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Molecular systematics of the digenean community parasitising the cerithiid gastropod *Clypeomorus batillariaeformis* Habe & Kusage on the Great Barrier Reef

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ABSTRACT

A rich fauna of digenetic trematodes has been documented from the Great Barrier Reef (GBR), yet little is known of the complex life-cycles of these parasites which occur in this diverse marine ecosystem. At Heron Island, a small coral cay at the southern end of the GBR, the intertidal marine gastropod *Clypeomorus batillariaeformis* Habe & Kusage (Cerithiidae) is especially abundant. This gastropod serves as an intermediate host for 12 trematode species utilising both fish and avian definitive hosts. However, 11 of these species have been characterised solely with morphological data. Between 2015–2018 we collected 4,870 *C. batillariaeformis* from Heron Island to recollect these species with the goal of using molecular data to resolve their phylogenetic placement. We found eight of the 12 previously known species and two new forms, bringing the total number of digenean species known to parasitise *C. batillariaeformis* to 14. The families of this trematode community now include the Atractotrematidae Yamaguti, 1939, Bivesiculidae Yamaguti, 1934, Cyathocotylidae Mühling, 1898 , Hemiuridae Looss, 1899, Heterophyidae Leiper, 1909, Himasthlidae Odhner, 1910, Microphallidae Ward, 1901, and Renicolidae Dollfus, 1939. Molecular data (ITS and 28S rDNA) were generated for all trematode species, and the phylogenetic position of each species was determined. The digenean community parasitising *C. batillariaeformis* includes several common species, as well as multiple species which are uncommon to rare. Although most of those trematodes in the community which exploit fishes as definitive hosts have remained common, the composition of those which utilise birds appears to have shifted over time.

Keywords: Trematode; Cercariae; Cerithiidae; Phylogenetics; Life-cycle; Coral Reef

1. Introduction

The Great Barrier Reef (GBR), off Australia's tropical north-eastern coast, is among the most biodiverse of all marine ecosystems, and correspondingly, has a rich diversity of parasite species [1–4]. Digenetic trematodes are the best known group of parasites on the GBR, but the largest gap in our understanding of this fauna is knowledge of their complex life-cycles [3]. Understanding trematode life-cycles can inform our taxonomic hypotheses, give us critical insights into evolutionary and ecological processes, and help us paint a more accurate and complete picture of trophic interactions and food-web dynamics [5–10]. However, connecting larval trematodes with their respective adult is no simple task because larval trematodes typically bear little morphological resemblance to their adult forms.

The traditional method of elucidating trematode life-cycles requires experimental infection. In an ecosystem as complex as the GBR, however, with countless numbers of potential hosts which might be implicated in a trematode life-cycle, such methodology is impractical. In recent years, matching various stages of helminth life-cycles with molecular markers has become the method of choice for elucidating such life histories [11]. Although this method has been employed effectively on the GBR to elucidate some full and partial helminth life-cycles [12–17], life-cycle data for many GBR trematodes remains scarce.

Heron Island, a small coral cay on the southern GBR, has been a focal point for parasitology research in the region for many years. The earliest report of larval trematodes from the island was made in 1962 by Moulton [18] who, while studying the clustering behaviour of the cerithiid gastropod *Clypeomorus batillariaeformis* Habe & Kosuge, observed among others, dark tailed magnacercous heterophyid cercariae parasitising a large proportion of the *C. batillariaeformis* population. In 1968, Pearson [19] became the first to both formally describe

a cercaria from *C. batillariaeformis* and elucidate a trematode life-cycle at Heron Island, those of *Paucivitellosus fragilis* Coil, Reid & Kuntz, 1965 (Bivesiculidae Yamaguti, 1934). A decade passed before Cannon [20] described an additional 10 trematode cercariae from *C. batillariaeformis* from Heron Island, giving them each a placeholder designation, i.e. *Cercaria queenslandae* I–X. Some years later Beuret and Pearson [21] described a new heterophyhid cercariae from *C. batillariaeformis* from Heron Island, bringing the number of species known to parasitise *C. batillariaeformis* to 12. In 2000, Beuret et al. [22] used infection experiments to identify *Cercaria queenslandae* IX as the cercaria of the heterophyid *Galactosomum bearupi* Pearson, 1973, an intestinal parasite of piscivorous marine birds. More recently, in 2017, Huston et al. [17] described a new species of the family Atractotrematidae Yamaguti, 1939, *Isorchis cannoni* Huston, Cutmore & Cribb, 2017 from rabbitfishes caught off Heron Island and used molecular methods to demonstrate that *Cercaria queenslandae* II was its larval form.

Here, we continue the study of larval digeneans parasitising *C. battilariaeformis* from Heron Island. We have attempted to collect all cercariae previously known from this gastropod and, using molecular data, determine the phylogenetic position of each species in the greater digenean phylogeny. Although not all trematodes previously known from *C. batillariaeformis* at this location were found, we discovered infections of two previously uncharacterised cercariae, which are described herein.

2. Methods

2.1. Specimen collection

During four separate periods between 2015 and 2018 (5–13 Oct 2015; 26–31 Oct 2015; 21–29 Oct 2016; 13–19 Jan, 2018), specimens of *C. batillariaeformis* (n = 4,870) were collected

from beach rock along Heron Island, southern Great Barrier Reef, Queensland, Australia (23° 26'S, 151° 55'E). Snails were isolated in a small amount of seawater in individual 10 ml wells and left for 24–48 hr to allow for cercarial emergence. Snail-wells were examined daily for emerged cercariae. Snails from which no cercariae had emerged within 48 hr were released at the site of capture. Upon discovery, some emerged cercariae were studied live with the aid of neutral red vital stain and preliminarily identified using the key of Cannon [20]; the remainder were fixed in near-boiling saline and preserved in 70% ethanol for subsequent parallel morphological and molecular analyses. For each trematode species observed in this study, up to five of the respectively infected *C. batillariaeformis* were dissected for the collection of intramolluscan trematode larval stages; infected snails found in excess of project needs were released at the site of capture. Additionally, random samples of 100 snails in 2016 and 180 snails in 2018 were collected and dissected to better determine total trematode prevalence. Intramolluscan trematode stages were fixed and preserved as above.

2.2. Morphological analyses

Trematode specimens used for morphological examination were removed from their preservative, washed in fresh water, overstained in Mayer's haematoxylin, destained in a solution of 1.0% hydrochloric acid, then neutralized in 0.5% ammonium hydroxide solution. Specimens were then dehydrated in a graded ethanol series, cleared in methyl salicylate and mounted in Canada balsam. Drawings for new material recognised in this study were made using an Olympus BX-53 compound microscope with attached drawing tube, and illustrations were digitized in Adobe Illustrator. Measurements were made with cellSens standard imaging software paired with an Olympus SC50 digital camera mounted on an Olympus BX-53 compound microscope. Measurements are given in µm as the range followed by the mean in parentheses. Where length is followed by breadth, the two measurements are separated by '×'. All vouchers are lodged in the Queensland Museum (QM), Brisbane, Australia.

Accession numbers for lodged vouchers are presented in the taxonomic section of this manuscript.

2.3. Molecular sequencing

For each species studied, two ribosomal DNA markers were targeted: the internal transcribed spacer 2 (ITS2) and the 28S rRNA coding region. The ITS2 region is the most widely used marker for the delineation of trematode species, whereas the 28S rRNA region is the most common marker for constructing phylogenetic hypotheses of relationships in the Digenea [11, 23]. Additionally, sequence data for the internal transcribed spacer 1 (ITS1) were generated for the single species of Echinostomatoidea Looss, 1902 found in this study. Larval trematode sequence replicates were obtained from different individual snails where infection numbers permitted. PCR products were obtained from total genomic DNA extracted from trematodes using phenol/chloroform extraction techniques [24], or using a direct PCR method. PCR using genomic DNA was performed using Bioline MyTaq™ DNA Polymerase and Reaction Buffer following the manufacturer recommendations [16, 17]. For direct PCR, trematodes were first removed from their preservative and cleaned in fresh 70% ethanol. With a micropipette, individual sporocysts, rediae or cercariae were transferred to a 600 µl PCR tube in 5 µl of 70% ethanol. Tubes were incubated with lids open in a drying oven at 50°C for 30 min to allow evaporation of the ethanol, upon which 13.25 µl of Invitrogen™ ultraPURE™ distilled water was added to each tube. Tubes were then sealed and incubated for a further 20 min at 95°C. After this incubation period, 6.75 µl of a PCR master-mix made up without the H_2O component was added to each tube and PCR was performed as normal. For both PCR methods employed, the entire ITS1, entire ITS2 and partial 28S rRNA regions were amplified with the primers and cycling conditions used by Huston et al. [16, 17]. Amplified DNA was purified using a Bioline ISOLATE II PCR and Gel purification kit, as per the manufacturer's protocol. Cycle sequencing of purified DNA was carried out using

ABI Big Dye™ v.3.1 chemistry following the manufacturer's recommendations at the Australian Genome Research Facility, using an AB3730x1 capillary sequencer. Primers used for sequencing the ITS1, ITS2 and 28S regions are listed in Huston et al. [16, 17]. Sequencher™ version 4.5 (GeneCodes Corp.) was used to assemble and edit contiguous sequences. Collection data and GenBank accession numbers for taxa sequenced are presented in the taxonomic section of this manuscript.

2.4. Phylogenetic analyses

The original cercarial classification of Cannon [20], along with BLAST analyses of the new ITS2 and 28S sequence data were used to preliminarily assign each distinct species sequenced in this study to a superfamily or family-level group, resulting in six individual molecular datasets for analysis. The newly generated partial 28S sequences were aligned with selected ingroup and outgroup taxa available on GenBank (Table 1) using MUSCLE [25] as implemented in MEGA 7 [26]. Alignments were trimmed to the shortest sequence length, except in the alignment for the Diplostomoidea Poirier, 1886 (see section 3.9 of the results below). Outgroup choice was based upon the molecular phylogenies of Olson et al. [27] and Littlewood et al. [28].

