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Overlooked Parasite Diversity in Staurozoa: Two Species of Lepocreadiidae (Trematoda: Digenea) Parasitic in *Haliclystus tenuis*

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Running header: New parasite records from Staurozoa

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Abstract

Three non-encysted digenean metacercariae were found parasitic in the mesoglea of two of 13 individuals of the staurozoan cnidarian *Haliclystus tenuis* Kishinouye, 1910 from Yoichi, Hokkaido, Japan. The metacercariae comprised two morphospecies (one oval, the other elongate) for which the gross morphology is described. Partial sequences of the 18S and 28S rRNA genes and the internal transcribed spacer 2 region were determined for both species, and a partial sequence of the cytochrome *c* oxidase subunit I gene was determined for the oval species. Molecular phylogenetic analyses revealed both digeneans to be in the family Lepocreadiidae, a taxon not previously reported from staurozoans. The oval species was a member of the "*Diploproctodaeum* Clade." The elongate species was identified as *Prodistomum orientale* (Layman, 1930), known to use marine fishes in the genus *Scomber* Linnaeus, 1758 as definitive hosts. Second intermediate hosts of *P. orientale* known around Australia include five hydrozoan, one scyphozoan (Cnidaria), and one ctenophoran species. This is the first information on a second intermediate host from Japan; however, we cannot rule out the possibility that staurozoans may also be dead-end hosts. Including an opecoelid species previously reported, three digenean species in two families are now known from a single staurozoan species in the small region comprising the northwestern coast of Hokkaido. Future surveys for parasites in other staurozoan species and regions will likely detect additional digeneans and other parasite groups utilizing staurozoans.

Key words: Cnidaria, gelatinous animal, life cycle, parasite, Platyhelminthes, stalked jellyfish, Stauromedusa

INTRODUCTION

Gelatinous animals such as cnidarians and ctenophorans were traditionally considered as a “trophic dead end” or “survival food” in marine food webs (Verity and Smetacek, 1996; Ruiz et al., 2024). Recent research, however, has revealed that they are more frequently consumed than previously thought by a variety of organisms, underscoring their importance as a food source (Arai, 2005; Hays et al., 2018). This has drawn increased attention to the role of these groups as hosts for parasites, a topic actively investigated in recent years (e.g., D’Ambra and Graham, 2009; Riascos et al., 2012; Killi and Mariottini, 2018; Browne et al., 2020).

Digenea (Platyhelminthes: Trematoda) is one of the most actively studied groups parasitic in Cnidaria. Digeneans typically utilize three host species in their life cycle: a first intermediate host (typically gastropod), a second intermediate host (a wide range of invertebrate and vertebrate), and a definitive host (vertebrate). Cnidarians and ctenophorans have previously been reported as second intermediate hosts for digeneans, though most previous studies dealt with hydrozoan cnidarians or ctenophorans (Nogueira et al., 2015).

Staurozoans, or stalked jellyfishes, are sessile cnidarians. Their body is composed of calyx and peduncle, the latter of which is used to attach to substrates such as algae, seagrasses, and rocks (Miranda et al., 2018). Epiphytic staurozoans can occur offshore, attached to drifting seaweeds (Tsutsui, 2024). Little is known about their role in the ecosystem. Staurozoans consume arthropods (mainly small crustaceans), gastropods,

annelids, and sessile ctenophores (Zagal, 2004; Falconer, 2013). Reports of predation on staurozoans are quite limited, with sea spiders, sea slugs, fish, sea anemones, and scyphozoan polyps known as consumers (Miranda et al., 2018; Sun et al., 2021; Wells et al., 2021). That regenerating and injured individuals are commonly found in the field, however, suggests that grazing predation occurs more frequently than has been reported, possibly by sea slugs (Mills and Hirano, 2007). Recent metabarcoding approaches have frequently detected staurozoan prey in fish stomachs (e.g., Ruiz et al., 2024), suggesting staurozoans are not uncommonly eaten by fishes. Staurozoans are thus likely utilized as intermediate hosts for various parasites, but the sole report to date of a parasite in a staurozoan (Kakui, 2022) was the digenean Opecoelidae sp. in *Haliclystus tenuis* Kishinouye, 1910.

Here we report additional parasites in a staurozoan, non-encysted digenean metacercariae in *H. tenuis* from Hokkaido, northern Japan. Our sample contained two morphospecies: an oval species (hereafter, "Parasite O") and an elongate species (hereafter, "Parasite E"). Here we describe their gross morphology and the results of molecular phylogenetic analyses by which we attempted to identify them.

MATERIALS AND METHODS

Thirteen *Haliclystus tenuis* individuals were collected from *Sargassum* sp. at 0−1 m depth in Yoichi, Hokkaido, Japan (43.242562°N, 140.726170°E) on 15 June 2024. They were

examined for parasite infection with an Olympus TG-7 set to microscope mode. Infected individuals were photographed alive. Parasites were extracted from the living hosts by using sharpened needles, observed live and photographed under an Olympus BX53 microscope, and fixed in 99% ethanol. Before extraction, the ethanol-fixed parasites were observed and photographed under an Olympus BX53 microscope. Measurements of the length of fixed parasites were made from digital images by using ImageJ (Schneider et al., 2012).

