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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 and 2018 we collected 4870*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.

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

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(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* ($n = 4870$) 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 h to allow for cercarial emergence. Snail-wells were examined daily for emerged cercariae. Snails from which no cercariae had emerged within 48 h 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 mastermix 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 1000 bootstrap psuedoreplicates. Bayesian inference was performed using MrBayes v3.2.6 [32]. Four chains were sampled every 1000 generations for 10,000,000 generations with the first 2500 samples being discarded as burn-in, at which point average standard deviation of split frequencies were < 0.01 for all analyses.