respectively. Reaction solutions comprised 5 µl of 5 × MyTaq™ Reaction Buffer (Bioline, Australia), 0.75 µl of each primer (10 µM), 0.25 µl of Taq polymerase (Bioline MyTaq™ DNA Polymerase, Australia) and 2 µl (ITS2 and 16S, approximately 10 ng) or 4 µl (28S, approximately 20 ng) of DNA template, completed to a total volume of 20 µl with Invitrogen™ ultraPURE™ distilled water. The ITS2 region was amplified using primers 3S (5'-GGT ACC GGT GGA TCA CGT GGC TAG TG-3'; Bowles *et al.*, 1993) and ITS2.2 (5'-CCT GGT TAG TTT CTT TTC CTC CGC-3'; Cribb *et al.*, 1998) under the following denaturation–annealing–extension procedure: 1 × (3 min at 95°C, 2 min at 45°C, 90 s at 72°C); 4 × (45 s at 95°C, 45 s at 50°C, 90 s at 72°C); 30 × (20 s at 95°C, 20 s at 52°C, 90 s at 72°C); and 1 × (5 min extension only at 72°C). The 28S region was amplified using external primers LSU5 (5'-TAG GTC GAC CCG CTG AAY TTA AGC A-3'; Littlewood, 1994) and 1200R (5'-GCA TAG TTC ACC ATC TTT CGG-3'; Lockyer *et al.*, 2003) under the following denaturation–annealing–extension procedure: 1 × (4 min denaturation only at 95°C); 30 × (1 min at 95°C, 1 min at 56°C, 2 min at 72°C); and 1 × (1 min at 95°C, 45 s at 55°C, 4 min at 72°C). The partial 16S region was amplified using primers 16SARL-CBU (5'-CGC CTG TWT ADC AAA AAC ATG-3') and 16SBRH (5'-CCG GTC TGA ACT CAG ATC ACG-3') or 16SBRH-ALT (5'-CCG GTC TGA ACT CAG ATC AYG T-3') (all modified from Palumbi (1996)) under the denaturation–annealing–extension procedure described by Cribb *et al.* (2020).

Amplicons were run on 1% m/v agarose gels supplemented with 0.01% v/v SYBR™ Safe (Invitrogen, Australia). Amplified DNA was sequenced by Sanger cycle sequencing at the Australian Genome Research Facility (Brisbane) using the same primers as for amplification for ITS2 and 16S, and internal primers 300 F (5'-CAA GTA CCG TGA GGG AAA GTT-3'; Littlewood *et al.*, 2000) and ECD2 (5'-CTT GGT CCG TGT

TTC AAG ACG GG-3'; Littlewood *et al.*, 1997) for 28S. Sequences were checked for quality, contiged and trimmed in Geneious v.11.0.5. High-similarity Basic Local Alignment Search Tool searches were made against the United States National Center for Biotechnology Information nucleotide collection (nr/nt). Sequences produced in this study were lodged in GenBank.

Sequences of the vermetid infections were aligned with selected GenBank sequences and sequences of didymozoid adults produced in this study (table 1) using Muscle v3.7 (Edgar, 2004) in MEGA7 (Kumar *et al.*, 2016) for 28S (gap opening penalty = -400, gap extension penalty = -100) and on the Cyberinfrastructure for Phylogenetic Research (CIPRES) portal for ITS2, with unweighted pair group method with arithmetic mean clustering for iterations 1 and 2. Sequences of both alignments were trimmed in MEGA7 to the maximal length of the 50% shortest sequences. In-partition gaps were removed (Martin *et al.*, 2018a) if affecting >25% of sequences. Alignments were further curated using Gblocks v.0.91b (Castresana, 2000; Dereeper *et al.*, 2008) with parameters of least-stringent selection (Kück *et al.*, 2010). After these procedures, 31.1% of the original 28S alignment (representing 965 base pairs (bp) of 28S region) and 15.9% of the original ITS2 alignment (470 bp of ITS2 region) could be kept for phylogenetic analyses.

For Bayesian inference (BI) and maximum likelihood (ML) analyses, the best evolutionary model selected in jModelTest v.2.1.10 (Darriba *et al.*, 2012) using a corrected Akaike information criterion (Akaike, 1974; Hurvich & Tsai, 1993) was the general time reversible model with estimates of invariant sites and gamma-distributed among-site variation (GTR + I + Γ) for the 28S dataset, and the TVM model with estimates of invariant sites and gamma-distributed among-site variation (TVM + I + Γ) for the ITS2 dataset. Alignments were converted into an appropriate format in Mesquite v.3.6 (Maddison, W.P., Maddison, D.R., 2018. Mesquite: A modular system for evolutionary analysis. Version 3.6. <http://www.mesquiteproject.org>). MrBayes 3.2.7a (Ronquist *et al.*, 2012) and RAxML-Blackbox (Stamatakis, 2014) were accessed through the CIPRES portal for BI and ML analyses, respectively. BI analyses used the values for amino-acid fixed substitution rate (revmatpr), gamma shape fixed parameter (shapepr), number of discrete categories to approximate the gamma distribution (ngammacat) and fixed proportion of invariable sites (prinvarpr) calculated in jModelTest. Other specified parameters were: ngen = 10,000,000; no discarding of sampled values as burn-in when calculating the convergence diagnostic; sumt burn-in = 3000; consensus tree with all compatible groups and tree probabilities; and sump burn-in = 3000. The trees were rooted in FigTree v1.4.3 (Rambaut, A., 2017. FigTree version 1.4.3, a graphical viewer of phylogenetic trees. Computer program distributed by the author, <http://tree.bio.ed.ac.uk/software/figtree>) and edited in Adobe Illustrator CC 2018.