Phylogenetic trees for each 28S dataset were constructed with maximum likelihood and Bayesian inference analyses on the CIPRES portal [29]. Best-fit nucleotide substitution models were selected using jModelTest 2 [30] with the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). Maximum likelihood analyses were performed using RAxML [31] with 1,000 bootstrap psuedoreplicates. Bayesian inference was performed using MrBayes v3.2.6 [32]. Four chains were sampled every 1,000 generations for 10,000,000 generations with the first 2,500 samples being discarded as burn-in, at which point average standard deviation of split frequencies were <0.01 for all analyses.

3. Results and Discussion

3.1. Overview

From the 4,870 C. *batillariaeformis* examined, a total of 134 infections were detected during the course of this study. These infections included representatives of eight of the 12 species of trematode previously reported from *C. batillariaeformis* on the Great Barrier Reef, as well as two new species, the intramolluscan stages of which are characterised here. Of these infections, just 55 were detected via cercarial emergence (indicative of 1% total trematode infection prevalence). The subset of 100 *C. batillariaeformis* dissected in 2016 yielded eight infections (8% prevalence) and the subset of 180 *C. batillariaeformis* dissected in 2018 yielded 69 infections (38% prevalence). The previously characterised larval trematodes of Cannon [20] *Cercaria queenslandae* IV, VII, VIII, and X were not detected in this study. Molecular data were successfully generated for representatives of all trematode species obtained, and the phylogenetic position for each species was determined. With the exception of the Diplostomoidea dataset (see section 3.9 below), maximum likelihood and Bayesian inference analyses generated identical tree topologies for all 28S rDNA datasets.

3.2. Taxonomic summary

Superfamily Bivesiculoidea Yamaguti, 1934

Family Bivesculidae Yamaguti, 1934

Genus *Paucivitellosus* Coil, Reid & Kuntz, 1965

Paucivitellosus fragilis Coil, Reid & Kuntz, 1965

Prevalence *via* cercarial emergence: 0.06–0.6% (11/1,890 in 2015; 1/1,700 in 2016; 6/1,000 in, 2018).

Prevalence *via* dissection: 2–4% (2/100 in 2016; 8/180 in 2018).

Voucher material: QM G237390–237393.

Representative DNA sequences: ITS2, three identical replicates (one sequence submitted to GenBank, MH257758). Partial 28S rRNA, two identical replicates (one sequence submitted to GenBank, MH257768).

3.2.1. Molecular analyses

In phylogenetic analyses for Bivesiculidae based on 28S rDNA data (Fig. 1), this species clusters with others of the genus *Paucivitellosus*, the molecular data for which were all generated from specimens from off Vietnam [33]. Strong support was found for the monophyly of *Paucivitellosus*, but our analyses recovered *Bivesicula unexpecta* Cribb, Bray & Barker, 1994 as sister to *Paucivitellosus*, suggesting the genus *Bivesicula* Yamaguti, 1934, may not be a natural grouping. No ITS2 data had been previously generated for a species of *Paucivitellosus*; BLAST analyses of these data suggested a close affiliation with species of *Bivesicula*.

3.2.2. Remarks

This was the first larval trematode to be described from *C. batillariaeformis* from Heron Island. Pearson [19] described the life-cycle and reported adults from the blenny *Istiblennius meleagris* (Valenciennes) and the mullets *Crenimugil crenilabis* (Forsskål) and *Mugil cephalus* Linnaeus. Cercariae emerge from the snail and adhere to the substratum with the anterior end of the cercarial body while waving the tail vigorously. The cercariae are directly ingested by the definitive hosts.

3.3. Taxonomic summary

Superfamily Echinostomatoidea Looss, 1902

Family Himasthlidae Odhner, 1910

Genus *Acanthoparyphium* Dietz, 1909

Acanthoparyphium sp.

(= *Cercaria queenslandae* I Cannon, 1978)

Prevalence *via* cercarial emergence: 0–0.2% (3/1,890 in 2015; 0/1,700 in 2016; 0/1,000 in 2018).

Prevalence *via* dissection: 0–1.0% (0/100 in 2016; 2/180 in 2018).

Voucher material: QM G237394–237397.

Representative DNA sequences: ITS1, three identical replicates (one sequence submitted to GenBank, MH257759). ITS2, three identical replicates (one sequence submitted to GenBank, MH257760). Partial 28S rRNA, two identical replicates (one sequence submitted to GenBank, MH257769).

3.3.1. Molecular analyses

Phylogenetic analyses using 28S rDNA (Fig. 2) confidently place this species in the genus *Acanthoparyphium* Dietz, 1909 within the recently elevated family Himasthlidae Odhner, 1910 [see 34]. The ITS2 sequences obtained for this species were unusually long and we were unable to detect the 28S motif at the end of the sequence via annotation with the ITS2 database [35, 36]; BLAST analyses of these sequences were inconclusive. However, ITS1 sequences generated for this species aligned well with other ITS1 sequences of *Acanthoparyphium* spp. available on GenBank, although identity scores did not exceed 98%. Both ITS1 and 28S data suggest this species is closely related to, but distinct from, *Acanthoparyphium spinulosum* Johnston, 1917.

3.3.2. Remarks

Cannon [20] suggested that this cercaria was that of a species of *Acanthoparyphium*, and perhaps even that of *A. spinulosum*. The presence of 25 collar spines in *C. queenslandae* I is inconsistent with the diagnosis of the genus *Acanthoparyphium*, which states species of this genus possess 23 collar spines [37]. Although it is possible that the cercariae which we sequenced were in fact not *C. queenslandae* I, and rather those of a morphologically similar species which we have mistaken for that described by Cannon [20], we think this unlikely. We note that three of the five genera in the family Himasthlidae include species with variable numbers of collar spines [37]. There are clearly many species of *Acanthoparyphium* yet undiscovered, and we think it likely that future diagnoses will need to be adjusted to accommodate a broader concept of the genus.

Acanthoparyphium spinulosum was described from the Pacific Golden Plover *Pluvialis fulva* (Gmelin) (Charadriidae) [reported as *Pluvialis dominica* (Müller)] from near Sydney, New South Wales, Australia [38]. The species has also been reported from the Grey Plover *Pluvialis squatarola* (Linnaeus) and Greater Sand Plover *Charadrius leschenaultia* (Lesson) (Charadriidae) [34, 39, 40]. Our phylogenetic analyses using 28S rRNA demonstrated a close relationship between the species of *Acanthoparyphium* sequenced in this study and the sequence of *A. spinulosum* from a Grey Plover provided by Tkach et al. [34], suggesting that the definitive host of the Heron Island species might also be a plover. Grey Plovers and Pacific Golden Plovers are known summer visitors to Heron Island, along with the Red-capped Plover *Charadrius ruficapillus* Temminck, and the Lesser Sand Plover *Charadrius mongolus* Pallas [41]. During the 2018 collection period in this study, we observed large numbers of Pacific Golden and Lesser Sand Plovers on Heron Island, but saw no Grey or Red-capped Plovers.

With the notable exception of *Acanthoparyphium tyosenense* Yamaguti, 1939 which infects naticid gastropods [42], first intermediate hosts for species of *Acanthoparyphium* are all cerithioid gastropods [39, 40, 43–47]. Second intermediate hosts include a variety of gastropods, bivalves and polychaetes, and multiple studies have reported the first intermediate host also serving as the second intermediate host [39, 40, 43, 44, 46]. However, Cannon [20] made no mention of observing metacercariae of *Acanthoparyphium* sp. in any of the *C. batillariaeformis* he examined from Heron Island, and we did not observe any metacercariae in this gastropod either.

3.4. Taxonomic summary

Superfamily Haploporoidea Nicoll, 1914

Family Atractotrematidae Yamaguti, 1939

Genus *Isorchis* Durio & Manter, 1969

Isorchis cannoni Huston, Cutmore & Cribb, 2017

(= *Cercaria queenslandae* II Cannon, 1978)

Prevalence *via* cercarial emergence: 0.2–1.1% (3/1,890 in 2015; 0/1,700 in 2016; 11/1,000 in 2018).

Prevalence *via* dissection: 0–30% (0/100 in 2016; 54/180 in 2018).

Voucher material: QM G236340–236344 [see 17].

Representative DNA sequences: ITS1: MF803155; ITS2: MF803156; partial 28S rRNA: MF803154 [see 17].

3.4.1. Remarks

We previously described the sexual adult of this species, elucidated its life-cycle, and determined its phylogenetic position [17]. Cercariae of *I. cannoni* emerge from *C. battilariaeformis* and encyst in the environment where they are then incidentally consumed by the browsing definitive hosts *Siganus fuscescens* (Houttuyn) and *Siganus lineatus* (Valenciennes) (Siganidae). During the last collection period of this study (Jan., 2018) we observed a large number of *I. cannoni* infections in the *C. battilariaeformis* collected. Cannon [48], in an ecological study of the trematodes of *C. battilariaeformis* on Heron Island, collected across all seasons and observed no seasonality of parasitism. Although our data show a dramatic difference in prevalence of *I. cannoni* between spring 2016 and summer 2018, this was across two years and such differences may be due to a variety of factors beyond seasonality.

3.5. Taxonomic summary

Superfamily Microphalloidea Ward, 1901

Family Renicolidae Dollfus, 1939

Genus *Renicola* Cohn, 1904

Renicola sp.

(= *Cercaria queenslandae* III Cannon, 1978)

Prevalence *via* cercarial emergence: 0–0.05% (1/1890 in 2015; 0/1,700 in 2016; 0/1,000 emerged in 2018).

Prevalence *via* dissection: 0–1.0% (1/100 in 2016; 0/180 in 2018).

Voucher material: QM G237398 –237401.