DNA was extracted from the whole body of Parasite O and Parasite E by using a NucleoSpin Tissue XS Kit (Macherey–Nagel, Germany). Table 1 lists the primers used for PCR and cycle sequencing in the 18S rRNA (18S), 28S rRNA (28S), cytochrome *c* oxidase subunit I (COI) genes, and the region containing the 3′ part of 5.8S rRNA (5.8S), internal transcribed spacer 2 (ITS2), and the 5′ part of 28S (hereafter, "ITS2 cluster"). PCR amplification conditions for 18S and 28S with KOD ONE PCR Master Mix (Toyobo, Japan) were 45 cycles of 98°C for 10 s, 60°C for 5 s, and 68°C for 10 s. PCR amplification conditions for the ITS2 cluster and COI with TaKaRa Ex Taq polymerase (Takara Bio, Japan) were 94°C for 1 min; 35 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 30 s (ITS2 cluster) or 40 s (COI); and 72°C for 2 min. All nucleotide sequences were determined with a BigDye Terminator Kit ver. 3.1 and a 3730 DNA Analyzer (Life Technologies, USA). Fragments were concatenated by using MEGA7 (Kumar et al., 2016). The sequences we generated were deposited in the International Nucleotide Sequence Database (INSD) through

the DNA Data Bank of Japan.

The 28S dataset for phylogenetic analysis included the two sequences we generated, and 64 lepocreadiid sequences and four outgroup sequences taken from the INSD; the dataset for ITS2 included one sequence we generated, and 51 *Lepocreadium*-clade sequences and four *Diploproctodaeum*-clade sequences taken from the INSD. The datasets were aligned independently by using the online version of MAFFT ver. 7 (Katoh and Standley, 2013; Katoh et al., 2019) with the "Q-INS-i" strategy (for 28S; Katoh and Toh, 2008) or "Auto" strategy (for ITS2; "L-INS-i" selected; Katoh et al., 2005) and then trimmed with MEGA7 to the shortest length among them. The regions corresponding to 5.8S and 28S were removed from the ITS2 dataset based on positional information from the sequence with the INSD accession number KU527433 (https://www.ncbi.nlm.nih.gov/nuccore/KU527433; Claxton et al., 2017). Alignment-ambiguous sites were removed with Gblocks ver. 0.91b (Castresana, 2000) in NGPhylogeny.fr (Lemoine et al., 2019), under the "relaxed" parameters described in Talavera and Castresana (2007). The aligned datasets contained 1177 (28S) and 289 (ITS2) characters, respectively (Supplementary Files 1−5). Methods for selecting the optimal substitution models (GTR + F + R3 for 28S; TVM + F + G4 for ITS2), the maximum likelihood (ML) analyses, estimation of nodal support (analyses of 1000 pseudoreplicates for both Shimodaira–Hasegawa-like approximate likelihood ratio tests [SH-aLRT] and ultrafast bootstraps [UFBoot]), and drawing the trees were as described by Shimada et al. (2023).

RESULTS

Two of 13 staurozoans collected were infected. One with eight arms contained one Parasite O individual in the mesoglea of the peduncle (Fig. 1A, C). The other with 15 arms (an abnormal number) contained one Parasite E individual each in the mesoglea of the calyx and peduncle (Fig. 1B, D, E); the parasite in the peduncle was lost.

All the found digeneans were non-encysted metacercariae. Parasite O was oval (Figs. 1C, 2A, B; Supplementary Movies S1, S2); the body length of the fixed specimen was 198 μm. Parasite E was elongate (Figs. 1D, E, 2C, D; Supplementary Movies S3, S4); the body length of the fixed specimen was 273 μm. In both Parasites O and E, oral sucker, ventral sucker, pigmented eyespots, and pharynx were observed (Fig. 2B, D).

The following partial sequences were determined from the two morphospecies: for 18S, INSD accession number LC850121 (1605 bp, Parasite O) and LC850120 (1605 bp, Parasite E); for 28S, LC850123 (1692 bp, Parasite O) and LC850122 (1691 bp, Parasite E); and for the ITS2 cluster, LC850125 (465 bp, Parasite O) and LC850124 (463 bp, Parasite E). A partial COI sequence (LC850126; 482 bp, encoding 160 amino acids) was determined from Parasite O. The most similar previously known sequences to ours, as determined by BLAST searches (Altschul et al., 1990), are summarized in Table 2.