Results

Morphological analyses

A single hemiuroid trematode infection was found from dissections of 612 specimens (0.16% prevalence) of a *Thylacodes* sp. (Gastropoda: Vermetidae) collected from MB (see Corner *et al.*, 2022). The infected vermetid specimen was found at Polka Point, NSI, MB. Sporocysts were localized in the tissue at the base of the host's gills. Cystophorous cercariae characteristic of

the Hemiuroidea were found in the sporocysts. Both cercariae and sporocysts are described below.

Molecular analyses

Forty-six representative 28S rDNA hemiuroid sequences from GenBank (table 1) and one novel 28S sequence produced in this study (that of *Metadidymozoon branchiale* Yamaguti, 1970 from *Istiophorus platypterus* (Shaw) (Carangiformes: Istiophoridae); GenBank number: OP793494, QM numbers: G240312–G240314) (table 1) were used to determine the family of the vermetid infection. The position of the vermetid taxon was the same in both ML and BI analyses, so phylogenetic trees are shown for the latter only (figs 1 and 2). In both BI (fig. 1) and ML (not shown) 28S analyses, the sequence from the vermetid infection was part of a clade within the Didymozoidae. Subsequently, 42 representative ITS2 sequences of didymozoid taxa were used to further explore the identity of the infection (table 1). In both BI and ML analyses of 28S and ITS2 datasets (figs 1 and 2; ML trees not shown), the sequence of the vermetid-infecting taxon formed a strongly supported clade with that of an uncharacterized didymozoid from the pharyngeal teeth area of *Lethrinus miniatus* (Forster) (Lethrinidae) from HI (Queensland, Australia) (see Louvard *et al.* (2022)). In all the analyses, the sequence of that uncharacterized taxon was itself part of a strongly supported clade comprising *S. magnacetabula*, described from the fin membrane of *Elops hawaiiensis* Regan from MB, and another uncharacterized didymozoid from the same site, host and geographical location as *S. magnacetabula* (see Louvard *et al.* (2022)).

In the BI 28S analysis (fig. 1), the clade formed by the vermetid infection, *S. magnacetabula* and the uncharacterized didymozoids from *E. hawaiiensis* and *L. miniatus* is sister to a clade containing all other available didymozoid sequences. In the ML 28S analysis (not shown), however, that clade is sister to a clade formed by a single taxon, Nematobothriinae sp. 4 from black marlin, *Istiompax indica* (Cuvier), itself sister to a clade containing all the remaining didymozoids. In the BI ITS2 analysis (fig. 2), the clade [*S. magnacetabula* + vermetid infection + unidentified taxa from *E. hawaiiensis* and *L. miniatus*] is sister to Nematobothriinae sp. 4 to the exclusion of all the other didymozoid sequences. In the ML ITS2 analysis (not shown), however, that clade is sister to all the other didymozoid clades except that of Nematobothriinae sp. 4.

Morphological description

Family Didymozoidae

Didymozoidae sp. A

Locality: Polka Point, North Stradbroke Island, Queensland, Australia (27°29'46.3"S 153°23'54.6"E)

Host: *Thylacodes* sp. (Gastropoda: Vermetidae)

Site: Tissue at base of gills

Specimens deposited: QM G240302–G240311

Representative DNA sequences: GenBank no. OP793495 (28S rDNA) and OP793498–793500 (ITS2 rDNA)

Sporocyst (fig. 3A)

Measurements and description based on nine specimens. Sporocysts interpreted as the second intramolluscan generation contained abundant cystophorous cercariae.

Sporocysts in fleshy tissue at base of host gills. Body contractile, cylindrical to irregular elongate, tapering towards pointed

Table 1. Molecular sequences used in the present study and Queensland Museum voucher identifiers for sequences produced in this study.

