Finding a Needle in a Haystack: larval Stages of Didymozoidae (Trematoda: Digenea) Parasitizing Marine Zooplankton

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Abstract

Larval didymozoids (Trematoda, Digenea) were discovered parasitizing the hemocoel of the heteropod *Firoloida desmarestia* (redia mean intensity = 13) and the chaetognaths *Flaccisagitta enflata* and *Flaccisagitta hexaptera* (metacercaria mean intensity = 1) during a 2014–2016 systematic study of parasites of zooplankton collected in the central and southern regions of the Gulf of California, Mexico. Didymozoid infection route during the early life cycle was inferred combining morphological and molecular evidence (light and scanning electron microscopy and mitochondrial cytochrome c oxidase subunit I gene, *cox1*, respectively). Didymozoid rediae parasitizing *F. desmarestia* were observed alive releasing hundredths of cystophorous cercariae at a mean rate of one cercariae every 12 s. Cercariae developed into young metacercariae in 1 d at 22°C. Molecular analysis of *cox1* showed that rediae found in *F. desmarestia* belong to two distinct didymozoid species (Didymozoidae sp. 1 and sp. 2). Metacercariae parasitizing chaetognaths were morphologically identified as Didymozoidae type Monilicaecum and *cox1* sequences showed that metacercariae of chaetognaths matched with these two Didymozoidae sp.1, and sp. 2 species found parasitizing *F. desmarestia*, plus a third distinct Didymozoidae sp. 3. These are the first DNA sequences of *cox1* gene from didymozoid larvae for any zooplankton taxonomic group in the world. We concluded that *F. desmarestia* is the first intermediate host of rediae and second intermediate host cercariae and chaetognaths are the third intermediate hosts of didymozoid metacercariae demonstrating a potential overlap distribution of these two carnivorous zooplankton taxonomic groups. The didymozoid specimens were not identified to species level because any of the *cox1* sequences generated here matched with the sequences of adult didymozoids currently available in GenBank and Bold System databases. This study provides valuable information for the morphological and molecular understanding of the Didymozoidae larvae that has been previously based on the recognition of the 12 known morphotypes.

Introduction

The family Didymozoidae Monticelli, 1888 (Trematoda: Digenea) is one of the 13 families included in the Superfamily Hemiuroidea Loss, 1899, that parasitize marine animals and share, in a broad sense, similar life cycles (Pozdnyakov and Gibson 2008). Although the didymozoid life cycle has not been fully investigated and therefore poorly understood, it has been indirectly inferred as follows: the first intermediate hosts are gastropods, the second intermediate hosts are crustaceans, particularly Cirripedia, the third intermediate or paratenic hosts are zooplanktonic invertebrates and small fishes, and the definitive hosts are large predatory fishes (Nikolaeva 1965; Køie and Lester 1985; Al-Bassel and Ohaida 2006). Adult didymozoids usually infect the digestive tract of carnivorous fishes, mostly species of the family Scombridae Rafinesque, 1815 (Yamaguti 1971; Cribb et al. 2000; Mladineo 2006; Rodríguez-Ibarra et al. 2011; Melo et al. 2013; Chero et al. 2015; Schrandt et al. 2016; Bárcenas-de los Santos et al. 2021). Didymozoid metacercaria have been recorded in several marine invertebrates, such as cephalopods (Overstreet and Hochberg 1975; Hochberg 1980, 1990), copepods, cnidarians, ctenophores, polychaeta, chaetognaths (Madhavi 1968; Reimer et al. 1971, 1975; Shimazu 1978; Yip 1984; Øresland and Bray 2005; Gómez del Prado-Rosas et al. 1999, 2007; Lozano-Cobo et al. 2017a, 2018), and fish larvae (Køie and Lester 1985; Cribb et al. 2000; Tolonen and Karlsbakk 2003; Felizardo et al. 2011), as well as, infecting elasmobranch fishes as paratenic hosts (Rodríguez-Ibarra et al. 2011). Understandably, none of the reports of didymozoids parasitizing marine zooplankton have been identified to species level, partially because their small body size and the lack of morphological diagnostic features (Gómez del Prado-Rosas et al. 1999, 2007; Lozano-Cobo et al. 2017a, 2018) and plus lack of molecular studies at early development stages.