In the optimal ML tree based on 28S sequences (Fig. 3), Parasite O lies within a

highly supported (SH-aLRT/UFBoot = 98.1%/97%) "*Diploproctodaeum* Clade" (Cribb et al., 2024) and is the sister group to a clade comprising *Echeneidocoelium indicum* Simha and Pershad, 1964 and *Deraiotrema platacis* Machida, 1982, though with low nodal support (89.6%/75%). In the 28S tree, Parasite E belongs to an unsupported (< 65%/< 70%) "*Lepocreadium* Clade" (Cribb et al., 2024) and forms a highly supported (99.5%/100%) clade with *Prodistomum* sp. Type 3, *Prodistomum orientale* (Layman, 1930) and *Opechonoides opisthoporus* Duong, Cutmore, Cribb, Pitt, Wee, and Bray, 2022. In the optimal ML tree based on ITS2 sequences (Fig. 4), Parasite E lies in a largely unresolved clade (89.9%/95%) containing *Prodistomum* sp. Type 3 and Type unknown, Lepocreadiidae sp. 3, *P. orientale*, and *O. opisthoporus*.

DISCUSSION

We found non-encysted metacercariae of two digenean species previously unknown in staurozoans. Our molecular data suggest both of them belong in Lepocreadiidae, species of which use fish as definitive hosts (Bray et al., 2018a).

Parasite O was identified molecularly as a member of the *Diploproctodaeum* Clade in Lepocreadiidae. Many lepocreadiid species not yet sequenced have been reported from Japan (e.g., Goto and Ozaki, 1929; Machida and Uchida, 1987). Parasite O may correspond to one of those species. Parasite O represents the first molecularly confirmed *Diploproctodaeum*-

Clade species that utilizes gelatinous animals as a second intermediate host (Cribb et al., 2024; but see also Kondo et al., 2016). The two-host life cycle containing cercariae encysting as metacercariae on algae, which was reported in *Diploproctodaeum arothroni* Bray and Nahhas, 1998 by Hassanine (2006), is suggested to be common in this clade (e.g., Bray et al., 2009; Huston et al., 2025); however, our present finding indicates that this clade contains species having a three-host life cycle.

Our 28S and ITS2 phylogenies and the BLAST results indicated that Parasite E is *Prodistomum orientale* in the *Lepocreadium* Clade. *Prodistomum orientale* uses the chub mackerel (*Scomber japonicus* Houttuyn, 1782) and blue mackerel (*Scomber australasicus* Cuvier, 1832) as definitive hosts, occurs in Japan and Australia, and contains two lineages (Types 1 and 2) (Cribb et al., 2024). The two lineages differ slightly in COI sequence and adult morphology, and currently show allopatric distributions, Type 1 in Australia and Type 2 in Japan. Unfortunately, we failed to determine a COI sequence for Parasite E, but based on its sampling locality, Parasite E is likely the Type-2 lineage of *P. orientale*.

Investigations around Australia have found the second intermediate hosts of *P. orientale* to include seven species of cnidarians and ctenophorans: five hydrozoan cnidarians—*Aequorea* sp.; *Aldersladia magnificus* Gershwin, 2006; *Geryonia proboscidalis* (Forsskål, 1775); and two unidentified species—, one scyphozoan cnidarian *Chrysaora pentastoma* Péron and Lesueur, 1810, and one ctenophoran *Pukia falcata* Gershwin, Zeidler,

and Davie, 2010. Our discovery of *P. orientale* metacercariae (probably the Type-2 lineage) is the first information on a second intermediate host around Japan and the first known occurrence of any lepocreadiid parasitic in a staurozoan. Given the broad taxonomic range of the second intermediate hosts reported around Australia (Cribb et al., 2024), *P. orientale* around Japan highly likely infects various cnidarian and ctenophoran groups in addition to staurozoans.

There has been only one record of fish predation on staurozoans. Davenport (1998) found *Haliclystus antarcticus* Pfeffer, 1889 in stomachs of the rockcod *Notothenia rossii* Richardson, 1844. The paucity of observations of predation by fishes may simply reflect the fact that gelatinous animals are rapidly digested (Arai, 2005) and thus rarely observable in fish stomachs (Hays et al., 2018; Brodeur et al., 2021; Ruiz et al., 2024). This is supported by recent DNA metabarcoding studies; Ruiz et al. (2024), for example, obtained staurozoan (*H. antarcticus*) sequences from the stomach contents of several individuals of *N. rossii* and *Notothenia coriiceps* Richardson, 1844 and suggested that staurozoans are a relatively common fish prey. Fishes of *Scomber* Linnaeus, 1758 have not been reported to consume staurozoans, but as they frequently prey on jellyfish such as scyphozoan medusae, hydrozoan medusae, and siphonophores (e.g., Takano, 1954; Lamb et al., 2019; Sasaki et al., 2023), they may also prey on infected staurozoans on drifting seaweeds and become parasitized. However, we cannot rule out the possibility that staurozoans are not the regular hosts for the

digeneans. Considering that the host-specificity of metacercariae is often low in *Lepocreadium* Clade (Cribb et al., 2024), staurozoans may also be dead-end hosts (i.e., not eaten by the definitive hosts).