Family	Taxon	GenBank accession number 28S/internal transcribed spacer 2	Reference
Accacoeliidae	<i>Accacoelium contortum</i>	AY222190	Olson et al. (2003)
Bunocotylidae	<i>Bunocotyle progenetica</i>	DQ354365	Pankov et al. (2006)
Bunocotylidae	<i>Robinia aurata</i>	DQ354367	Pankov et al. (2006)
Derogenidae	<i>Derogenes varicus</i>	AY222189	Olson et al. (2003)
Didymozoidae	<i>Annulocystis</i> cf. <i>auxis</i>	OL336029/OL846595	Louvard et al. (2022)
Didymozoidae	<i>Annulocystis</i> sp. 2	OL336030/OL846596	Louvard et al. (2022)
Didymozoidae	<i>Brasicystis bennetti</i>	–/JX074770	Louvard et al. (2022)
Didymozoidae	<i>Colocytotrema</i> cf. <i>auxis</i>	–/OL846597	Louvard et al. (2022)
Didymozoidae	<i>Didymocylindrus</i> sp.	OL336001/OL846563	Louvard et al. (2022)
Didymozoidae	<i>Didymocystis scomberomori</i>	KU341979	Schrandt et al. (2016)
Didymozoidae	<i>Didymocystis</i> sp. 3	–/OL846565	Louvard et al. (2022)
Didymozoidae	<i>Didymocystis</i> sp. 4	OL336005	Louvard et al. (2022)
Didymozoidae	<i>Didymocystis</i> sp. 5	–/OL846567	Louvard et al. (2022)
Didymozoidae	<i>Didymocystis</i> sp. 7	OL336009	Louvard et al. (2022)
Didymozoidae	Didymodiclininae sp. 1	OL335995/OL846556	Louvard et al. (2022)
Didymozoidae	<i>Didymodiclinus</i> cf. <i>pacificus</i>	OL335998/OL846560	Louvard et al. (2022)
Didymozoidae	<i>Didymosulcus</i> sp. 2	–/OL846572	Louvard et al. (2022)
Didymozoidae	<i>Didymosulcus</i> sp. 3	OL336012	Louvard et al. (2022)
Didymozoidae	Didymozoidae sp.	–/AB326109	Yagi et al. (2007)
Didymozoidae	Didymozoidae sp.	–/OL846622	Louvard et al. (2022)
Didymozoidae	Didymozoidae sp.	–/OL846623	Louvard et al. (2022)
Didymozoidae	Didymozoidae sp.	–/OL846624	Louvard et al. (2022)
Didymozoidae	Didymozoidae sp.	–/OL846625	Louvard et al. (2022)
Didymozoidae	Didymozoidae sp.	–/OL846626	Louvard et al. (2022)
Didymozoidae	Didymozoidae sp.	–/OL846627	Louvard et al. (2022)
Didymozoidae	Didymozoidae sp.	–/OL846628	Louvard et al. (2022)
Didymozoidae	Didymozoidae sp.	–/OL846629	Louvard et al. (2022)
Didymozoidae	Didymozoidae sp.	OL336036/OL846604	Louvard et al. (2022)
Didymozoidae	Didymozoidae sp.	OL336037/OL846605	Louvard et al. (2022)
Didymozoidae	Didymozoidae sp. ex <i>Thylacodes</i> sp.	OP793495/OP793497	Present study
Didymozoidae	Didymozoini sp.	–/OL846576	Louvard et al. (2022)
Didymozoidae	Didymozoini sp.	–/OL846577	Louvard et al. (2022)
Didymozoidae	<i>Didymozoon</i> sp. 1	OL336015/OL846579	Louvard et al. (2022)
Didymozoidae	<i>Didymozoon</i> sp. 3	OL336017	Louvard et al. (2022)
Didymozoidae	<i>Didymozoon</i> sp. 4	–/OL846585	Louvard et al. (2022)
Didymozoidae	<i>Helicodidymozoon helcis</i>	–/AJ224758	Anderson (1998)
Didymozoidae	<i>Helicodidymozoon tortor</i>	OL336042	Louvard et al. (2022)
Didymozoidae	<i>Indodidymozoon metridion</i>	–/AJ224756	Anderson (1998)
Didymozoidae	Koellikeriinae sp. 1	OL336038/OL846606	Louvard et al. (2022)
Didymozoidae	Koellikeriinae sp. 2	–/OL846607	Louvard et al. (2022)
Didymozoidae	<i>Lobatozoum</i> sp.	OL336021/OL846587	Louvard et al. (2022)
Didymozoidae	Nematobothriinae sp. 1	OL336048/OL846617	Louvard et al. (2022)
Didymozoidae	Nematobothriinae sp. 2	OL336049	Louvard et al. (2022)

(Continued)

Table 1. (Continued.)