Representative DNA sequences: ITS2, three identical replicates (one sequence submitted to GenBank, MH257761). Partial 28S rRNA, two identical replicates (one sequence submitted to GenBank, MH257770).

3.5.1. Molecular analyses

Phylogenetic analyses for the Renicolidae using 28S rDNA data (Fig. 3) demonstrated that the species sequenced in this study is a member of the genus *Renicola*. Although 28S sequence data have been provided for three fully identified species of the genus *Renicola* [49], these sequences fail to align properly with those produced for renicolids in this or any other study [27, 50–53]. Because of this, we did not incorporate these 28S rDNA data into our phylogenetic analyses. ITS2 data for the Renicolidae has been produced only by Heneberg et al. [49] and the present study. BLAST analyses of the ITS2 data generated in this study suggest a closer relationship with microphallids, than with the ITS2 data generated for the species of *Renicola* studied by Heneberg et al. [49]. This may be an indication that for the Renicolidae, utility of the ITS2 marker is limited across large geographic distances.

3.5.2. Remarks

There is a paucity of molecular data for fully identified renicolid species, thus within-family phylogenetic relationships are currently difficult to explore. However, as only two genera are currently recognised in the Renicolidae, phylogenetic placement of newly sequenced material (such as the cercariae sequenced here) is relatively straightforward. Adult renicolds are kidney parasites of birds with the typical life-cycle involving marine prosobranchs as first intermediate hosts and bivalves and fishes as second intermediate hosts [54]. Ching [55] described adults of two species of *Renicola* from black noddies, *Anous minutus* (Boie) (Laridae), collected at Heron Island, as well as a renicolid metacercariae from the muscle tissue of a local atherinid fish, and although she indicated that her metacercariae was likely

that of one of the adults she described, she made no indication that any of these trematodes represented the adult or metacercarial stage of *Cercaria queenslandae* III.

3.6. Taxonomic summary

Family Microphallidae Ward, 1901

Genus *Maritrema* Nicoll, 1907

Maritrema sp.

(= *Cercaria queenslandae* V Cannon, 1978)

Prevalence *via* cercarial emergence: 0% (0/1,890 in 2015; 0/1,700 in 2016; 0/1,000 in 2018).

Prevalence *via* dissection: 0–1.0% (1/100 in 2016; 0/180 in 2018).

Voucher material: QM G237402–237407.

Representative DNA sequences: ITS2, two identical replicates (one sequence submitted to GenBank, MH257762). Partial 28S rRNA, two identical replicates (one sequence submitted to GenBank, MH257771).

3.6.1. Molecular analyses and remarks

See section 3.7.1. and 3.7.2. below.

3.7. Taxonomic summary

Genus *Microphallus* Ward, 1901

Microphallus sp.

(= *Cercaria queenslandae* VI Cannon, 1978)

Prevalence *via* cercarial emergence: 0–0.06% (0/1,890 in 2015; 1/1,700 in 2016; 0/1,000 in 2018).

Prevalence *via* dissection: 0–1.0% (1/100 in 2016; 0/180 in 2018).

Voucher material: QM G237408–237411.

Representative DNA sequences: ITS2, three identical replicates (one sequence submitted to GenBank, MH257763). Partial 28S rRNA, two identical replicates (one sequence submitted to GenBank, MH257772).

3.7.1. Molecular analyses

Phylogenetic analyses with 28S rDNA data (Fig. 4) placed *Cercaria queenslandae* V and VI within the Microphallidae in the genera *Maritrema* and *Microphallus*, respectively. Although these relationships had strong support, these analyses did not provide any further insight into the specific identity of these two cercariae. Likewise, BLAST analyses of the ITS2 sequence data demonstrate a close relationship between *Cercaria queenslandae* V and species of *Maritrema* and between *Cercaria queenslandae* VI and species of *Microphallus*, but the ITS2 sequences do not match any previously produced sequences.

3.7.2. Remarks

Species of the genus *Maritrema* are known for typical three host life-cycles, with cercariae emerging from gastropods, penetrating crustaceans to form metacercariae, and awaiting ingestion by avian definitive hosts, [e.g. 56–58]. In our phylogenetic analyses (Fig. 4), *Cercaria queenslandae* V fell near *Maritrema novaezealandense* Martorelli, Fredensborg, Mouritsen & Poulin, 2004, a species which utilises the red-billed gull *Chroicocephalus novaehollandiae scopulinus* (Forster) (subspecies of the silver gull, family Laridae) as a

definitive host. As *C. novaehollandiae* is an abundant species on Heron Island [41; personal observations, 2015–2018], it may well be the definitive host of *Cercaria queenslandae* V. Many species of the genus *Microphallus* exhibit a derived two-host life-cycle in which cercariae develop to the metacercarial stage within the sporocyst inside the first intermediate host, rather than emerging as free swimming cercariae [59, 60]. This is thought to be an evolutionary adaptation to the utilisation of birds which make short stops on long migratory routes as their definitive hosts [61]. *Cercaria queenslandae* VI apparently does not fit this life-cycle pattern, as this species has a free living cercarial stage and no metacercariae have been observed within the sporocyst of this species by us or any other workers [20, 48, 62]. Although the specific identity of the definitive host remains unclear, it seems most likely to be a resident rather than a migratory species of bird.

3.8. Taxonomic summary

Superfamily Opisthorchioidea Braun, 1901

Family Heterophyidae Leiper, 1909

Genus *Galactosomum* Looss, 1899

Galactosomum bearupi Pearson, 1973

(= *Cercaria queenslandae* IX Cannon, 1978)

Prevalence *via* cercarial emergence: 0–0.4% (8/1,890 in 2015; 0/1,700 in 2016; 1/1,000 in 2018).

Prevalence *via* dissection: 0–1.1% (0/100 in 2016; 2/180 in 2018).

Voucher material: QM G237412–237415.

Representative DNA sequences: ITS2, three identical replicates (one sequence submitted to GenBank, MH257764). Partial 28S rRNA, two identical replicates (one sequence submitted to GenBank, MH257773).

3.8.1 Molecular results and remarks

See section 3.8.5. and 3.8.6. below

3.8.2. Taxonomic summary

Galactosomum sp. 'Heron Island Zygocercaria' of Beuret and Pearson [21].

Prevalence *via* cercarial emergence: 0–0.2% (3/1,890 in 2015; 0/1,700 in 2016; 2/1,000 in 2018).

Prevalence *via* dissection: 2.2–3.0% (3/100 in 2016; 4/180 in 2018).

Voucher material: QM G237416–237418.

Representative DNA sequences: ITS2, three identical replicates (one sequence submitted to GenBank, MH257765). Partial 28S rRNA, two identical replicates (one sequence submitted to GenBank, MH257774).

3.8.3. Molecular results and remarks

See section 3.8.5. and 3.8.6. below

3.8.4. Taxonomic summary

Galactosomum sp. 'Heron Island Magnacercaria' (Fig. 5A, B)

Prevalence *via* cercarial emergence: 0–0.3% (6/1,890 in 2015; 0/1,700 in 2016; 0/1,000 in 2018).

Prevalence *via* dissection: 0% (0/100 in 2016; 0/180 in 2018).

Location of infection: Gonad and digestive gland.

Voucher material: QM G237419–237427.

Representative DNA sequences: ITS2, three identical replicates (one sequence submitted to GenBank, MH257766). Partial 28S rRNA, two identical replicates (one sequence submitted to GenBank, MH257775).

3.8.4.1. Descriptions

Description of redia (Fig. 5A)

(Based on 10 excised specimens, fixed and mounted). Body elongate, cylindrical with rounded ends, containing numerous developing cercariae, 732–1153 × 82–122 (938 × 97). Body length divided by width 7.0–13.1 (9.7). Mouth terminal. Pharynx ellipsoid, 22–26 × 25–30 (24 × 27). Oesophagus absent; gut sac-like, 12–19 × 12–21 (15 × 16). Birth pore not observed.

Description of cercaria (Fig. 5B)

(Based on 15 emerged cercariae, fixed and mounted, supplemented with live observations). Oculate magnacercous opisthorchioid cercaria. Behaviour consists of motionless periods broken by short swimming bursts via lateral undulation of post-medial portion of tail. Body elongate, oval, lacking pigmentation, tightly packed with cystogenous gland-cells, 113–129 × 35–44 (120 × 39). Tegument spinose. Eye-spots two, premedian, 6–9 × 6–8 (8 × 7). Eye-spot pigment dispersed around eye-spots. Penetration organ at anterior extremity, composed of enlarged spines; penetration glands obscured by cystogenous gland cells. Oral sucker

globular, subterminal, 22–24 × 22–24 (23 × 23). Alimentary system obscured. Ventral sucker not developed. Excretory bladder elliptical, 18–25 × 14–20 (21 × 19). Reproductive primordia at anterior margin of excretory bladder, botryoidal, 16–22 × 12–21 (19 × 17). Flame-cell formula not determined. Tail lanceolate, 420–473 × 21–31 (proximal), 81–138 (medial), 6–12 (distal) (449 × 24, 118, 10); 3.3–4.1(3.8) times longer than body. Tail tissue vacuolate; anterior portion unpigmented; posterior portion with lattice of minute pigment granules.

3.8.5. Molecular results

In our phylogenetic analyses using 28S rDNA (Fig. 6), the three larval heterophyids sequenced in this study formed a well-supported monophyletic clade with *Galactosomum lacteum* (Jägerskiöld, 1896), implying the presence of three distinct species of *Galactosomum* as parasites of *C. batillariaeformis* at Heron Island. No previous ITS2 data were available for species of *Galactosomum*, however BLAST analyses of the ITS2 data generated confirmed the placement of these cercariae in the family Heterophyidae.