The two lepocreadiid species reported here and one opecoelid species reported by Kakui (2022) were discovered in *H. tenuis* from a small area in Hokkaido, Japan. This degree of digenean diversity detected from limited samples suggests that more digenean species utilize staurozoans than was previously thought, and that the importance of staurozoans as second intermediate hosts has been underestimated. Future surveys of parasites in other staurozoan species and regions will likely detect additional digeneans and other parasitic groups utilizing staurozoans.

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COMPETING INTERESTS

We declare no competing interests.

AUTHOR CONTRIBUTIONS

KT collected and observed the staurozoans. KK conducted the molecular analysis.

KT and KK conceived and designed the study, observed the morphology of the digeneans, wrote the manuscript, and read and approved the final draft.

Supplementary materials

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

Supplementary File S1. Aligned digenean 28S sequences, before trimming to the shortest length among them and the removal alignment-ambiguous sites.

Supplementary File S2. Aligned digenean 28S sequences used for the maximum-likelihood analysis, after trimming in MEGA7 to the shortest length among them and removing alignment-ambiguous sites with Gblocks under "relaxed" parameters.

Supplementary File S3. Aligned digenean sequences of the 5.8S−ITS2−28S region (ITS2 cluster), before trimming to the shortest length among them and the removing alignment-ambiguous sites.

Supplementary File S4. Aligned digenean ITS2 sequences, before the removing alignment-ambiguous sites.

Supplementary File S5. Aligned digenean ITS2 sequences used for the maximum-likelihood analysis, after the removing alignment-ambiguous sites with Gblocks under "relaxed" parameters.

Supplementary Movie S1. Fresh Parasite O individual observed with light microscopy.

Supplementary Movie S2. Ethanol-fixed Parasite O individual observed with light microscopy.

Supplementary Movie S3. Fresh Parasite E individual observed with light microscopy.

Supplementary Movie S4. Ethanol-fixed Parasite E individual observed with light microscopy.

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FIGURE LEGENDS, AND TABLE AND SUPPLEMENTARY MATERIAL CAPTIONS

Fig. 1. *Haliclystus tenuis* parasitized by digeneans, living animals. **(A)** Parasite O (arrowhead) in a host with eight arms. **(B)** Parasite E (arrowheads) in a host with 15 arms. **(C)** Parasite O in the peduncle. **(D, E)** Parasite E in the calyx (D) and peduncle (E). Scale not available for (C−E).

Fig. 2. Digenean metacercariae extracted from *Haliclystus tenuis*, light microscopic images. **(A, B)** Parasite O, living (A) and fixed (B) individual. **(C, D)** Parasite E, living (C) and fixed (D) individual. Abbreviations: ep, pigmented eyespot; os, oral sucker; ph, pharynx; vs, ventral sucker.

Fig. 3. ML tree for 28S sequences (1177 characters) from digeneans, including the metacercariae from Parasites O and E (bold font) found in *Haliclystus tenuis*. Numbers near nodes are Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT; left of slash) and ultrafast bootstrap (UFBoot; right of slash) values as percentages; only values of SH-aLRT ≥65% and UFBoot ≥70% are shown. Scale at bottom indicates branch length in substitutions per site.

Fig. 4. ML tree for ITS2 sequences (289 characters) from digeneans, including the metacercaria from Parasite E (bold font) found in *Haliclystus tenuis*. Numbers near nodes are Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-aLRT; left of slash) and ultrafast bootstrap (UFBoot; right of slash) values as percentages; only values of SH-aLRT

≥65% and UFBoot ≥70% are shown. Scale at bottom indicates branch length in substitutions per site. Shading indicates sequences from *Prodistomum orientale* (dark gray, Type 1; light gray, Type 2).

Table 1. List of PCR and cycle sequencing (CS) primers used in this study. *unsuccessful for two species; †unsuccessful for Parasite E.

Table 2. Similarity of digenean nucleotide sequences from this study to those having the highest identity score in public databases, as determined by BLAST searches. IS, identity score; QC, query cover; Ac#, accession number.

Supplementary File S1. Aligned digenean 28S sequences, before trimming to the shortest length among them and removing alignment-ambiguous sites.

Supplementary File S2. Aligned digenean 28S sequences used for the maximum-likelihood analysis, after trimming in MEGA7 to the shortest length among them and removing alignment-ambiguous sites with Gblocks under "relaxed" parameters.

Supplementary File S3. Aligned sequences of the 5.8S–ITS2–28S region (ITS2 cluster), before trimming to the shortest length among them and removing alignment-ambiguous sites.

Supplementary File S4. Aligned ITS2 sequences, before removing alignment-ambiguous sites.

Supplementary File S5. Aligned ITS2 sequences used for the maximum-likelihood analysis, after removing alignment-ambiguous sites with Gblocks under "relaxed" parameters.

Supplementary Movie S1. Fresh Parasite O individual observed with light microscopy.

Supplementary Movie S2. Ethanol-fixed Parasite O observed with light microscopy.

Supplementary Movie S3. Fresh Parasite E individual observed with light microscopy.