Family	Taxon	GenBank accession number 28S/internal transcribed spacer 2	Reference
Didymozoidae	Nematobothriinae sp. 3	–/OL846618	Louvard <i>et al.</i> (2022)
Didymozoidae	Nematobothriinae sp. 4	OL336052/OL846619	Louvard <i>et al.</i> (2022)
Didymozoidae	Nematobothriinae sp. 5	–/OL846620	Louvard <i>et al.</i> (2022)
Didymozoidae	<i>Nematobothrium scombri</i>	AY222195	Olson <i>et al.</i> (2003)
Didymozoidae	Neodidymozoini sp.	–/OL846592	Louvard <i>et al.</i> (2022)
Didymozoidae	<i>Neodidymozoon cf. macrostoma</i>	OL336027/OL846593	Louvard <i>et al.</i> (2022)
Didymozoidae	<i>Neometadidymozoon elusivum</i>	OL336023/OL846589	Louvard <i>et al.</i> (2022)
Didymozoidae	<i>Neonematobothrium cf. kawakawa</i>	–/KU216180	Mele <i>et al.</i> (2016)
Didymozoidae	<i>Metadidymozoon branchiale</i>	OP793494/OP793498–793500	Present study
Didymozoidae	<i>Oesophagocystis cf. dissimilis</i>	OL336022/OL846588	Louvard <i>et al.</i> (2022)
Didymozoidae	<i>Philopinna higai</i>	MH628312	Sokolov <i>et al.</i> (2019)
Didymozoidae	<i>Saccularina magnacetabula</i>	OL336033/OL846599	Louvard <i>et al.</i> (2022)
Didymozoidae	<i>Wedlia retrorbitalis</i>	OL336041/OL846610	Louvard <i>et al.</i> (2022)
Gonocercidae	<i>Gonocerca oshoro</i>	KY197013	Sokolov <i>et al.</i> (2018)
Gonocercidae	<i>Gonocerca phycidis</i>	KY197009	Sokolov <i>et al.</i> (2018)
Gonocercidae	<i>Hemipera manteri</i>	AY222196	Olson <i>et al.</i> (2003)
Hemiuridae	<i>Aponurus laguncula</i>	KU527430	Claxton <i>et al.</i> (2017)
Hemiuridae	<i>Dinurus longisinus</i>	AY222202	Olson <i>et al.</i> (2003)
Hemiuridae	<i>Hemius luehei</i>	MH628316	Sokolov <i>et al.</i> (2019)
Hemiuridae	<i>Machidatrema chilostoma</i>	AY222197	Olson <i>et al.</i> (2003)
Hemiuridae	<i>Merluciotrema praeclarum</i>	AY222204	Olson <i>et al.</i> (2003)
Hemiuridae	<i>Opisthadena dimidia</i>	AY222198	Olson <i>et al.</i> (2003)
Hemiuridae	<i>Thometrema lotzi</i>	KC985236	Calhoun <i>et al.</i> (2013)
Hirudinellidae	<i>Hirudinella ventricosa</i>	KC985232	Calhoun <i>et al.</i> (2013)
Isoparorchidae	<i>Isoparorchis eurytremus</i>	MH628315	Sokolov <i>et al.</i> (2019)
Lecithasteridae	<i>Lecithaster gibbosus</i>	AY222199	Olson <i>et al.</i> (2003)
Lecithasteridae	<i>Lecithophyllum botryophoron</i>	MH628301	Sokolov <i>et al.</i> (2019)
Sclerodistomidae	<i>Prosogonotrema bilabiatum</i>	AY222191	Olson <i>et al.</i> (2003)
Syncoeliidae	<i>Copiatestes filiferus</i>	AY222188	Olson <i>et al.</i> (2003)
Outgroups			
Azygiidae	<i>Azygia longa</i>	KC985234	Calhoun <i>et al.</i> (2013)
Azygiidae	<i>Otodistomum cestoides</i>	AY222187	Olson <i>et al.</i> (2003)