Metacercariae of didymozoids have been classified in 12 morphotypes based on their internal morphological structures, such as, presence or absence of a ventral sucker, pharynx, ‘Drusenmagen’ and gland cells around the esophagus and/or anterior parts of caeca (Kurochkin and Nikolaeva 1978; Podznyakov and Gibson 2008). The diagnostic morphological structures of the sporocyst, redia, and cercaria to identify species are even scarcer, particularly in comparison with other families of Digenea, such as Hemiuridae, that also parasitize marine invertebrate and vertebrate hosts (Køie and Lester

1985; Køie 1995). Based on the information obtained from other digeneans, such as Hemiuroidea and Lepocreadiidae, it has been proposed that sporocysts, rediae, and cercariae of didymozoids would parasitize marine benthonic and zooplanktonic mollusks (Sharmeem et al. 1990; Morales-Ávila et al. 2018); however, to date no records of the intermediate hosts have been documented.

In previous studies it has been possible to distinguish between species of *Didymozoon* and *Helicodidymozoon helicis* (Lester, 1979) Anderson and Cribb, 1994 of marine fishes from Australia using nuclear DNA sequences (ITS 1, ITS 2, 5.8S, 28S) (Anderson and Baker 1993, 1998; Baker et al. 1993). Other studies, also has been used another region of DNA to identified the species of didymozoids from marine fishes (ITS 2, 18S and 28S) (Olson et al. 2003; Pascual et al. 2006; Mladineo et al. 2010; Abe et al. 2014). It is worth mentioning that only a previous study included mitochondrial markers (cytochrome c oxidase subunit I, *cox1*) to identified species of Didymozoidae (Mladineo et al. 2010).

In the present study, we showed the morphology and behavior of the early larval stages of didymozoids using light and scanning electron microscopy, and we also generated mitochondrial DNA sequences (*cox1*) of each of the larval stages to infer how many didymozoid biological species infect gelatinous zooplankton. This taxonomical and molecular information represents the first integrative characterization of larval stages of didymozoids parasitizing marine zooplankton (holoplanktonic mollusks and chaetognaths) and also represents new geographic records for these parasites in the Eastern Pacific, Gulf of California, Mexico.

Material And Methods

Field sampling

Zooplankton samples were collected from 42 sampling stations obtained during three CAPEGOLCA oceanographic cruises carried out during February 2014, June–July 2016, and June 2017 in the central and southern region of the Gulf of California on board the R/V “El Puma” (Universidad Nacional Autónoma de México) (Table S1, Supplementary information). Samples were collected at night (22:00–02:00 h) with a zooplankton net of 1 m mouth diameter, black 250 µm mesh, equipped with a large lathe hard closed cod-end 15 L volume; 21 cm diameter, 71 cm length, to observe live zooplankton (Live net, Table S1). This zooplankton net was hauled typically between 10–50 m depth during 10 min while the ship drifted to avoid the damage of the captured zooplankton (Table S1). Each zooplankton sample was emptied into a cooler filled with the 15 L *in situ* seawater contained in the closed cod-end to observe zooplankton alive onboard the R/V El Puma. Zooplankton also was collected using a standard Bongo net (0.6-m mouth diameter provided with a cylindrical–conical 505 µm mesh net), hauled obliquely from 300 m depth to surface (modifying depth to about 50 m above the seafloor, in locations with < 300 m seafloor depth) following standard methods (Smith and Richardson 1977) (Bongo net, Table S1). These Bongo zooplankton samples were fixed in 70% ethanol with a total three change of ethanol at the end of each oceanographic cruise. Additionally, six surface zooplankton samples were collected during August 2016 at Punta Lobos (PL, 23°38’ N, -110°15’ W) and 48 zooplankton samples were collected during January-December 2016 at Cabo Pulmo National Park (CPNP, 23°27’ N, -109°25’ W), Baja California Sur, Mexico. Zooplankton samples at Punta Lobos and Cabo Pulmo National Park were collected between 0–10 depth during 10 min in both coastal regions using a surface conical net of 1-m mouth diameter, black 333 µm mesh, equipped with a calibrated digital flowmeter (General Oceanic R2030) attached to the mouth of the net to estimate the volume of seawater filtered by the net during each zooplankton tow (Smith and Richardson 1977). Zooplankton parasitized with Didymozoidae larval stages collected in the three oceanographic cruises in the central Gulf of California, Punta Lobos and Cabo Pulmo National Park are shown in Table S1.

