

Digenean Metacercariae Parasitic in a Staurozoan Cnidarian

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I report digenean metacercariae from Staurozoa, which were not previously known as digenean hosts. The host species, *Haliclystus tenuis* Kishinouye, 1910, was collected from algae in Oshoro Bay, Hokkaido, Japan, and contained metacercariae in the mesoglea. The metacercariae were encysted; cysts were oval, 93 µm long by 64 µm wide in one live individual. For the digenean, I generated partial sequences for the 18S rRNA (1585 bp) and 28S rRNA (1672 bp) genes, and the region spanning the 3′ end of the cytochrome *c* oxidase subunit gene and the 5′ end of the 16S rRNA gene, including the threonine tRNA gene (868 bp in total). Phylogenetic reconstructions based on combined 18S+28S datasets showed the digenean to belong in Opecoelidae, members of which utilize marine or freshwater teleost fishes as definitive hosts, and placed it in Plagioporinae (sensu lato) clade C within Opecoelidae.

Key words: COI, fluke, intermediate host, Medusozoa, Platyhelminthes, Scyphozoa, stalked jellyfish, Stauromedusa, Trematoda

INTRODUCTION

It can be challenging to ascertain the intermediate host for a particular parasitic species, and this information is lacking for many parasites. This is especially the case for parasites utilizing unexpected taxa, where novel hosts are generally discovered only fortuitously (e.g., Escobar-Briones et al., 1999; Kakui, 2014; Kakui et al., 2021).

Digenea (Platyhelminthes: Trematoda), a group of endoparasitic metazoans with more than 12,000 described species (Littlewood et al., 2015), utilize second intermediate hosts from diverse animal groups (cf. Lefebvre and Poulin, 2005), and many unexpected host groups may remain undiscovered. In digenean intermediate host surveys, Cnidaria (e.g., corals, jellyfishes, and sea anemones) had not received much attention (Browne et al., 2020). Recent studies revealed that more digeneans utilize cnidarians as their intermediate hosts than expected (e.g., Martin et al., 2018b; Browne et al., 2020), but still many cnidarian groups remain untouched.

Staurozoa (Cnidaria: Medusozoa) is a group of sessile “jellyfishes”, members of which remain attached by a stalk to the substratum throughout their life (Mills and Hirano, 2007). Fifty species have been reported worldwide (Miranda et al., 2018). Staurozoans prey on small crustaceans and in turn are consumed by pycnogonids (sea spiders), nudibranch mollusks (sea slugs), and fishes (Miranda et al., 2018). To my knowledge, there have been no reports of organisms parasitic on staurozoans.

Here I report the first case of a digenean parasitic in a staurozoan, in this case *Haliclystus tenuis* Kishinouye, 1910. Because the parasites I found were encysted metacercariae, I attempted to identify them using a molecular phylogenetic approach, determining partial sequences for the 18S rRNA (18S) and 28S rRNA (28S) genes, and the region from the 3′ end of cytochrome *c* oxidase subunit 1 gene to the 5′ end of the 16S rRNA gene, including the threonine tRNA gene (here termed the “COI cluster”).

MATERIALS AND METHODS

One infected *Haliclystus tenuis* individual was collected among algae in the intertidal zone at Oshoro, Hokkaido Island, Japan (43°12′33.5″N 140°51′34.3″E) on 19 July 2021. After use in a feeding experiment (Kakui, in press), the staurozoan host was anesthetized with menthol, dissected to extract parasites, and fixed and preserved in 99% ethanol on 15 August 2021. A total of 10 parasites were extracted, all of which were put in 30% ethanol for ca. 15 min, transferred to 50% ethanol (ca. 15 min), and finally to 70% (for morphology) or 99% ethanol (for DNA extraction). Before fixation, one of the parasites was observed live with an Olympus BX51 microscope and photographed, and its length and width were measured from a digital image by using ImageJ (Rasband, 2021).

Five of six parasites fixed for morphological observation were transferred into a 1:9 mixture of glycerin and 70% ethanol and placed in a thermostatic chamber at 40°C for 4 h, after which they were mounted on glass slides in glycerin and observed with an Olympus BX51 microscope. The other was kept intact and preserved in 70% ethanol for future study. The material studied was deposited in the Invertebrate Collection of the Hokkaido University Museum (ICHUM), Sapporo under catalog number ICHUM6297.