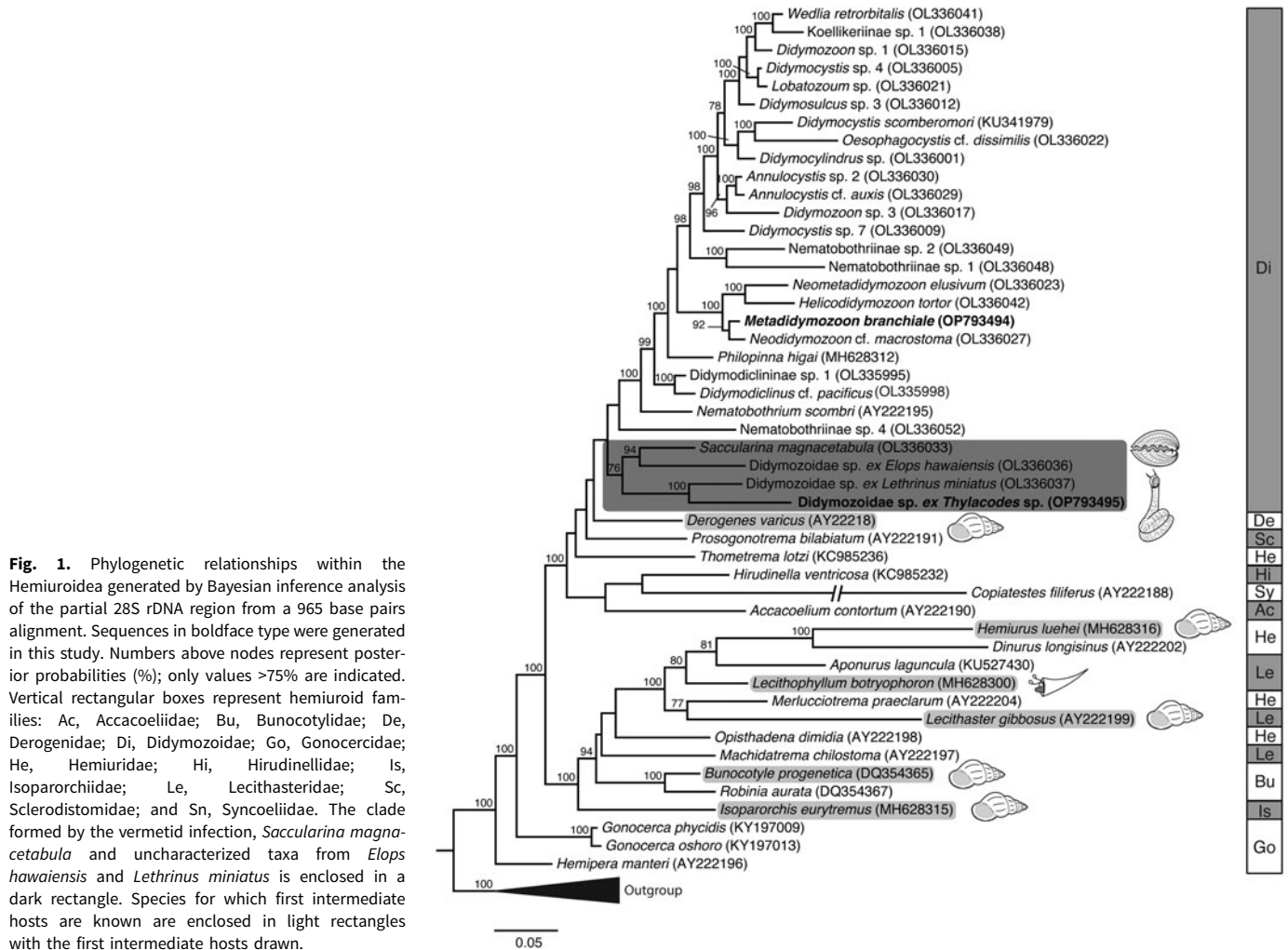
conical anterior extremity and rounded posterior extremity, 1449–3356 (2385) × 183–268 (232). Tegument with fine striations most visible at anterior extremity. Birth pore at anterior extremity. Birth canal straight, sometimes scarcely visible, sometimes invaginates inside anterior extremity anteriorly to brood chamber. Brood chamber filled with active, developing cercariae.

Cercaria (fig. 3B)

Cercariae observed only inside sporocysts. None with cercarial body retracted into caudal chamber. No natural emergence observed. Measurements and descriptions based on ten cercarial bodies, 11 internal caudal cysts, six external caudal cysts and four excretory appendages.

Cystophorous cercaria. Body variable in shape, but typically short cylindrical with bluntly rounded extremities, frequently

detached from tail cyst, 42–94 (65) × 14–26 (21). Suckers, mouth and digestive tract not recognizable. Excretory bladder and pore not seen. Caudal chamber rounded, composed of internal and external parts, bearing excretory appendage; internal part thick-walled, with ridge separating concave sides from convex middle, with rounded conical anterior extremity, 33–41 (36) × 31–41 (36); external part thin-walled, irregularly shaped, covering internal part, thickening and darkening at apex at junction with conical extremity of internal part, becoming indifferentiable from internal part at posterior extremity of chamber, 42–46 (43) × 42–47 (45). Delivery tube coiled tightly inside internal part of caudal chamber. Excretory appendage long, widening at posterior extremity into small paddle, 76–180 (116) × 2–4 (3.5).



Discussion

The Didymozoidae are among the most speciose hemiuroid families with 262 species currently recognized (World Register of Marine Species, 2022a). Yet, this study is only the third report of a didymozoid asexual infection host, and just the second for a gastropod. Given the rarity of hemiuroid infections in bivalves relative to other trematode groups that use bivalves as main first intermediate hosts, Louvard *et al.* (2022) hypothesized that the majority of didymozoid species use gastropod hosts as do most other hemiuroid families. The present findings, together with those of Lozano-Cobo *et al.* (2022), confirm that didymozoids do infect both mollusc lineages. Whether gastropods are the dominant host group remains to be determined.