3.8.6. Remarks

Because Prévot [63] had previously elucidated the first life-cycle of a species of *Galactosomum*, Cannon [20] had inferred that his *Cercaria queenslandae* IX was a likely member of the genus *Galactosomum*. Pearson [64] had reported adults and metacercariae of *Galactosomum bearupi*, from the noddy *Anous minutus* and halfbeaks *Hyporhamphus* sp., respectively, at Heron Island, and later Beuret et al. [22] confirmed the species identity of this cercariae as *G. bearupi* and elucidated its complete life-cycle via experimental infection. The cercariae of *Galactosomum bearupi* have large red-pigmented tails and are strongly photopositive [20, 22]. They swim actively at the surface of the water in a figure eight pattern. This is presumed to facilitate consumption by the second intermediate hosts,

planktivorous fishes [20, 22]. The brief description of the heterophyid cercariae found from *C. batillariaeformis* from Heron Island given by Moulton [18] appears to match this species, thus the earliest record of *G. bearupi* from Heron Island can be dated to November of 1960.

The second species of *Galactosomum* collected in this study (the 'Heron Island Zygocercariae') was previously described and studied in detail by Beuret and Pearson [21], but was not reported by Cannon [20]. These cercariae exhibit a remarkable behaviour in which they join together by the tail in aggregates of several hundred up to a thousand and swim together in unison, thus attracting the second intermediate host fishes [21]. Beuret and Pearson [21] thought it likely that these cercariae were that of a species of *Galactosomum*, and our molecular analyses confirm this (Fig. 6). The definitive host for this species remains unknown, but is almost undoubtedly a piscivorous bird as for other species of the genus.

Although a single sentence of their paper suggests that Beuret and Pearson [21] were aware of an additional magnacercous cercariae exploiting *C. batillariaeformis* at Heron Island, it was never described. We found multiple infections in 2015 of what is clearly a third species, and have now formally described it and confirmed it as a species of *Galactosomum* (Fig. 5A, B; Fig. 6). The morphology and behaviour of this cercariae suggests that it attracts and is consumed directly by the second intermediate host, as for other species of the genus. The second intermediate and definitive hosts of this species are currently unknown, but it is expected that they are fishes and piscivorous birds as with other species of *Galactosomum*. Although heterophyid cercariae of other lineages have adapted a variety of strategies for infecting their second intermediate hosts, e.g. penetrating the gills after being inhaled incidentally [65–67], or active penetration of the host epidermis [65, 68], all known cercariae of *Galactosomum* appear to seek direct ingestion by small fishes [21, 22, 63, present study).

Pearson [64] recognised five species of *Galactosomum* in Australia: *G. angelae* Pearson, 1973, *G. bearupi*, *G. renincola* Pearson, 1973, *G. ussuriense* Oshmarin, 1963, and *G. sinuilactis* Pearson, 1973. Of these species, *G. angelae* is known only from South Australia, and the northern most report of *G. sinuilactis* came from Moreton Bay, in southern Queensland [64]. Conversely, both *G. bearupi* and *G. renincola* have been reported from Heron Island, and *G. ussuriensis* was reported from Townsville in north-eastern Queensland (adjacent to the GBR north of Heron Island) [64]. As there are three species of *Galactosomum* known from piscivorous marine birds in northern Queensland, it is likely more than coincidence that we have detected three species of *Galactosomum* parasitising *C. batillariaeformis* on the GBR, one of which has already been associated with *G. bearupi* [22]. Although there may well be further species of *Galactosomum* in Australia, it is entirely possible that the Heron Island Zygocercaria and Magnacercaria are the larval forms of *G. renincola* and *G. ussuriensis*. There is an issue in this speculation however, as Rekharani and Madhavi [69] characterised the cercariae and metacercariae of what they determined was *G. ussuriensis* from cerithiid gastropod *Cerithium coralium* Keiner and the grunter *Terapon jarbua* (Forsskål), respectively, from India. Rekharani and Madhavi [69] described the cercariae of *G. ussuriensis* as having a darkly pigmented tail more like that of *G. bearupi* than that of the Heron Island Magnacercaria characterised here, indicating that these cercariae presumably relate to two distinct species. Additionally, Rekharani and Madhavi [69] made no mention of aggregating behaviour in their cercariae, and the morphology is quite distinct from that of the Heron Island Zygocercaria. It is possible that the adults of *G. ussuriensis* reported by Pearson [64] actually represent an undescribed species, or that the metacercarial-based identification of *G. ussuriensis* of Rekharani and Madhavi [69] was incorrect. It is also possible that these *Galactosomum* cercariae from Heron Island that remain unconnected with an adult are those of yet undiscovered species. New, and preferably molecular, data from

adult trematodes would do much to illuminate the species identities and life-cycles in the genus *Galactosomum*.

3.9. Taxonomic summary

Superfamily Diplostomoidea Poirier, 1886

Family Cyathocotylidae Mühling, 1898

Cyathocotylidae sp. 'Heron Island'

Prevalence *via* cercarial emergence: 0% (0/1,890 in 2015; 0/1,700 in 2016; 0/1,000 in 2018).

Prevalence *via* dissection: 0–0.6% (0/100 in 2016; 1/180 in 2018).

Location of infection: Gonad and digestive gland.

Voucher material: QM G237428–237433.

Representative DNA sequences: ITS2, three identical replicates (one sequence submitted to GenBank, MH257767). Partial 28S rRNA, two identical replicates (one sequence submitted to GenBank, MH257776).

3.9.1. Descriptions

Description of sporocyst (Fig. 5C).

(Based on 10 excised specimens, fixed and mounted). Body allantoid with acute ends, containing many immature and near-mature cercariae, 1024–2285 × 152–246 (1471 × 197). Length divided by width 5.4–11.4 (7.5). Birth pore not observed.

Description of cercaria (Fig. 5D).

(Based on 10 ‘mature’ excised specimens, fixed and mounted). Nonoculate furcocercous cercaria. Body elliptical, slightly concave ventrally, tightly packed with cystogeneous gland-cells, 262–352 × 131–205 (294 × 168). Body length divided by width 1.4–2.1 (1.8). Oral sucker subterminal, ovoid, 25–41 × 31–48 (32 × 42). Pre-pharynx short; pharynx globular, 12–19 × 13–20 (16 × 17); oesophagus 0–21 (16) long. Intestine bifurcation 27–48 (43) from oral sucker; caeca sinuous, run parallel, terminating on either side of tail insertion. Post-caecal space 18–43 (33), representing 7–15 (11)% of body length. Ventral sucker not observed. Genital primordia intercaecal, darkly stained, compact, 9–15 × 12–20 (13 × 16). Tail insertion dorsal, 35–58 (46) from posterior body extremity. Tail muscular, stem 564–729 × 47–68 (648 × 56); furcae 236–384 (339) long, sharply attenuating, with thin membranous finfold arising midway along furcae and extending to just beyond furcal termination. Excretory vesicle obscured, situated in region of tail insertion.

3.9.2. Molecular results

The morphology of this cercaria suggested it belonged to the family Cyathocotylidae and preliminary BLAST analyses of the ITS2 sequence data generated for these cercariae indicated a relationship with *Braunina cordiformis* Wolf, 1903, the species forming the basis for the monotypic family Brauninidae Wolf, 1903. The Brauninidae was resolved as sister to the Cyathocotylidae in the molecular phylogeny of Hernández-Mena et al. [70]. To confidently place our cyathocotylid cercariae within the Diplostomoidea we added our sequence to an abbreviated version of the 28S rRNA dataset used by Hernández-Mena et al. [70]. Some species in this dataset were of significant interest but the sequences were very short (i.e. Cyathocotylid sp. HQ219208), so the alignment was trimmed to the length of the sequence generated for the cyathocotylid cercariae in this study. Although our tree differed somewhat from that of Hernández-Mena et al. [70] in the topology of the Diplostomidae + Proterodiplostomidae + Strigeidae clade (due to low bootstrap/posterior probabilities for

these nodes in both studies, and our abbreviated dataset), the Heron Island cyathocotylid cercariae formed a well-supported clade with the other cyathocotylid species + *B. cordiformis*.

3.9.3. Remarks

In our molecular analyses, the Heron Island cyathocotylid cercaria fell between the two representatives of the Cyathocotylidae (Cyathocotylidae sp. and *Mesostephanus microbursa* Caballero, Grocott & Zerecero, 1953) and *B. cordiformis*. *Braunina cordiformis* is the only species of its family, and as our sequences are not a match to this species, this cercaria appears to be a cyathocotylid rather than a brauninid. Beyond this family level designation however, we can gain no further insight into the identity of this species. Many cyathocotylid cercariae have been described, and some attempts have been made to divide them morphologically into subfamilies and genera. Dubois [71] divided the known cyathocotylid cercariae into four groups on the basis of the flame-cell formulae, however there is no clear relationship between these groups and the systematics of the family [72]. We were unable to determine the flame-cell formula of the Heron Island cyathocotylid, and as was found with the molecular data, are unable to assign this species to a subfamily or genus. However, we do note that the flame-cell formulae for described cyathocotylid cercariae can both vary among species of genera or be the same between genera across subfamilies [see 72], thus attempts to identify these cercariae on the basis of the excretory system may be inadvisable.

Only one infection of this cyathocotylid species was found during the course of this study, upon dissection during the last collection period in January of 2018. The rarity of this species could be the result of a variety of factors. The species could have recently become established, but this speculation could only be tested by further collection. It is well known that detecting trematode infections in gastropod hosts via cercarial emergence routinely fails

to detect all infections [e.g. 73], an assertion strongly supported by the data from this study. Thus, additional infections of this species might have been present, but were not detected via emergence and were not represented in the subsamples dissected. The 2018 collection period was in the summer while all other collection periods were in the spring, thus seasonality might explain why no cyathocotylid infections were detected in 2015 and 2016. However, neither Cannon [48] nor Rohde [62] observed any seasonal effects on parasitism of gastropods from Heron Island. Furthermore, no other study of the trematodes of *C. battilarieformis* from Heron Island [20–22, 48, 74, 75] has reported a cyathocotylid cercariae. The rarity of cyathocotylid cercariae at Heron Island suggests that the definitive host may be a rare or only occasional visitor to the island. Many migratory birds might fit this scenario, as would those which live along the coast of mainland Australia.