An attempt was made to extract total DNA from the whole body of four parasites by using a NucleoSpin Tissue XS Kit (Macherey-Nagel, Germany), but only one of the four extracts allowed successful PCR amplification (see below). Primers used for PCR and sequencing are listed in Table 1. PCR amplification conditions for the COI cluster, 18S, and 28S with KOD One polymerase (Toyobo, Japan) were 45 cycles of 98°C for 10 s, 51°C (COI cluster) or 60°C (18S and 28S) for 5 s, and 68°C for 1 s (COI cluster) or 5 s (18S and 28S). Nucleotide sequences were determined by direct sequencing with a Big Dye Terminator Kit ver. 3.1 and a 3730 DNA Analyzer (Life Technologies, USA). Sequencing fragments were concatenated by using MEGA7 (Kumar et al., 2016). Final sequences were

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Table 1. List of PCR and cycle sequencing (CS) primers used in this study.

Gene	Primer	Sequence	Reaction	Source
COI cluster	JB3	TTTTTTGGGCATCCTGAGGTTTAT	PCR, CS	Bowles et al. (1993)
	cox1rrnL	AATCATGATGCAAAAGGTA	PCR, CS	Králová-Hromadová et al. (2008)
18S	SR1	TACCTGGTTGATCCTGCCAG	PCR	Nakayama et al. (1996)
	SR3	AGGCTCCCTGTCCGGAATC	CS	Nakayama et al. (1996)
	SR12	CCTTCCGCAGGTTCACCTAC	PCR	Nakayama et al. (1996)
	18S-b3F	CCTGAGAAACGGCTACCACAT	CS	Kakui & Shimada (2017)
	18S-b4F	TGCGGTTAAAAAGCTCGTAGTTG	CS	Kakui et al. (2011)
	18S-b4R	TCCAACTACGAGCTTTTTAACC	CS	Kakui et al. (2011)
	18S-b5F	GATCGAAGGCGATYAGATACC	CS	Kakui et al. (2021)
	18S-b6F	CCTGCGGCTTAATTTGACTC	CS	Kakui et al. (2011)
	18S-a6R	AACGGCCATGCACCAC	CS	Kakui et al. (2011)
	18S-b8F	GGTCTGTGATGCCCTTAGATG	CS	Kakui et al. (2011)
28S	U178	GCACCCGCTGAAYTTAAG	PCR, CS	Lockyer et al. (2003)
	300F	CAAGTACCGTGAGGGAAAGTTG	CS	Lockyer et al. (2003)
	300R	CAACTTTCCCTCACGGTACTTG	CS	Lockyer et al. (2003)
	U1148	GACCCGAAAGATGGTGAA	CS	Lockyer et al. (2003)
	L1642	CCAGCGCCATCCATTTTCA	PCR, CS	Lockyer et al. (2003)

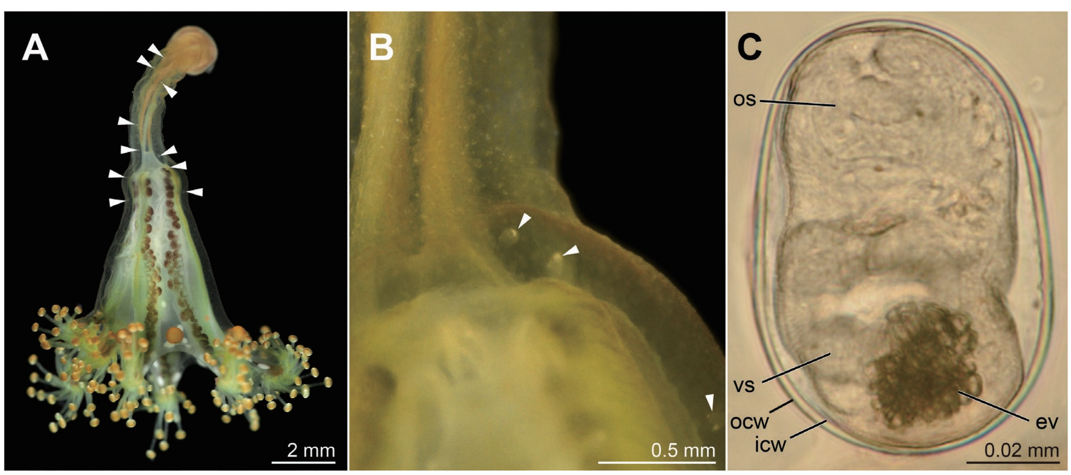

Fig. 1. *Haliclystus tenuis* parasitized by digeneans. **(A)** habitus of *H. tenuis*, living animal. **(B)** base of calyx of *H. tenuis*, living animal. **(C)** encysted metacercaria extracted from *H. tenuis*, living animal, light microscopic image. Arrowheads, metacercariae in mesoglea. Abbreviations: ev, excretory vesicle; icw, inner cyst wall; ocw, outer cyst wall; os, oral sucker; vs, ventral sucker.

deposited in the International Nucleotide Sequence Database (INSD) through the DNA Data Bank of Japan.