Live observations of zooplankton

Live zooplankton from samples were observed on board R/V El Puma, immediately after collection, using a light stereoscope (Carl Zeiss, Stemi SV11 model, 0.6–6.6× magnification) to recover parasites of zooplankton. Specimens preliminarily assigned to Didymozoidae family were observed parasitizing heteropods and chaetognaths. Standard taxonomic keys were used to identify heteropods (Seapy et al. 2003) and chaetognaths (Alvariño 1963; Bieri 1991). Live parasitized heteropods were dissected with entomological needles to recover live rediae from the hemocoel for photo and video documentation. Cercaria recovered were incubated onboard in a Petri dish at constant 22°C (same temperature than *in situ* sea surface temperature at time of collection) under dark conditions and periodically photographed with a digital camera fitted to a light microscope (Carl Zeiss, 10–40× magnification). Rediae and cercaria recovered from the hemocoel of the heteropods were preserved in ethanol 96% for genetic analysis and other in formalin 4% for further morphological observations using light microscopy and scanning electron microscope (SEM) following the standard methods described in Lozano-Cobo et al. (2017b). SEM images were obtained using a Hitachi S-300N scanning electron microscope at 20 kv in Laboratorio de Microscopía Electrónica at Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, Baja California Sur, Mexico.

Didymozoid metacercariae parasitizing the coelom of the trunk of chaetognaths were observed and photographed alive. Each parasitized host (and the parasite once extracted from their host) was photographed and videotaped with a Canon G11 digital camera fixed to the optical stereoscope (Carl Zeiss SV11). Digital images of SEM and digital camera were edited using Adobe Photoshop (Adobe Systems Incorporated) software adding the scale bars (µm) in Adobe Illustrator (Adobe Systems Incorporated).

Morphological identification of parasitic larval stages

No identification keys are currently available for the first larval stages of digeneans (rediae and cercariae). Therefore, our specimens were only identified to superfamily Hemiuroidea using the morphological descriptions of parasites reported from marine invertebrates and marine fish hosts (Cort 1920; Cable 1956; Lester and Newman 1986; Newman 1990; Køie et al. 2002; Morales-Ávila et al. 2018). Metacercariae were assigned to their respective morphotype group according to Pozdnyakov and Gibson (2008), taxonomic keys of adult didymozoids parasitizing marine fishes (Yamaguti 1971) and previous reports of metacercariae parasitizing marine zooplankton or other marine invertebrates (Nikolaeva 1965; Madhavi 1968; Reimer et al. 1971, 1975; Overstreet and Hochberg 1975; Shimazu 1978; Køie and Lester 1985; Gómez del Prado-Rosas et al. 1999, 2007; Felizardo et al. 2011; Rodríguez-Ibarra et al. 2011; Lozano-Cobo et al. 2017a, 2018; Morales-Ávila et al. 2018).