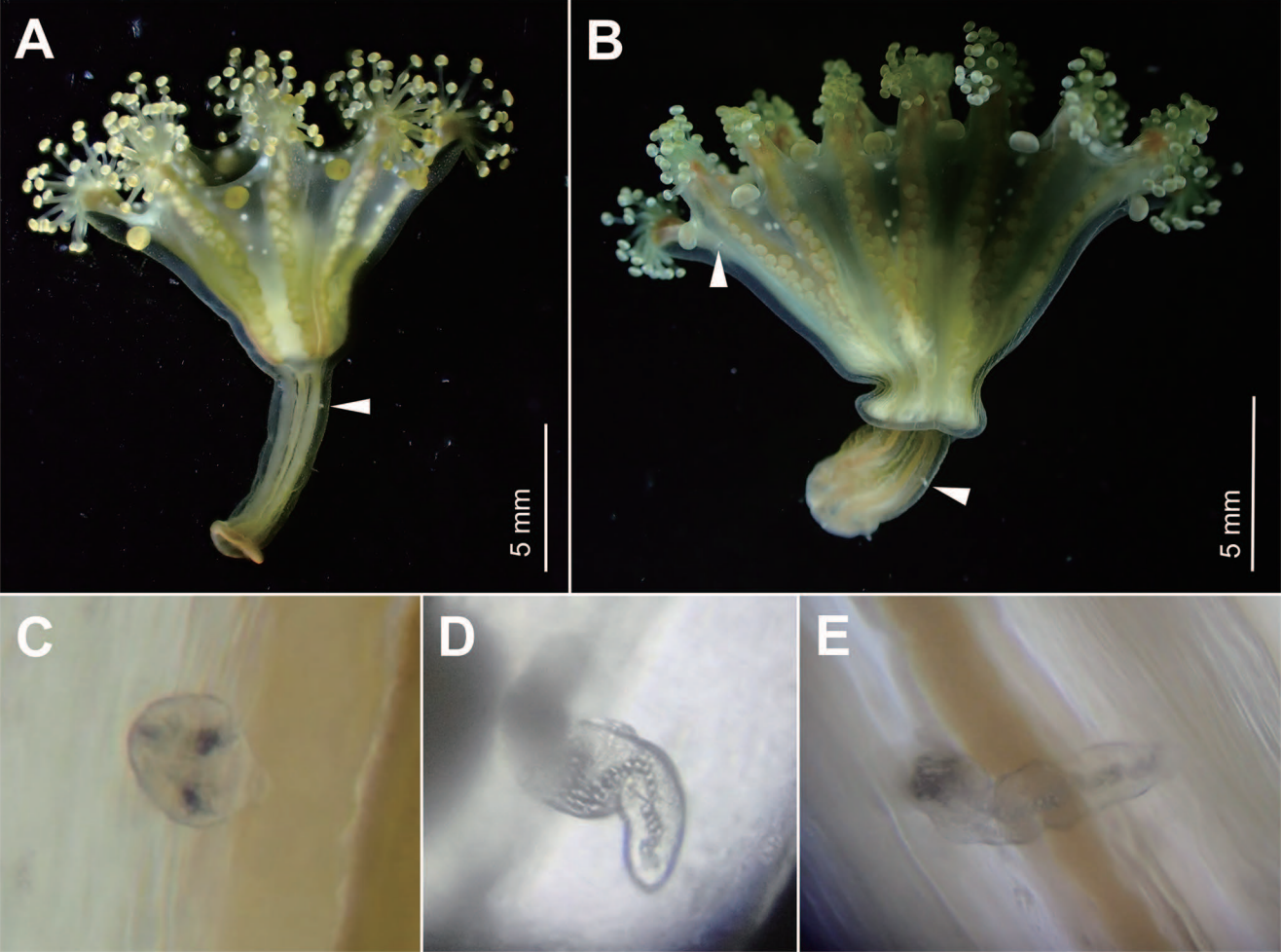
Supplementary Movie S4. Ethanol-fixed Parasite E observed with light microscopy.

Table 1. List of PCR and cycle sequencing (CS) primers used in this study. *unsuccessful for two species; †unsuccessful for Parasite E.