The result of BLAST (Altschul et al., 1990) for our 18S sequence indicated that the parasite was a member of the digenean family Opecoelidae, and so a combined 18S+28S dataset that included sequences from 106 opecoelids and five outgroup taxa (see Supplementary Table S1) was analyzed by maximum likelihood (ML) to place the parasite into a subfamily or clade within Opecoelidae. To construct the 18S+28S nucleotide dataset, the 18S and 28S data were first aligned independently by using the "Q-INS-i" strategy (Katoh and Toh, 2008) in MAFFT ver. 7 (Katoh and Standley, 2013) and then trimmed with MEGA7 to match the shortest length for each gene (1578 aligned positions for 18S; 1002 for 28S). The optimal substitution model for both genes was GTR+I+G, determined under the corrected Akaike information criterion (AICc) with PartitionFinder 2.1.1 (Lanfear et al., 2017) using a greedy algorithm (Lanfear et al., 2012). An ML analysis of the 18S+28S dataset was conducted by using RAxML v.8.2.10 (Stamatakis, 2014), with nodal support values obtained by analysis of 1000 bootstrap pseudoreplicates. The ML tree was drawn by using FigTree v1.4.4 (Rambaut, 2021). The subfamily/clade nomenclature follows Martin (2020a, b) and Sokolov et al. (2020).

RESULTS

More than 10 metacercariae were observed in the mesoglea of the host staurozoan (Fig. 1A, B). In one living metacercaria, the oral and ventral suckers, excretory vesicles, and inner and outer cyst walls (Fig. 1C) were evident; the cyst was 93 μm long and 64 μm wide. In four ethanol-fixed metacercariae, the oral and ventral suckers and inner and outer cyst walls were evident.

Sequences for the COI cluster (868 bp; INSD accession number LC651419) and parts of the 18S (1585 bp; LC651417) and 28S (1672 bp; LC651418) genes were determined from