DNA analysis

Total DNA from four rediae recovered from the heteropod *Firoloida desmarestia*, four metacercariae from the chaetognath *Flaccisagitta enflata,* and two metacercariae from *F. hexaptera* was extracted using the automated Glass Fiber protocol for animals (Ivanova et al. 2006). DNA extraction was done at Barcode of Life Laboratory located at Centro de Investigaciones Biológicas del Noroeste (CIBNOR), La Paz, Baja California Sur, Mexico. A 651-base pair (bp) fragment of the mitochondrial cytochrome c oxidase subunit gene (*cox1*) was amplified for each specimen through the polymerase chain reaction (PCR) using the forward primer JB3 5´ TTTTTTGGGCATCCTGAGGTTTAT 3´ (Bowles et al. 1992) and CO1-R-Trema 5´ CAACAAATCATGATGCAAAAGG 3´ (Miura et al. 2005). Amplification reactions were performed in a thermo-cycler Eppendorf (Master cycler Pro) following the next profile: 3 min at 96 °C, then 35 cycles of denaturation for 30 s each at 94 °C, annealing 2 min at 56 °C for 90 s, elongation for 90 s at 72 °C with final extension of 5 min at 72 °C. The final volume of each PCR sample was 18 µl including: 1 µl of genomic DNA (10-30 ng/µl), 3.6 µl of 5X PCR Buffer, 0.9 µl of each primer (10 µM), 0.15 µl of MyTaq (5U/µl, Bioline) and 11.45 µl of ddH_2O. The PCR products were visualized on a 2% agarose gel and the most intense bands were purified. Sequencing reactions were carried out in an Applied Biosystem 3500x1 sequencer of 24 capillaries (Life Technology Corporation, Thermo Fisher Scientific,

Singapore) at the Laboratorio Nacional de Biodiversidad (LANABIO, IB-UNAM, Mexico City). DNA sequences of both directions were assembled, edited and aligned using GENEIOUS 11.1.4 software (Kearse et al. 2012).

A Basic Local Alignment Search Tool analysis (BLAST) (Altschul et al. 1990) was first used to exclude sequences of the host or co-amplifications and then to select and compare with sequences publicly available in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and BOLD Systems (http://www.boldsystems.org) for comparative purposes (Table S2). We added to our dataset *cox1* sequences available on GenBank and BOLD Systems of Didymozoidae that parasitizing different host species to compare with the *cox1* sequences of the Didymozoidae parasitizing heteropods and chaetognaths (Table S2). Three sequences of species of the genus *Lecithaster* Lühe, 1901 (Family Lecithasterinae Odhner, 1905) were selected as an outgroup based on a comprehensive phylogenetic study of Digenea (Pérez-Ponce de León and Hernández-Mena 2019) and one more of *Brasicystis bennetti* Thatcher, 1979 as other outgroup based on the common use of this didymozoid species in the DNA analyses with Kimura two-parameter (K2P) model (Melo et al. 2013) as in this study. All sequences were aligned and the genetic distances among pairs of sequences were calculated using Kimura two-parameter model in the software PAUP* v.4.0 (Swofford 2002). Maximum Likelihood phylogenetic analyses were performed in the command line version of RaxML v. 8.2 (Stamatakis 2014), using the general time reversible model (GTR) with gamma distributed rate parameter and invariable regions model. Bootstrap support values (BS) were obtained with 10000 replicates using RaxML v. 8.2 program. Additionally, genetic distances (K2P method) within and between groups shown in the neighbor-joining tree were obtained.