Marker	Primer	Reaction	Primer sequence	Source
18S	SR1	PCR	TACCTGGTTGATCCTGCCAG	Nakayama et al. (1996)
	18S-b3F	CS	CCTGAGAAACGGCTACCACAT	Kakui and Shimada (2017)
	18S-b5F	CS	GATCGAAGGCGATYAGATACC	Kakui et al. (2021)
	18S-a6R	CS	AACGGCCATGCACCAC	Kakui et al. (2011)
	18S-b8F	CS	GGTCTGTGATGCCCTTAGATG	Kakui et al. (2011)
	SR12	PCR	CCTTCCGCAGGTTCACCTAC	Nakayama et al. (1996)
28S	U178	PCR & CS	GCACCCGCTGAAYTTAAG	Lockyer et al. (2003)
	300F	CS	CAAGTACCGTGAGGGAAAGTTG	Lockyer et al. (2003)
	300R	CS	CAACTTTCCCTCACGGTACTTG	Lockyer et al. (2003)
	900F	CS	CCGTCTTGAAACACGGACCAAG	Lockyer et al. (2003)
	U1148	CS	GACCCGAAAGATGGTGAA	Lockyer et al. (2003)
	L1642	PCR	CCAGCGCCATCCATTTTCA	Lockyer et al. (2003)
COI	Dig cox1Fa	PCR*	ATGATWTTYTTYTTYYTDATGCC	Wee et al. (2017)
	Dig cox1R	PCR*	TCNGGRTGHCCRAARAAYCAAAA	Wee et al. (2017)
	LCf	PCR & CS†	CGAGCTTTTCTTGATGCCKGT	This study
	LCr	PCR & CS†	AATACACCTCCGGATGMCCAA	This study
ITS2 cluster	3S	PCR & CS	GGTACCGGTGGATCACGTGGCTAGTG	Morgan and Blair (1995)
	ITS2.2	PCR & CS	CCTGGTTAGTTTCTTTTCCTCCGC	Cribb et al. (1998)

Table 2. Similarity of digenean nucleotide sequences from this study to those having the highest identity score in public databases, as determined by BLAST searches. IS, identity score; QC, query cover; Ac#, accession number.