The paucity of records of didymozoid first intermediate hosts, the chaotic state of the phylogeny of the Didymozoidae (see Louvard *et al.*, 2022), and the early divergent position of the clade containing both *S. magnacetabula* and the new vermetid infection relative to all other didymozoids, preclude definitive conclusions about the evolution of host usage within the Didymozoidae. Still, it is noteworthy that sequences of the vermetid infection from *Thylacodes* sp. are phylogenetically closer to those of *S. magnacetabula* than to those of any other didymozoid taxon except the unidentified species from *L. miniatus*, despite the distant relationship of their respective intermediate hosts.

Unfortunately, the cytochrome c oxidase I sequences of the two species from *F. desmarestia* produced by Lozano-Cobo *et al.* (2022) are not from the same locus as those produced for *S. magnacetabula* and the unidentified taxa from *L. miniatus* and *Elops hawaiiensis* from Louvard *et al.* (2022); thus, the phylogenetic position of the infection from *Thylacodes* sp. relative to that of those species is unknown.

Digenean families rarely infect more than one class of first intermediate hosts. Exceptions to this rule are understood to have arisen *via* host-switching and subsequent speciation. Convincing exceptions include two species of *Hurleytrema* Yamaguti, 1954 (Monorchioidea: Monorchioidea) in vermetid gastropods instead of the usual bivalves (Wee *et al.*, 2021); *Diploproctodaenum arothroni* Bray and Nahhas, 1998 (Lepocreadioidea: Lepocreadiidae) in an ostreid bivalve instead of the usual gastropods (Hassanine, 2006); *Allocreadium handiai* Pande, 1937 (Allocreadioidea: Allocreadiidae) in a bithyniid gastropod instead of the usual bivalves (Madhavi, 1980); and, within the Schistosomatoidea, the use of both gastropods and polychaetes by the Spirorchioidea (Pinto *et al.*, 2015; Cribb *et al.*, 2017b; de Buron *et al.*, 2018) and gastropods, polychaetes and bivalves by the Apocotylidae (Evans & Heckmann, 1973; K  ie, 1982; Cribb *et al.*, 2017a). The Hemiuroidea are exceptional as they use more mollusc classes (i.e. Bivalvia, Gastropoda and Scaphopoda) than any other trematode superfamily (Cribb