4. Conclusion

This study has brought the number of trematode species known to exploit *C. batillariaeformis* on the Great Barrier Reef to 14. This trematode species richness is clearly linked to the overall biodiversity of coral reefs coupled with the habitat niche of *C. batilliarieformis* which is at the interface of the reef lagoon and coral cay, exposing the snails to wide variety of marine and avian fauna. Although this system is rich, it does not exceed that of many others from the marine environment, and the species composition is comparable to other rich systems involving cerithiid gastropods [e.g. 43, 76–78]. Thus, none of the trematode species parasitising *C. batillariaeformis* are especially surprising or unexpected. A significant aspect of this trematode community is the morphological distinctiveness of each species and the lack of cryptic species. Application of molecular methods in the study of snail-trematode systems has led to a rapid accumulation of reports of cryptic species within these systems [e.g. 79–82]. In contrast, we found no evidence of interspecific variation in the molecular sequence data (i.e. cryptic species). It is thus the unremarkable characteristics of the trematode

community of *C. battilariaeformis* at Heron Island, and its similarity to other such communities, that make knowledge of this system so valuable. A more thorough understanding of the community composition of multiple similar snail-trematode systems lends strength to our ability to make sense of patterns arising from such systems, through the noise inherent in a complex natural environment. The present study reinforces our understanding of patterns in the life-cycles of multiple marine trematode lineages. For example, species of the himasthlid genus *Acanthoparyphium* and heterophyids of *Galactosomum* appear closely associated with cerithioid gastropods [20, 39, 40, 43–47, 63, 69, 83]. Conversely, congeneric microphallids may parasitise gastropods of multiple families [51, 56, 58, 59, 77, 84]

Although molecular techniques can introduce their own complications into the study of snail-trematode communities (e.g. detection of cryptic species), in most cases these methods can cut through the noise of complex systems. Molecular appraisal of larval trematode communities is both rapid and effective in resolving the phylogenetic placement of species, and in many cases, species or genera level identifications. Such an approach is especially useful for surveying the diversity of trematodes which utilise birds as definitive hosts, as it is difficult to obtain collecting permits for birds in many regions of the world, such as Australia.

Cannon [48] considered *Paucivitellosus fragilis*, *Acanthoparyhprium* sp. (*Cercaria queenslandae* I), *Isorchis cannoni* (*C. queenslandae* II), *Renicola* sp. (*C. queenslandae* III), *C. queenslandae* IV (Microphallidae), and *Maritrema* sp. (*C. queenslandae* V) to be the most common species at Heron Island, though he was not specific in regards to prevalence. Of the 12 trematode species previously known from *Clypeomorus batillariaeformis* at Heron Island, we failed to detect four species: *Cercaria queenslandae* IV and VII (Microphallidae), *C. queenslandae* VIII (family uncertain) and *C. queenslandae* X (Hemiuridae). Cannon [20] stated that *C. queenslandae* X was found only twice upon dissection (out of ~2000 snails), so

it is unsurprising that we did not detect this species. *Cercaria queenslandae* VII and VIII were not among the list of common species [48], so we presume they were rare at the time of their description as well [20]. Because it was listed as a common species by Cannon [48], failure to detect *C. queenslandae* IV was somewhat unexpected, though microphallids were detected only rarely in our study overall. Among the trematode species found in our study, only four were common (*P. fragilis, I. cannoni, G. bearupi,* and *Galactosomum* sp. 'Heron Island Zygocercaria), accounting for 88% of all infections detected. Although we failed to detect four of the 12 previously known digenean species in this system, we detected two previously unrecognised species: *Galactosomum* sp. 'Heron Island Magnacercaria' (Heterophyidae) and Cyathocotylidae sp. It appears thus, that while those trematode species utilising fish as definitive hosts (*P. fragilis* and *I. cannoni*) have maintained a high prevalence in the *C. batillariaeformis* population over time (with the exception of the hemiuroid, which remains rare), the prevalence of those species utilising birds has been far more dynamic. Our data indicate an increase in the prevalence of heterophyids and a decrease in the prevalence of echinostomatids, renicolids and microphallids since 1972 [20–22, 48, 62]. The Heron Island *C. batillariaeformis* population at times also plays host to several rare trematode species, likely due to the island receiving many migratory bird species annually. Obtaining a full understanding of this system will be difficult because of the dynamic nature of Heron Reef, but further exploration will likely lead to new insight into the life-cycles of trematodes in complex ecosystems.

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Conflict of interest

The authors declare they have no conflict of interest

Compliance with ethical standards

All applicable institutional, national and international guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Figure and Table Captions

Figures

Figure 1. Phylogram based on Bayesian inference and maximum likelihood analyses of the 28S rDNA dataset for the Bivesiculidae. Posterior probabilities are shown above the node, and bootstrap support values are shown below; values < 50 not shown.

Figure 2. Phylogram based on Bayesian inference and maximum likelihood analyses of the 28S rDNA dataset for the Echinostomatoidea. Posterior probabilities are shown above the node, and bootstrap support values are shown below; values < 50 not shown.

Figure 3. Phylogram based on Bayesian inference and maximum likelihood analyses of the 28S rDNA dataset for the Renicolidae. Posterior probabilities are shown above the node, and bootstrap support values are shown below; values < 50 not shown.

Figure 4. Phylogram based on Bayesian inference and maximum likelihood analyses of the 28S rDNA dataset for the Microphallidae. Posterior probabilities are shown above the node, and bootstrap support values are shown below; values < 50 not shown.

Figure 5. New cercariae and parthenitae found in this study. A) Redia of *Galactosomum* sp. 'Heron Island Magnacercaria'. B) Cercaria of *Galactosomum* sp. 'Heron Island Magnacercaria'. C) Sporocyst of Cyathocotylidae sp. 'Heron Island'. D) Cercaria of Cyathocotylidae sp. 'Heron Island'. Scale bars: A, C, D) 500 µm; B) 150 µm.

Figure 6. Phylogram based on Bayesian inference and maximum likelihood analyses of the 28S rDNA dataset for the Heterophyidae. Posterior probabilities are shown above the node, and bootstrap support values are shown below; values < 50 not shown.

Figure 7. Phylogram based on Bayesian inference and maximum likelihood analyses of the 28S rDNA dataset for the Diplostomoidea. Posterior probabilities are shown above the node, and bootstrap support values are shown below; values < 50 not shown.

Table 1. Sequence data from GenBank included in this study, subgrouped for each phylogram in which they were employed, with original references. Sequences references marked only with an '*' indicated direct submission to GenBank (i.e. no published manuscript associated with sequence).

Datas	GenBan	Referen	Datas	GenBan	Referen	Datas	GenBan	Referen
Fig. 1	AY22218	[27]	Fig. 4	AB52179	[85]	Fig. 6	AF18424	[50]
	AY22218	||		AY11687	[27]		AY11687	[27]
	AY22218	||		AY22222	||		AY11687	||
	AY22218	||		AY22222	||		AY22227	||
	KX18672	[86]		AY22222	||		KJ86821	[51]
	KX18674	||		AY22223	||		KM5050	[87]
	LN83171	[33]		FJ788496	[88]		KP71018	[53]
	LN83171	||		HQ83263	[89]		KP90341	[52]
	LN83171	||		JF823990	[90]		KP90341	||
	LN83171	||		KT87740	[91]	Fig. 7	AB55156	[92]
	LN83171	||		KU95148	[16]		AY22217	[27]
	LN86500	||		KU55955	[93]		AY22217	||
	LN86500	||		KU55956	||		AY22217	||
	LN86500	||		KU55956	||		HQ21920	*
	LN86500	||		KY35163	[94]		JF820607	[95]
Fig. 2	AY22221	[27]		KY36915	||		KF43476	[96]
	AY22221	||		KY36916	||		KM2586	[97]
	AY22222	||		KY36916	||		KT33416	[98]
	AY22222	||	Fig. 5	AB97436	[99]		MF39832	[70]
	AY22224	||		AF15192	[100]		MF39832	||
	AY22224	||		AY22061	[101]		MF39832	||
	AY22224	||		AY22062	||		MF39833	||
	AF15194	[100]		AY22062	||		MF39833	||
	AF15194	||		AY22062	||		MF39833	||
	EU02586	[102]		AY22062	||		MF39833	||
	EU02586	||		AY22062	||		MF39833	||
	EU02587	||		AY22063	||		MF39834	||
	EU02587	||		AY22063	||		MF39834	||
	JQ24643	[103]		AY22063	||		MF39834	||
	KF78130	*		AY22063	||		MF39834	||
	KP00962	[104]		HM5841	[60]		MF39834	||
	KP68312	[105]		HM5841	||			
	KT44752	*		HM5841	||			
	KT95691	[34]		HM5841	||			
	KT95691	||		HM5841	||			
	KT95691	||		HM5841	||			
	KT95692	||		HM5841	||			
	KT95692	||		HM5841	||			

KT95692	||	HM5841	||
KT95692	||	JF826247	[106]
KT95692	||	KJ14417	[57]
KT95693	||	KJ14417	||
KT95693	||	KJ14417	||
KT95693	||	KJ86821	[51]
KT95693	||	KT35581	[58]
KT95694	||	KT35582	||
KT95694	||	KT35582	||
KT95694	||	KT88022	[107]
KT95694	||	KX71208	[15]
KT95694	||		
KT95694	||		
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KT95694	||		
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KT95695	||		
KT95695	||		
KT95695	||		
KT95696	||		
KY85246	[108]		

Highlights

- *Clypeomorus batillariaeformis* was collected from the Great Barrier Reef
- Larval trematodes of *C. batillariaeformis* were studied with molecular data
- Ten species of trematode were detected and phylogenetically placed
- Two uncharacterised larval trematodes were detected and described
- The community composition of this host-parasite system has shifted over time