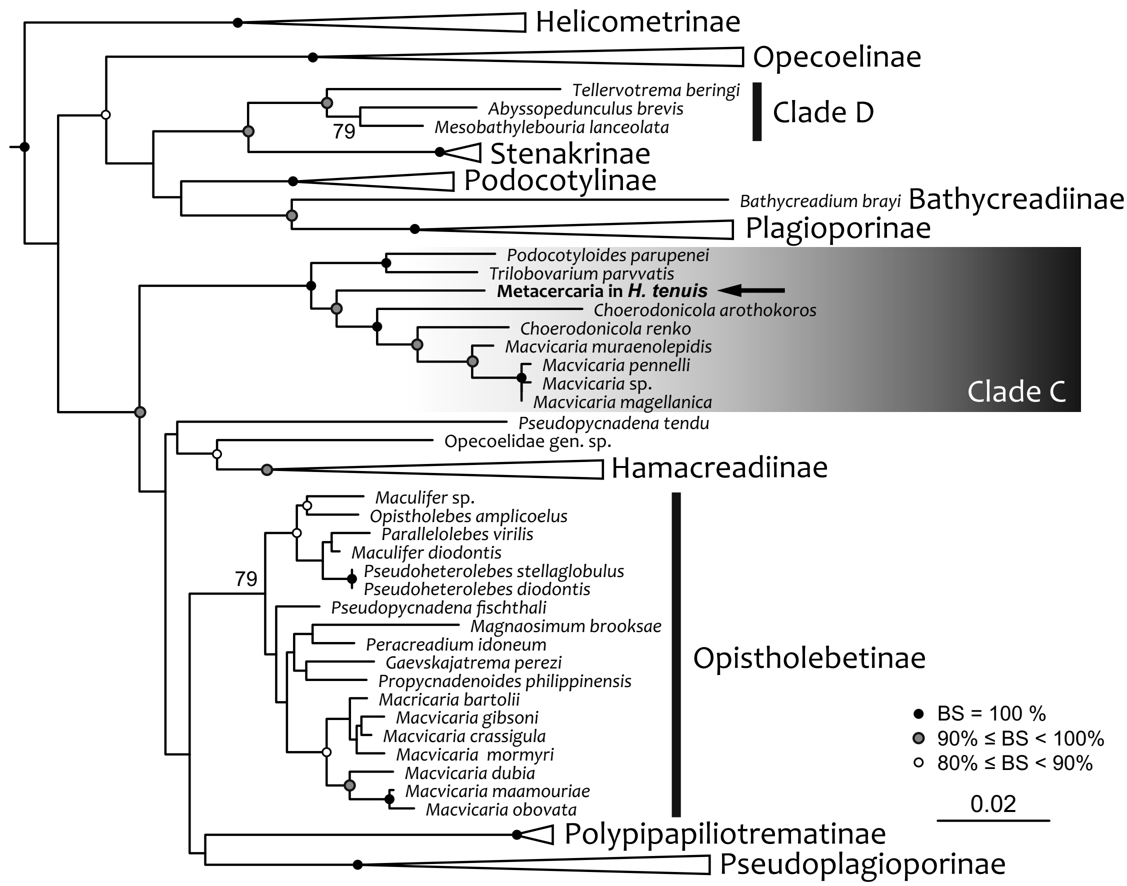

Fig. 2. ML tree for 18S+28S sequences (2580 positions) from digeneans, including the metacercaria from *Haliclystus tenuis*. Bootstrap values higher than 75% and lower than 80% given near nodes; those < 75% not shown. Outgroup taxa are not shown. Arrow indicates position of metacercaria from *Haliclystus tenuis*. Scale at bottom of the tree indicates branch length in substitutions per site.

a single metacercaria. In a BLAST search, the COI-cluster sequence most similar to the one I determined was from Opecoelidae sp. C (FJ765495; identity score 81.13%, query cover 100%), extracted from the first intermediate gastropod host *Diloma aethiops* in Otago Harbor, New Zealand (Leung et al., 2009).

In the ML tree based on the 18S+28S dataset (Fig. 2), the metacercaria lies in “Plagioporinae (sensu lato) clade C” sensu Martin et al. (2018a) with 100% bootstrap support.

DISCUSSION

My detection of encysted metacercariae in *Haliclystus tenuis* is the first example of a digenean utilizing a staurozoan as the second intermediate host, and also the first discovery of any parasite in staurozoans. The 18S+28S phylogenetic analysis strongly indicates that the digenean parasite is a member of Plagioporinae (sensu lato) clade C within Opecoelidae. Opecoelidae is the most species-rich digenean family and utilizes a highly diverse second intermediate host group, including scleractinian corals, crustaceans, aquatic insects, triclad flatworms, clitellate annelids (oligochaetes and leeches), gastropods, cephalopods, echinoids, and teleost and elasmobranch fishes (Martin et al., 2020b). Here I add staurozoans to this list.

Opecoelids utilize freshwater and marine teleost fishes as their definitive hosts (Martin et al., 2020b). There is one previous report of predation on staurozoans by teleost fishes, although this has been considered an instance of bycatch (Miranda et al., 2018). Information regarding predators on *H. tenuis* is lacking. The finding of opecoelids in *H. tenuis* indicates that certain marine teleosts may consume this staurozoan species as a diet item and be the definitive host for the opecoelid species I found.

Martin et al. (2020b) suggested that significant second intermediate host-switch events may have driven the evolution of (some) major lineages within Opecoelidae. For instance, most known opecoelid life cycles involve crustaceans as second intermediate hosts, but some species utilize corals for this role, all of which are contained in the subfamily Polypipapiliotrematinae (Martin et al., 2018b). *Haliclystus tenuis* is currently the sole example of second intermediate hosts for taxa in Plagioporinae (sensu lato) clade C (cf. Martin et al., 2020b). More data from other members in this clade are needed, but Plagioporinae (sensu lato) clade C may be the lineage defined by the use of staurozoans as their second intermediate host.

I fortuitously discovered the digenean parasite while observing *H. tenuis* during a feeding experiment (Kakui, in press). This underscores that contributions by non-parasitologists, like this study, can help elucidate the diversity of second intermediate host groups, many of which may remain undetected, for various parasite groups.

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COMPETING INTERESTS

I declare no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs210099)

Supplementary Table S1. Species included in 18S+28S dataset to infer the phylogenetic position within Opecoelidae of the metacercaria parasitic in *H. tenuis*.

Supplementary File S1. Aligned dataset used for phylogeny reconstruction (positions 1–1578, 18S rRNA gene; 1579–2580, 28S rRNA gene).

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