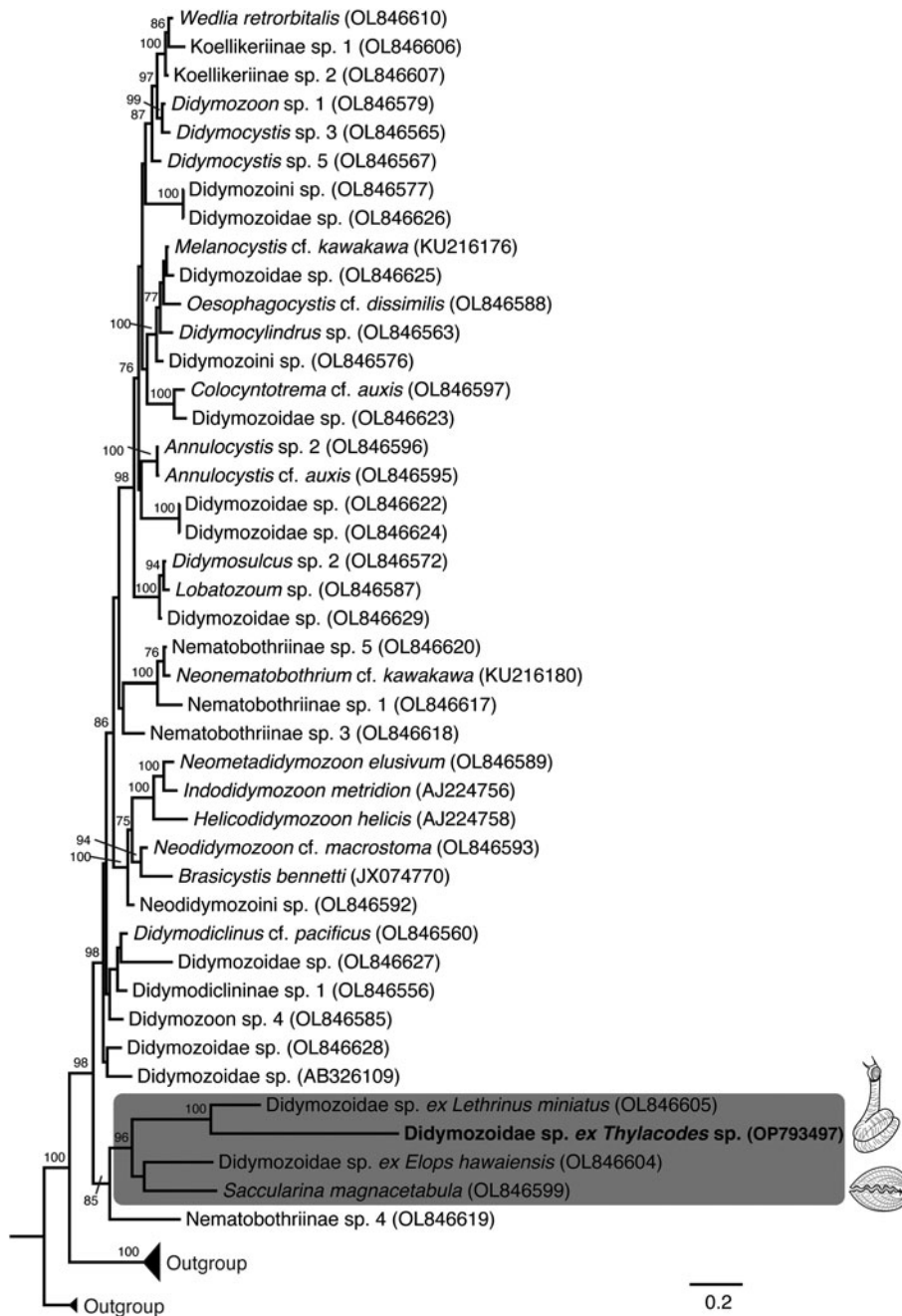


Fig. 2. Phylogenetic relationships within the Didymozoidae generated by Bayesian inference analysis of the partial internal transcribed spacer 2 rDNA region from a 470 base pairs alignment. Sequences in boldface type were generated in this study. Numbers above nodes represent bootstrap support values (%); only values >75% are indicated. The clade formed by the vermetid infection, *Saccularina magnacetabula* and uncharacterized taxa from *Elops hawaiiensis* and *Lethrinus miniatus* is enclosed in a dark rectangle. First intermediate hosts are drawn for the present vermetid infection and for *S. magnacetabula*.

et al., 2003; fig. 1). The use of both bivalves and gastropods by the Didymozoidae mirrors the use of both the Scaphopoda and the Gastropoda by the hemiurid family Lecithasteridae Odhner, 1905, with *Lecithophyllum botryophoron* (Olsson, 1868) using a scaphopod (Køie *et al.*, 2002) and *Lecithaster gibbosus* (Rudolphi, 1802) using a gastropod (Køie, 1989) (fig. 1). However, host use within the Lecithasteridae corresponds to a clear phylogenetic distinction between *Lecithophyllum* Odhner and *Lecithaster* Lühe (Sokolov *et al.*, 2019), whereas such distinction is absent in the Didymozoidae.

It is understood that the eggs of hemiurids, which are notoriously small, must be eaten by the first intermediate host for infections to develop (Szidat, 1956; Stunkard, 1973; Martorelli, 1989). The peculiar ecology of vermetid gastropods may provide clues to

the mechanism of their infection by didymozoid eggs. Vermetids deploy mucus nets (Hughes, 1978; Kappner *et al.*, 2000) that catch a wide variety of benthoplanktonic particles, which are ingested when the nets are retracted (Kusama *et al.*, 2021). Presumably vermetids become infected with didymozoids by ingesting eggs *via* net-feeding. Net-feeding specifically, and more generally filter-feeding, are strategies strikingly different from those of most other gastropod families harbouring hemiurids for which feeding behaviour is known (see review by Louvard *et al.*, 2022). Wee *et al.* (2021) hypothesized that a host-switching event from gastropods to bivalves inferred for monorchiids was enabled by convergence in feeding ecologies of bivalves and vermetid gastropods, allowing both groups to ingest monorchiid eggs. It can be inferred that multiple analogous, opportunistic