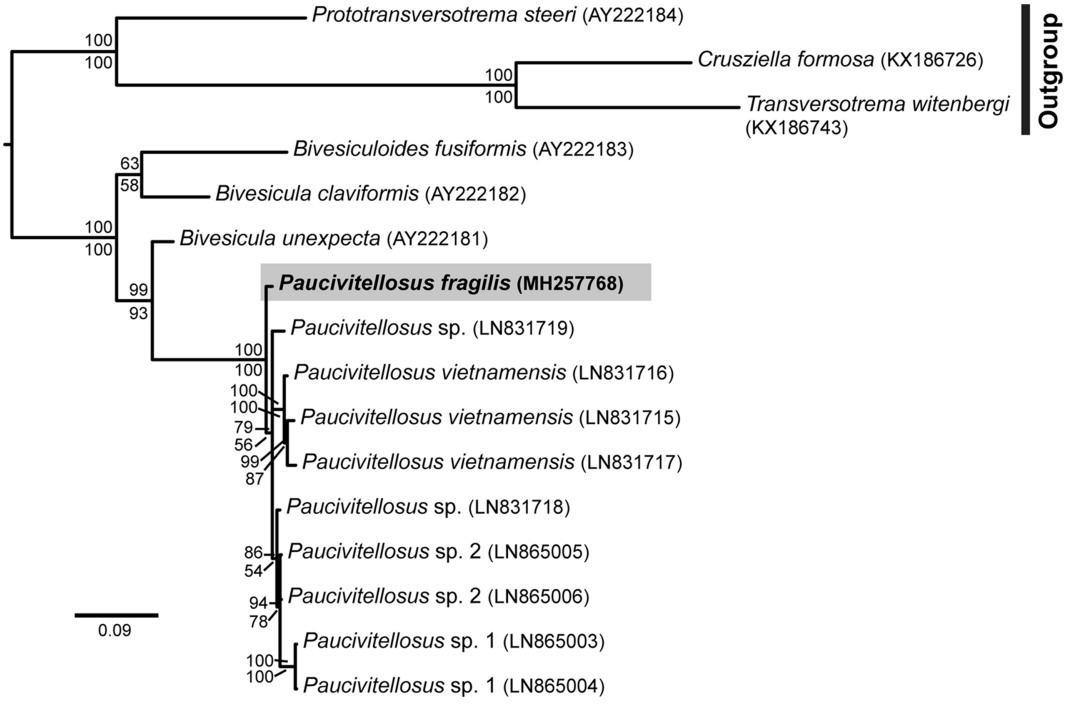
Prototransversotrema steeri (AY222184)
Crusziella formosa (KX186726)
Transversotrema witenbergi (KX186743)
Outgroup
Bivesiculoides fusiformis (AY222183)
Bivesicula claviformis (AY222182)
Bivesicula unexpecta (AY222181)
Paucivitellosus fragilis (MH257768)
Paucivitellosus sp. (LN831719)
Paucivitellosus vietnamensis (LN831716)
Paucivitellosus vietnamensis (LN831715)
Paucivitellosus vietnamensis (LN831717)
Paucivitellosus sp. (LN831718)
Paucivitellosus sp. 2 (LN865005)
Paucivitellosus sp. 2 (LN865006)
Paucivitellosus sp. 1 (LN865003)
Paucivitellosus sp. 1 (LN865004)
0.09

Figure 1

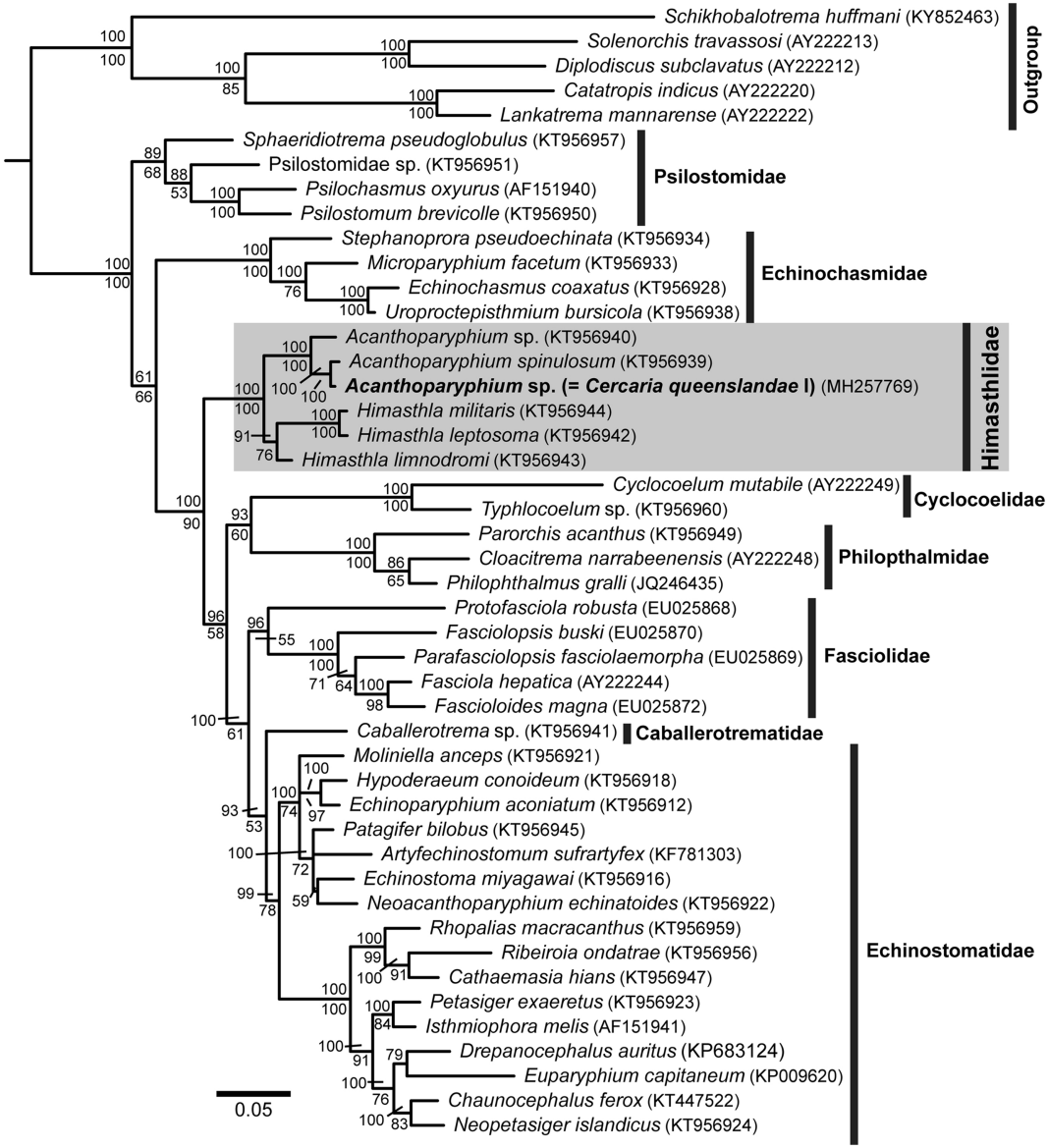
Schikhobalotrema huffmani (KY852463)
Solenorchis travassosi (AY222213)
Diplodiscus subclavatus (AY222212)
Catatropis indicus (AY222220)
Lankatrema mannarense (AY222222)
Outgroup
Sphaeridiotrema pseudoglobulus (KT956957)
Psilostomidae sp. (KT956951)
Psilochasmus oxyurus (AF151940)
Psilostomum brevicolle (KT956950)
Psilostomidae
Stephanoprora pseudoechinata (KT956934)
Microparyphium facetum (KT956933)
Echinochasmus coaxatus (KT956928)
Uroproctepisthmium bursicola (KT956938)
Echinochasmidae
Acanthoparyphium sp. (KT956940)
Acanthoparyphium spinulosum (KT956939)
Acanthoparyphium sp. (= Cercaria queenslandae I) (MH257769)
Himasthla militaris (KT956944)
Himasthla leptosoma (KT956942)
Himasthla limnodromi (KT956943)
Himasthlidae
Cyclocoelum mutabile (AY222249)
Typhlocoelum sp. (KT956960)
Cyclocoelidae
Parorchis acanthus (KT956949)
Cloacitrema narrabeenensis (AY222248)
Philophthalmus gralli (JQ246435)
Philopthalmidae
Protofasciola robusta (EU025868)
Fasciolopsis buski (EU025870)
Parafasciolopsis fasciolaemorpha (EU025869)
Fasciola hepatica (AY222244)
Fascioloides magna (EU025872)
Fasciolidae
Caballerotrema sp. (KT956941)
Caballerotrematidae
Moliniella anceps (KT956921)
Hypoderaeum conoideum (KT956918)
Echinoparyphium aconiatum (KT956912)
Patagifer bilobus (KT956945)
Artyfechinostomum sufrartyfex (KF781303)
Echinostoma miyagawai (KT956916)
Neoacanthoparyphium echinatoides (KT956922)
Rhopalias macracanthus (KT956959)
Ribeiroia ondatrae (KT956956)
Cathaemasia hians (KT956947)
Petasiger exaeretus (KT956923)
Isthmiophora melis (AF151941)
Drepanocephalus auritus (KP683124)
Euparyphium capitaneum (KP009620)
Chaunocephalus ferox (KT447522)
Neopetasiger islandicus (KT956924)
Echinostomatidae
0.05