Results

Parasites of Firoloida desmarestia **(Mollusca: Heteropoda)**

A total of 52 rediae of Didymozoidae were observed moving freely in the hemocoel from four out of 30 alive heteropods (mean intensity = 13) collected (Fig. 1A-B, Table 1). The heteropod hosts were identified as *Firoloida desmarestia* Lesueur, 1817 because the long and transparent cylindrical body, short ventral tail, absence of shell in the visceral mass, which is compressed into a terminal tear-drop shaped visceral nucleus (Fig. 1A). Rediae of two *F. desmarestia* specimens followed a slow zig-zag movement inside the host occupying most part of the hemocoel (Fig. 1A, B; Online Resource 1). Inside the hemocoel, several rediae released non-motile cystophorous cercariae throughout the mouth, located in the anterior end of the body (Fig. 1C, D; Online Resource 2). Released cystophorous cercariae were incubated at 22°C (Fig. 1D, 2A-F) and transformed into a young metacercaria after 24 h and cercariae died two hours later (Fig. 2G, H; Online Resource 3). Four of these rediae were characterized on the molecular basis obtaining three *cox1* sequences (Table 1, Tables S1, S2).

Table 1
Morphological and molecular information of larvae of Didymozoidae parasites (r = rediae, m = metacercariae), size of the parasites and the zooplankton host species (TL = total length, W = width), gonadal development stage of the chaetognath hosts [GDS I-IV; 0 = juvenile chaetognaths (without visible gonad)], intensity of infection, and microhabitat infection of parasites in each zooplanktonic host (Heteropoda and Chaetognatha)

BOLD	**Morphological / molecular**	**TL**	**W**	**Host**		**TL**	**W**	**Intensity**	**Microhabitat inside the host**
Systems code	identification of larvae parasite (r / m)	(mm)	(mm)	species	GDS	(mm)	(mm)	of infection	
ZPCR_170	Didymozoidae / Didymozoidae sp. 1 (r)	-	-	*F. desmarestia*	-	13.5	2.1	17	Hemocoel
ZPCR_171	Didymozoidae / Didymozoidae sp. 1 (r)	-	-	*F. desmarestia*	-	13.5	2.1	17	Hemocoel
ZPCR_218	Didymozoidae / Didymozoidae sp. 1 (r)	-	-	*F. desmarestia*	-	13.2	2.0	21	Hemocoel
ZPCR_216	Monilicaecum / Didymozoidae sp. 1 (m)	0.25	0.10	*F. enflata*	0	6.9	0.7	1	Trunk coelom
ZPCR_217	Didymozoidae / Didymozoidae sp. 2 (r)	-	-	*F. desmarestia*	-	13.2	2.0	21	Hemocoel
ZPCR_111	Monilicaecum / Didymozoidae sp. 2 (m)	0.18	0.08	*F. enflata*	0	7.7	0.5	1	Trunk coelom
ZPCR_062	Monilicaecum / Didymozoidae sp. 2 (m)	0.225	0.06	*F. enflata*	I	7.6	0.9	1	Trunk coelom
ZPCR_212	Monilicaecum / Didymozoidae sp. 2 (m)	0.165	0.06	*F. hexaptera*	IV	11.2	1.0	1	Trunk coelom
ZPCR_215	Monilicaecum / Didymozoidae sp. 2 (m)	0.15	0.05	*F. hexaptera*	I	5.8	1.0	2	Trunk coelom
ZPCR_112	Monilicaecum / Didymozoidae sp. 3 (m)	0.15	0.08	*F. enflata*	I	7.3	1.2	1	Trunk coelom

BOLD	Morphological / molecular	TL	W	Host		TL	W	Intensity	Microhabitat inside the host
-	Didymozoidae / sp. (r)	-	-	*F. desmarestia*	-	22	-	6	Hemocoel
-	Didymozoidae / sp. (r)	-	-	*F. desmarestia*	-	16	1.6	8	Hemocoel
-	Didymozoidae / sp. (r)	-	-	*F. desmarestia*	-	-	-	3	Hemocoel
-	Monilicaecum / sp. (m)	0.135	-	*F. enflata*	I	15.3	-	1	Trunk coelom
-	Monilicaecum / sp. (m)	0.15	-	*F. enflata*	I	12.0	-	1	Trunk coelom
-	Monilicaecum / sp. (m)	0.15	0.075	*F. enflata*	I	11.4	1.5	1	Trunk coelom
-	Monilicaecum / sp. (m)	0.17	0.045	*F. enflata*	0	5.64	0.7	1	Trunk coelom