Query		Taxon	IS (%)	QC (%)	Ac#	Host	Reference
Parasite O	18S	*Lepocreadium trulla*	98.13	100	AY222128	*Ocyurus chrysurus*	Olson et al. (2003)
	28S	*Lepotrema* sp.	97.94	80	MK648297	*Balistes vetula*	Pérez-Ponce de León and Hernández-Mena (2019)
	ITS2	*Lepotrema monile*	94.65	100	MH730009	*Pomacentrus wardi*	Bray et al. (2018b)
	COI	*Lepocreadium setiferoides*	82.32	64	MN272622	*Tritia obsoleta*	Blakeslee et al. (2020)
Parasite E	18S	*Lepocreadium trulla*	99.25	100	AY222128	*Ocyurus chrysurus*	Olson et al. (2003)
	28S	*Prodistomum orientale* Type 2	100	78	PP239553	*Scomber japonicus*	Cribb et al. (2024)
		Prodistomum orientale Type 1	100	78	PP239554	*Scomber australasicus*	Cribb et al. (2024)
	ITS2	*Prodistomum orientale* Type 1	100	100	PP239528	*Hydrozoa* sp.	Cribb et al. (2024)
		Prodistomum orientale Type 1	100	100	MT773350	*Scomber australasicus*	Browne et al. (2020); Cribb et al. (2024)
		Prodistomum orientale Type unknown	100	100	MT773351	*Aequorea eurodina*	Browne et al. (2020); Cribb et al. (2024)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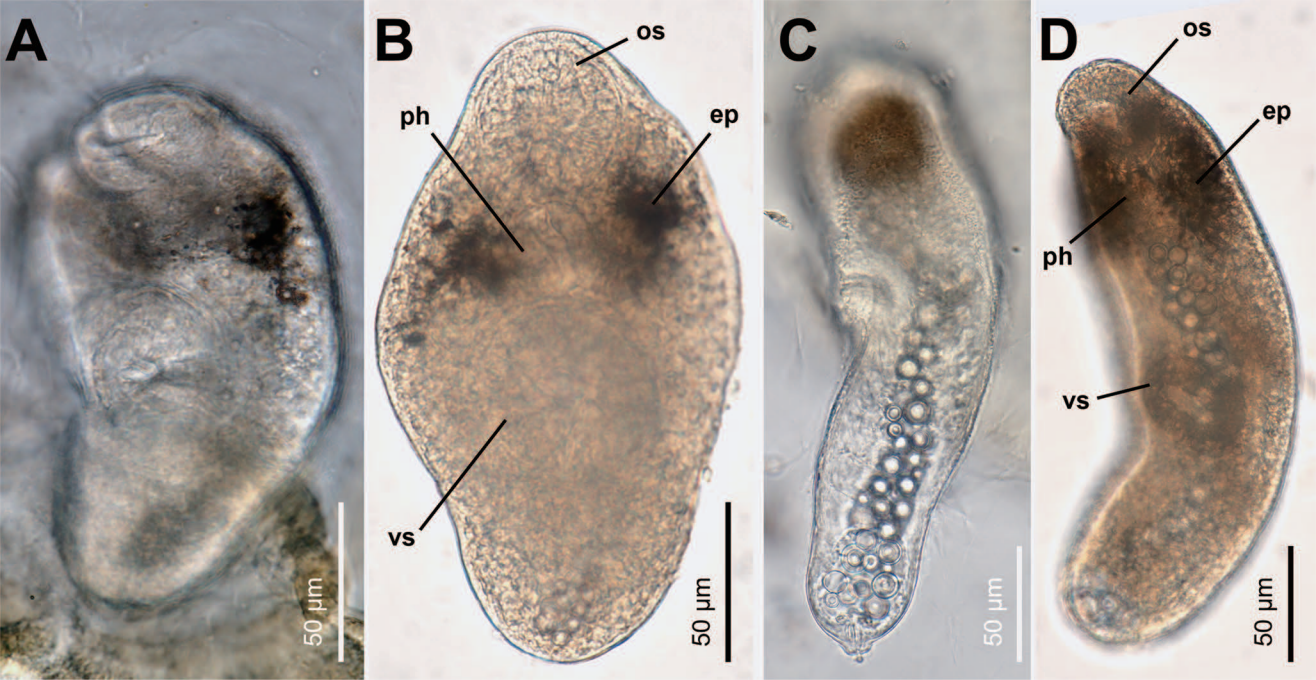
C
D
E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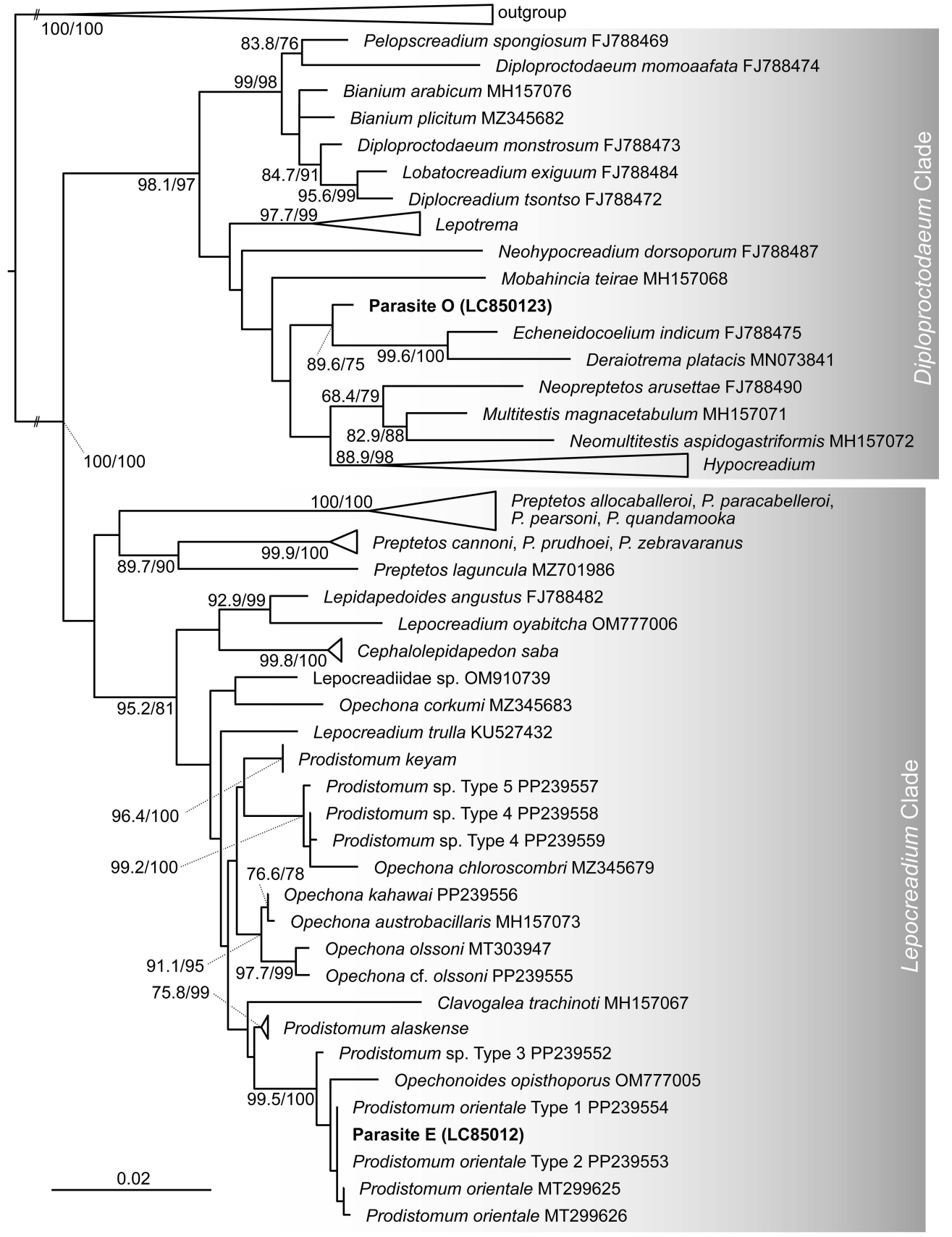
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99/98
Bianium arabicum MH157076
Bianium plicitum MZ345682
Diploproctodaeum monstrosum FJ788473
84.7/91
Lobatocreadium exiguum FJ788484
98.1/97
95.6/99