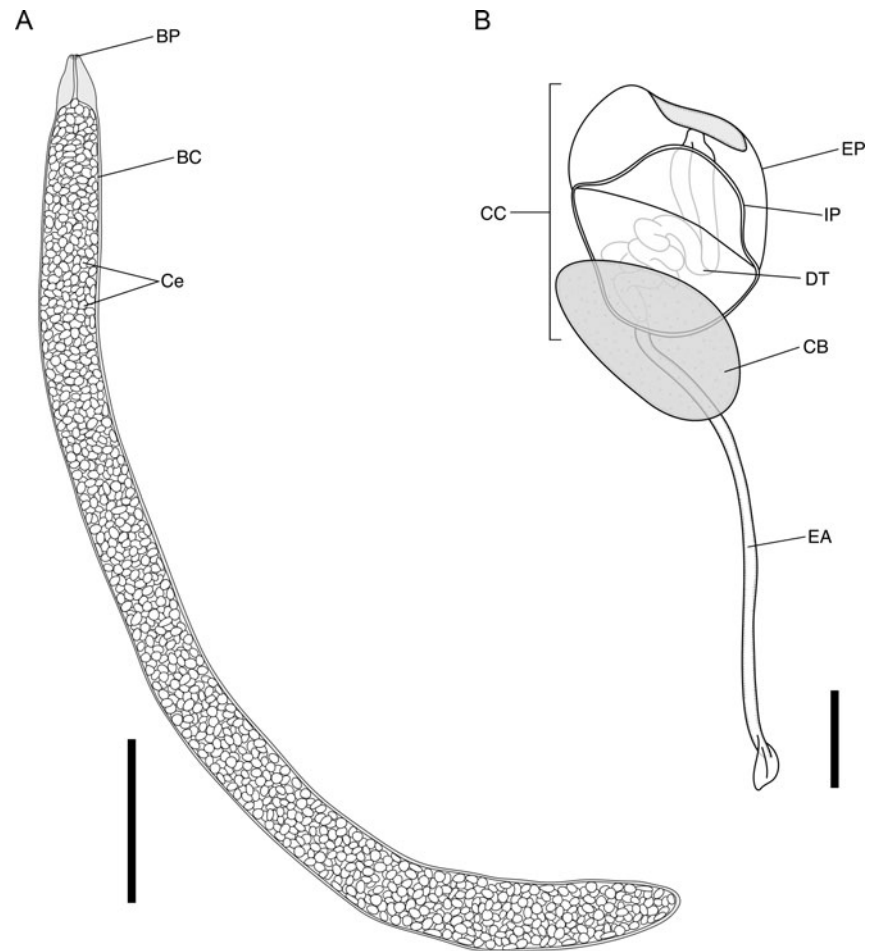


Fig. 3. Intramolluscan stages of the didymozoid infection from *Thylacodes* sp.: (A) sporocyst, scale: 500 μm ; and (B) immature cercaria inside the sporocyst, scale: 20 μm . Abbreviations: BC, brood chamber; BP, birth pore; CC, caudal chamber; CB, cercarial body; Ce, cercariae; DT, delivery tube; EA, excretory appendage; EP, external part of the caudal chamber; and IP, internal part of the caudal chamber.

host-switching events have occurred within the Didymozoidae. Specifically, it can be hypothesized that the similar feeding behaviours of vermetids and bivalves (i.e. particle feeding) allowed the phylogenetically closely related *S. magnacetabula* and the species from *Thylacodes* sp. to infect hosts from different mollusc classes. In contrast, how *F. desmarestia* becomes infected by didymozoids is mysterious. Gabe (1966) observed 'entire animals, perfectly identifiable' (p. 886) in the gut of some specimens, validating the interpretation of the radular and buccal structure of *F. desmarestia* as a clear indicator of carnivory (Buchmann, 1924; Gabe, 1966). Indeed, pterotracheoid gastropods become exclusively visual predators (Okutani, 1961; Seapy, 1980) after larval metamorphosis (Thiriôt-Quiévreux, 1969), catching and swallowing prey whole with their radula (Thiriôt-Quiévreux, 1973). Species of the closely related genus *Pterotrachea* Forsskål (Pterotracheoidea: Pterotracheidae) eat crustaceans, siphonophores and salps (Hirsch, 1915); species of *Carinaria* Lamarck (Pterotracheoidea: Carinariidae) eat crustaceans, chaetognaths (Okutani, 1961; Seapy, 1980), fish larvae, siphonophores, salps and other tunicates, polychaetes, pteropods and other pterotracheoids (Seapy, 1980); and species of *Atlanta* Lesueur (Pterotracheoidea: Atlantidae) eat pteropods, gastropod larvae (Thiriôt-Quiévreux, 1969) and other atlantids (Richter, 1968). Other than pteropods (Bonnievie, 1916) and pterotracheoids, such prey is not known to harbour first-stage trematode infections. Moreover, pterotracheoid radulas would not be able to grasp tiny hemiuroid eggs the way they seize other prey (Stunkard, 1973). In

the absence of a definitive explanation, the route of infection of *F. desmarestia* as first intermediate host for hemiuroids remains difficult to explain. However, there are at least two possible explanations: (1) *Firoloida* ingests eggs before larval metamorphosis; and (2) *Firoloida* ingests prey that have already ingested eggs, as Okutani (1961) said is most probably the case for *Carinaria japonica* which may have diatoms in its gut from eating salps.

Prévôt (1969) reported a hemiuroid infection from a vermetid, *Vermetus triquetrus* Bivona-Bernardi, from Marseille, France. This infection has not been attributed to any hemiuroid family. The cercariae from *Thylacodes* sp. resemble those of Prévôt (1969) in the division of the caudal vesicle between posterior internal and anterior external parts and in the thickening of the external part at its anterior extremity, but differ in the absence of concentric striae and two short posterior appendages, in having a single excretory appendage, and in the presence of a paddle at the appendage's end. The significance of these distinctions is not understood, and whether Prévôt's infection relates to the Didymozoidae or another hemiuroid family remains an open question.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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