Figure 2

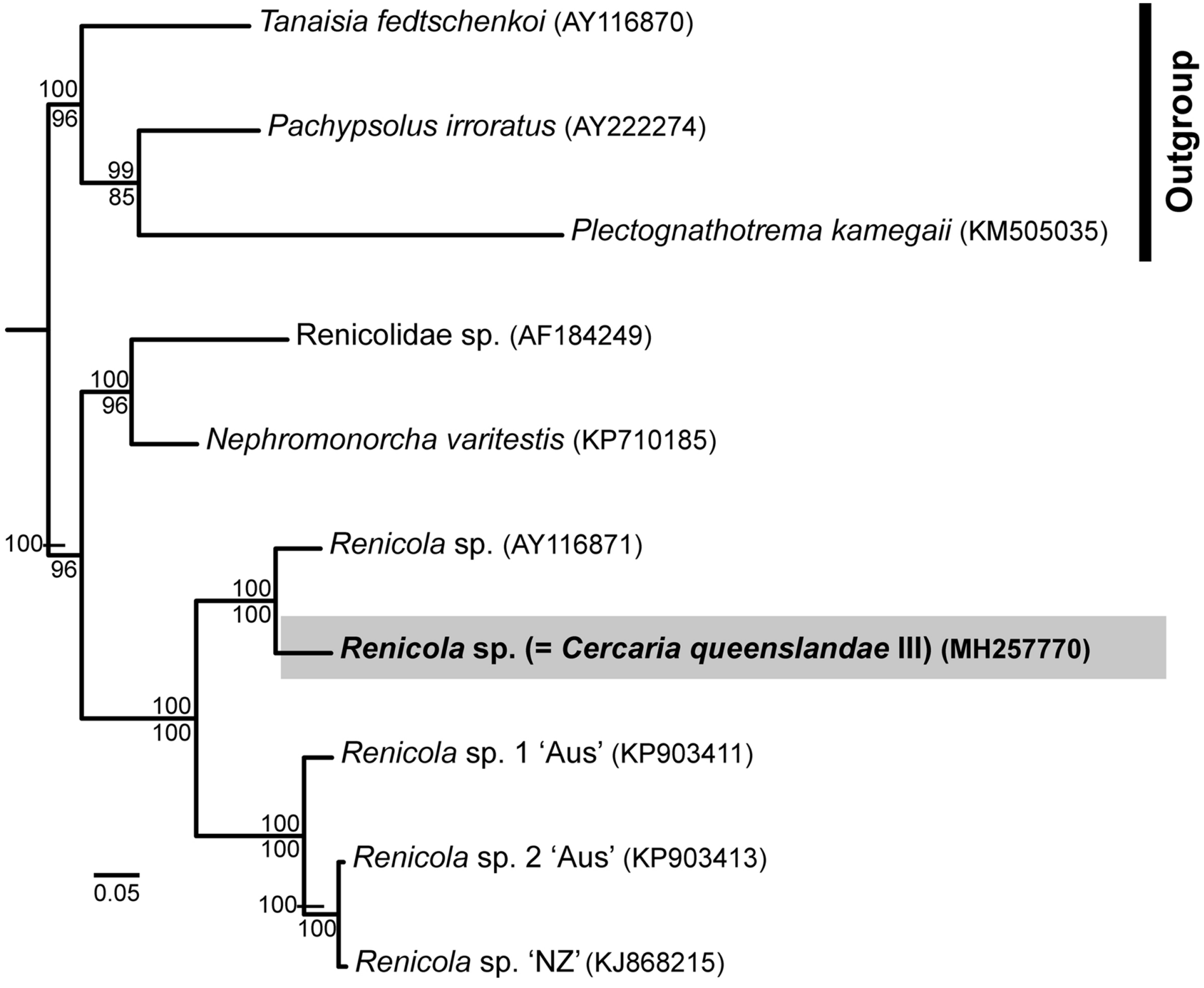
Tanaisia fedtschenkoi (AY116870)
Pachypsolus irroratus (AY222274)
Plectognathotrema kamegaii (KM505035)
Outgroup
Renicolidae sp. (AF184249)
Nephromonorcha varitestis (KP710185)
Renicola sp. (AY116871)
Renicola sp. (= Cercaria queenslandae III) (MH257770)
Renicola sp. 1 'Aus' (KP903411)
Renicola sp. 2 'Aus' (KP903413)
Renicola sp. 'NZ' (KJ868215)
0.05
100
96
99
85
100
96
100
96
100
100
100
100
100
100
100
100

Figure 3

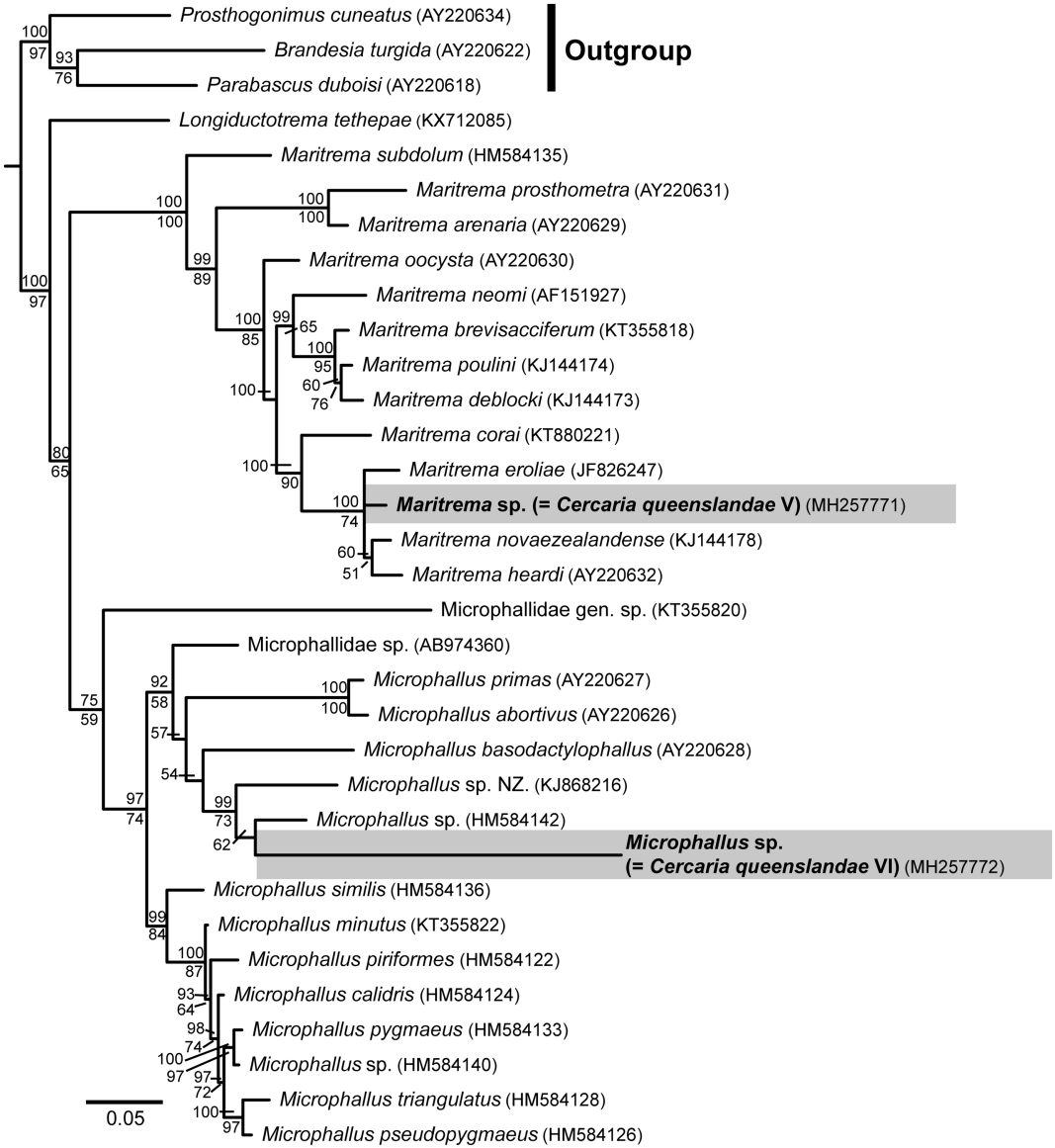
Prosthogonimus cuneatus (AY220634)
Brandesia turgida (AY220622)
Parabascus duboisi (AY220618)
Outgroup
Longiductotrema tethepae (KX712085)
Maritrema subdolum (HM584135)
Maritrema prosthometra (AY220631)
Maritrema arenaria (AY220629)
Maritrema oocysta (AY220630)
Maritrema neomi (AF151927)
Maritrema brevisacciferum (KT355818)
Maritrema poulini (KJ144174)
Maritrema deblocki (KJ144173)
Maritrema corai (KT880221)
Maritrema eroliae (JF826247)
Maritrema sp. (= Cercaria queenslandae V) (MH257771)
Maritrema novaezealandense (KJ144178)
Maritrema heardi (AY220632)
Microphallidae gen. sp. (KT355820)
Microphallidae sp. (AB974360)
Microphallus primas (AY220627)
Microphallus abortivus (AY220626)
Microphallus basodactylophallus (AY220628)
Microphallus sp. NZ. (KJ868216)
Microphallus sp. (HM584142)
Microphallus sp.
(= Cercaria queenslandae VI) (MH257772)
Microphallus similis (HM584136)
Microphallus minutus (KT355822)
Microphallus piriformes (HM584122)
Microphallus calidris (HM584124)
Microphallus pygmaeus (HM584133)
Microphallus sp. (HM584140)
Microphallus triangulatus (HM584128)
Microphallus pseudopygmaeus (HM584126)
0.05