Redia (Fig. 1A-D and Table 1). Alive rediae are brown opaque when observed with a light stereoscope illuminated with bottom transmitted light and with a pale whitish appearance inside the transparent heteropod host when observed with multispectral white light and black background (Fig. 1A-D). The rediae had cylindrical and elongated body (3.05–3.18 mm length and 0.18–0.24 mm width, n = 3) (Fig. 1C). The recovered redia also displayed slow wriggling movements (Online Resources 1 and 2). Each redia contained hundreds of cystophorous cercariae and each cercaria swam free in the hemocoel of their host (Online Resource 2). The cystophorous cercariae were expelled through the mouth of the redia at approximately one cercaria every 12 s (Fig. 1D; Online Resource 2).

Cercaria (Fig. 2A-F). Cystophorous cercaria have a caudal cyst enclosing the cercarial body with a delivery tube and bear an external plumage appendage. Cercarial body is oval (0.075 mm long and 0.030 mm in maximum width) just anterior to slight constriction between the body and tail, which appears segmented when the body is contracted (Fig. 2A). Body tegument segmented (Fig. 2B-C) with two pairs of papillae in lateral position of the body (papillae morphology not shown). Sub-terminal oral sucker and ventral sucker present. Excretory vesicle oval to cylindrical. Caudal cyst pyriform, transparent, closely adherent to inner cyst wall, which appears thick. Delivery tube 0.075 mm long and 0.008 mm wide (Fig. 2D). Free-swimming cercariae with a cercarial body and delivery tube introverted inside the cyst with the body folded to one side and delivery tube appearing as a collapsed tube (Fig. 2E-F; Online Resource 3).

Young metacercariae (Fig. 2G-H). Outside their cyst, young metarcercaria crawled at the bottom of the Petri dish transforming into metacercaria after 24 h at 22°C (Online Resource 3). The metacercaria body is oval, thick and segmented (Fig. 2G) with two pairs of papillae in an anterior-lateral position of the body (papillae morphology not shown). Oral sucker sub-terminal and ventral sucker situated in one third of the posterior body. Pharynx not observed. Excretory vesicle oval and only seen in the posterior part (Fig. 2H).

Rediae and cercariae ultrastructure (Fig. S1A-F). The tegument ultrastructure of the rediae observed with SEM showed transverse circumferential folds and the lateral body with several digitiform papillae (Fig. S1A). Mouth located at the end of the anterior region (Fig. S1C, D) and cystophorous cercariae with the tail coiled (Fig. S1E) and with a smooth cyst wall are shown (Fig. S1F).

Parasites Of Chaetognaths

A total of 51 out of 150 analyzed chaetognaths were found parasitized with at least one metacerariae (34% prevalence). Six of those 51 metacercaria specimens were morphologically described for further *cox1* molecular analysis. Initially all metacercaria specimens were identified using morphological criteria as type Monilicaecum, but species identification was not done because it was impossible to detect diagnostic morphological differences among the six analyzed specimens (Fig. 2I, J; Online Resource 4). Description based on the six non-encysted Didymozoidae specimens parasitizing the chaetognath *Flaccisagitta enflata* and *Flaccisagitta hexaptera* (Table 1): body oval to sub-cylindrical, elongate with rounded extremities, cuticle thick, without spines, transversely striated. Oral sucker slightly ellipsoidal in the anterior portion of body. Ventral sucker round, muscular, situated near level of anterior third portion of body. Mouth subterminal, pharynx not seen and esophagus not clearly visible. ‘Drusenmagen’ (or stomach) present, round located at intestinal bifurcation, dorsal and lateral to the ventral sucker. Intestinal caecum at each side occupies lateral regions of body, moniliform type, sinuous and each one composed with 4–8 inflated chambers oriented towards the posterior end of the body and near the posterior third of the hind-body. Excretory vesicle saccular, small or contracted, posterior to intestinal caecum. Excretory pore terminal. No reproductive structures were observed. Six metaceraria specimens were characterized on the molecular basis obtaining three distinct haplotypes of *cox1* sequences (Table 1, Tables S1, S2).