Diplocreadium tsontso FJ788472
97.7/99
Lepotrema
Neohypocreadium dorsoporum FJ788487
Mobahincia teirae MH157068
Parasite O (LC850123)
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89.6/75
99.6/100
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Neopreptetos arusettae FJ788490
68.4/79
Multitestis magnacetabulum MH157071
82.9/88
Neomultitestis aspidogastriformis MH157072
88.9/98
Hypocreadium
Diploproctodaeum Clade
100/100
100/100
Preptetos allocaballeroi, P. paracabelleroi, P. pearsoni, P. quandamooka
99.9/100
Preptetos cannoni, P. prudhoei, P. zebravaranus
89.7/90
Preptetos laguncula MZ701986
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Lepocreadium oyabitcha OM777006
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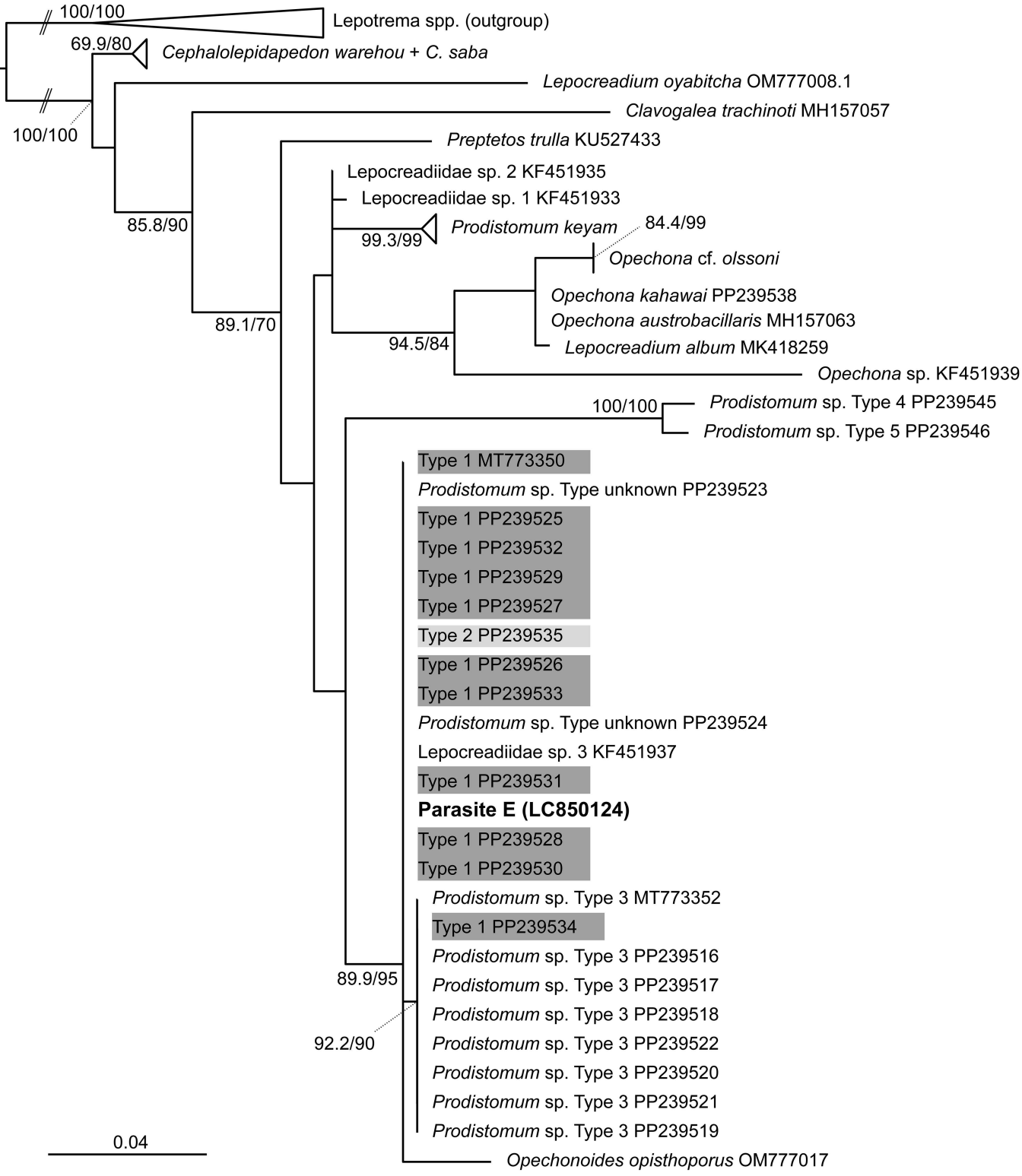
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100/100
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Lepocreadiidae sp. 2 KF451935
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85.8/90
99.3/99
Prodistomum keyam
84.4/99
Opechona cf. olssoni
Opechona kahawai PP239538
Opechona austrobacillaris MH157063
89.1/70
94.5/84
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100/100
Prodistomum sp. Type 4 PP239545
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Parasite E (LC850124)
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89.9/95
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