Figure 4

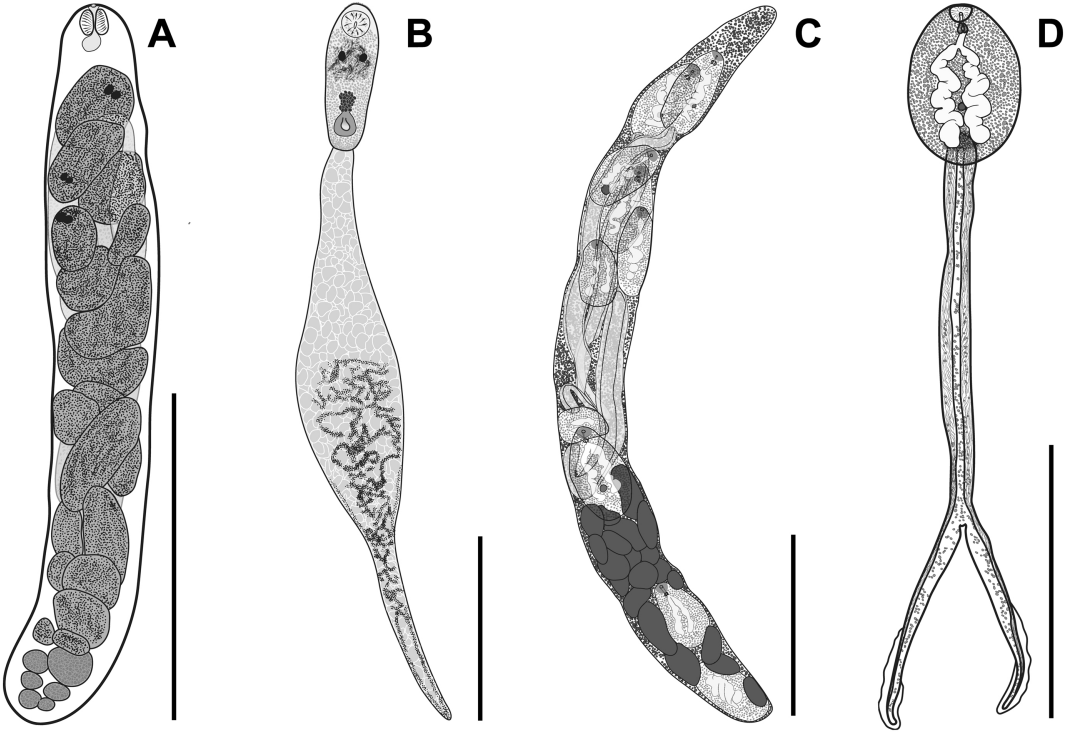

Figure 5

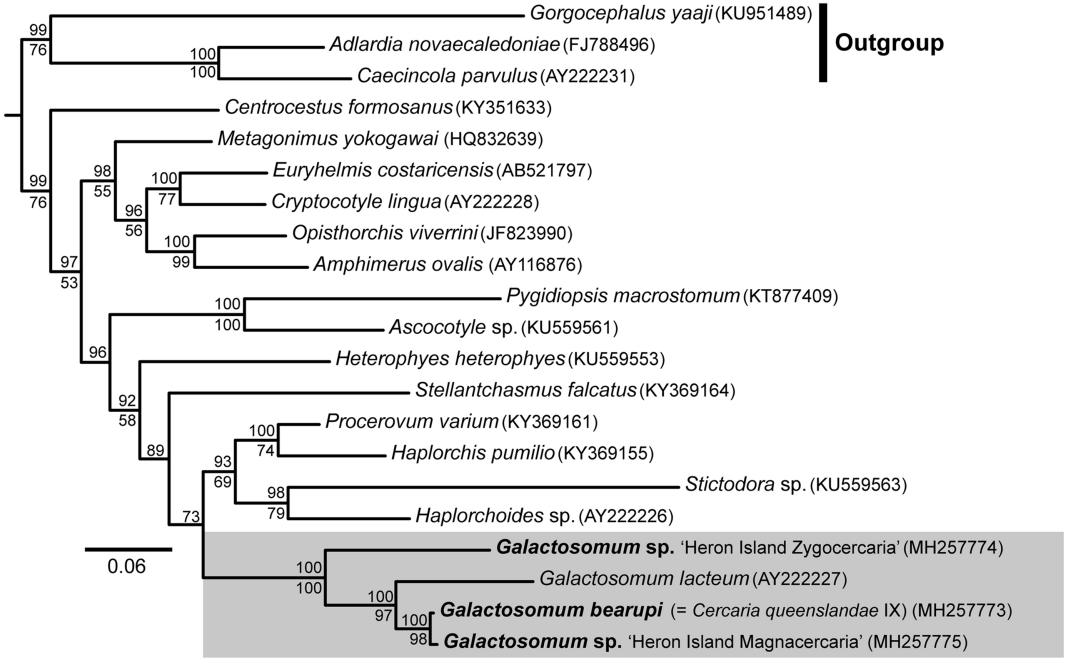
Gorgocephalus yaaji (KU951489)
Adlardia novaecaledoniae (FJ788496)
Caecincola parvulus (AY222231)
Outgroup
Centrocestus formosanus (KY351633)
Metagonimus yokogawai (HQ832639)
Euryhelmis costaricensis (AB521797)
Cryptocotyle lingua (AY222228)
Opisthorchis viverrini (JF823990)
Amphimerus ovalis (AY116876)
Pygidiopsis macrostomum (KT877409)
Ascocotyle sp. (KU559561)
Heterophyes heterophyes (KU559553)
Stellantchasmus falcatus (KY369164)
Procerovum varium (KY369161)
Haplorchis pumilio (KY369155)
Stictodora sp. (KU559563)
Haplorchoides sp. (AY222226)
0.06
Galactosomum sp. 'Heron Island Zygocercaria' (MH257774)
Galactosomum lacteum (AY222227)
Galactosomum bearupi (= Cercaria queenslandae IX) (MH257773)
Galactosomum sp. 'Heron Island Magnacercaria' (MH257775)

Figure 6

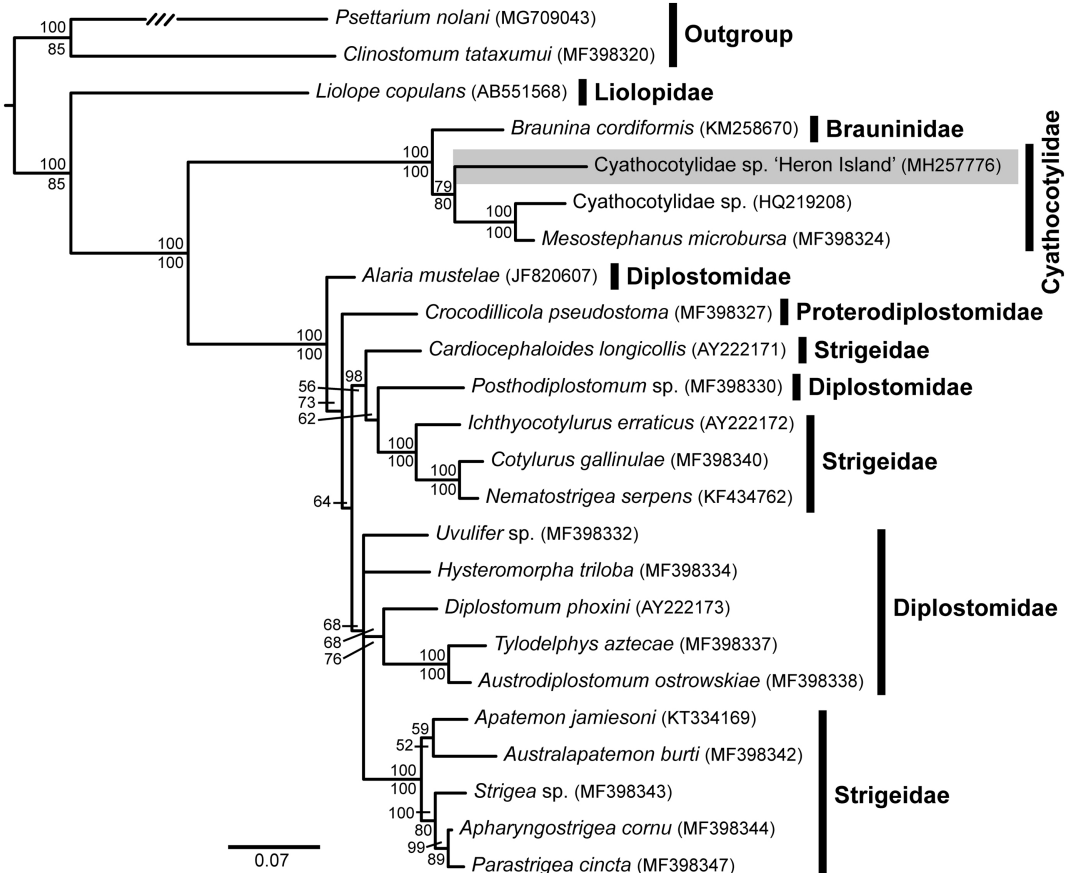
Psettarium nolani (MG709043)
Clinostomum tataxumui (MF398320)
Outgroup
Liolope copulans (AB551568)
Liolopidae
Braunina cordiformis (KM258670)
Brauninidae
Cyathocotylidae sp. 'Heron Island' (MH257776)
Cyathocotylidae sp. (HQ219208)
Mesostephanus microbursa (MF398324)
Cyathocotylidae
Alaria mustelae (JF820607)
Diplostomidae
Crocodillicola pseudostoma (MF398327)
Proterodiplostomidae
Cardiocephaloides longicollis (AY222171)
Strigeidae
Posthodiplostomum sp. (MF398330)
Diplostomidae
Ichthyocotylurus erraticus (AY222172)
Cotylurus gallinulae (MF398340)
Nematostrigea serpens (KF434762)
Strigeidae
Uvulifer sp. (MF398332)
Hysteromorpha triloba (MF398334)
Diplostomum phoxini (AY222173)
Tylodelphys aztecae (MF398337)
Austrodiplostomum ostrowskiae (MF398338)
Diplostomidae
Apatemon jamiesoni (KT334169)
Australapatemon burti (MF398342)
Strigea sp. (MF398343)
Apharyngostrigea cornu (MF398344)
Parastrigea cincta (MF398347)
Strigeidae
0.07

Figure 7