Molecular Identification And Phylogenetic Analyses

In total, 10 *cox1* sequences were generated; four metacercariae from redia parasitizing the heteropod *Firoloida desmarestia* (BOLD code = ZPCR 170, 171, 217 and 218), four from metacercariae parasitizing the chaetognath *Flaccisagitta enflata* (BOLD code = ZPCR 62, 111, 112, 216) and finally, two metacercariae specimens parasitizing the chaetognath *Flaccisagitta hexaptera* (BOLD code = ZPCR 212 and 215) (Table S2). BLAST analysis (Altschul et al. 1996) of *cox1* sequences obtained in the present study showed genetic similarity from 71.07 to 74.95% with available sequences of adult Didymozoidae (*B. bennetti*, *Koellikerioides apicali* Yamaguti, 1970, and *Didymocystis wedli* (Ariola, 1902)). Species identification is not possible to do, so far, with current available sequences deposited in public data bases. The ML phylogenetic analysis including the sequences generated in the present study, together with sequences selected from GenBank (Table S2) retrieved a tree with a log likelihood of -12212.44 (Fig. 3). The family Didymozoidae resulted as a monophyletic group with a bootstrap of 100% sister to a monophyletic Derogenidae Nicoll, 1910. All ten sequences obtained in the present study form a monophyletic group with a bootstrap of 92%, sister to *B. bennetti* and well nested within Didymozoidae family (Fig. 3).

The sequences obtained in the present study form three well-supported groups, labeled as Didymozoidae sp. 1– sp. 3, allegedly three distinct biological species. Didymozoidae sp. 1 includes three specimens obtained from rediae parasitizing the heteropod *Firoloida desmarestia* and one specimen obtained from a metacercaria parasitizing the chaetognath *Flaccisagitta enflata.* Didymozoidae sp. 2 includes one specimen obtained from a single redia parasitizing the heteropod *F. desmarestia* and two specimens obtained from metacercaria found in *F. enflata* and two specimens from *Flaccisagitta hexaptera.* Finally, the single Didymozoidae sp. 3 *cox1* sequence was obtained from a metacercaria parasitizing *F. enflata.* No genetic variation was found within of the groups of Didymozoidae sp. 1 and Didymozoidae sp. 2, but genetic distances between the cluster of Didymozoidae sp. 1 and the other two Didymozoidae (sp. 2 and sp. 3) showed a range from 0.36–0.43, and 0.15 between Didymozoidae sp. 2 and sp. 3. Finally, genetic distances between these tree groups in comparison with *B. bennetti* (sister cluster) were of 0.34–0.40 and with the genus *Lecithaster* (outgroup) showed a range of 0.50–0.67. The body size of the specimen labeled as “Didymozoidae sp. 1” based on *cox1* sequence (see latter) was 0.250 mm total length and 0.100 mm width at widest section, the four specimens labeled as “Didymozoidae sp. 2” based on *cox1* sequence were 0.150–0.220 mm of total length, 0.045–0.075 mm of width at widest section (Fig. 2J, Table S1), and the specimen labeled as “Didymozoidae sp. 3” based on *cox1* sequence was

0.150 mm of total length and 0.075 mm at widest section (Table S1). GenBank accession numbers of all the sequences generated in the present study are reported in Table S2.

Discussion

Molecular evidence (mitochondrial *cox1* gene) demonstrated that rediae and cercariae parasitizing the holoplanktonic mollusk *Firoloida desmarestia* and the metacercariae found in two chaetognath species (*Flaccisagitta enflata* and *F. hexaptera*) correspond to the same didymozoid species. This is the first study in the world of Didymozoid larvae that based on DNA sequences, demonstrates the infection of the same Didymozoid species in different life stages parasitize two distinct zooplanktonic taxonomic groups (Heteropoda and Chaetognatha), providing first evidence of the distinct taxonomic group of gelatinous zooplankton hosts participating in the life cycle of this group of didymozoid parasites in nature. Unfortunately, the taxonomic species identity of these didymozoids was not determined because no matches with currently available sequences of adult didymozoids in GenBank or BOLD Systems were found. Present available public genetic information of Dydimozoidae is incipient and therefore highly incomplete. All the *cox1* sequences downloaded from GenBank corresponding to Didymozoidae were generated from parasitized fishes of the order Perciformes (including the six families: Gobiidae, Haemulidae, Sciaenidae, Scombridae, Siganidae, and Sparidae) and Beloniformes (including the three families: Belonidae, Exocoetidae and Hemiramphidae) from several localities around the world ocean (Fig. 3, Table S2). All this indicates that the adult worms of the species studied here parasitizing *F. desmarestia*, *F. enflata*, and *F. hexaptera* may complete their parasite life cycle infecting species of similar group of fishes.

The metacercariae reported in the present study were identified among the 12 morphotypes distinguished by Pozdnyakov and Gibson (2008). All these specimens were identified as Monilicaecum larval type due the presence of a) ventral sucker round and muscular, b) round stomach ‘Drusenmagen’ located at intestinal bifurcation, lateral to the ventral sucker, and c) intestinal caecum in moniliform type, sinuous and each one composed with 4 to 8 inflated chambers occupying lateral regions of the posterior region. However, we were not able to observe the pharynx in any of the analyzed specimens and we were not able to observe evident morphological differences among the three Didymozoidae sp. 1, sp. 2, and sp. 3 biological species clearly distinguished with *cox1* sequences in the present study. However, minimum size differences among didymozoid and the chaetognath host species were detected. Metacercariae Didymozoidae sp. 3, found parasitizing *F. enflata*, was the smallest specimen, and Didymozoidae sp. 1 was on average the largest metacercariae specimen reported in the present study (Table 1). We agree with Pozdnyakov and Gibson (2008) that mentioned that the size of the parasites and the number of the chambers in the intestinal caecum should not be considered as the exclusive diagnostic characteristics to difference among didymozoid species. Based on the data of the present study, we also reject the idea that body size is a meaningful character to differentiate species. Our observations of the living rediae, cercariae and metacercaria showed clear and conclusive evidence of the large variability in their flexible body size (Online Resources 1 and 2).

The observation of living Didymozoidae allowed us to determine the development rate, ontogeny, and behavior of the redia, cercaria, and metacecaria parasitizing the Heteropoda and Chaetognatha holoplanktonic intermediate hosts. *Cox1* genetic sequences allowed us to infer the parasite species richness and to provide robust evidence to understand how the same Didymozoidae species infect different types of zooplankton invertebrate groups, heteropods and chaetognaths, each one with distinct life cycles, however, with similar trophic feeding strategies (carnivorous) and with overlapping vertical and biogeographic distribution range (Alvariño, 1963; Angulo-Campillo et al. 2011). Interestingly, Didymozoid *cox1* sequences (from redia or metacercariae) obtained in the present study represent an independent lineage of didymozoids for which adult stages are currently unknown (or at least without genetic sequences of adults) (Fig. 3, Table S2). In the taxonomic group of didymozoids, there is only one work of genetic divergence based on p-distance value of *cox*1 sequences and reported as highest level of interspecific genetic distance between *Didymocystis*

pectoralis (Yamaguti, 1970) Podznyakov, 1990 and *D. spirocauda* (0.087 – 0.086) (from *Thunnus orientalis* (Temminck & Schlegel, 1844) and *T. thynnus* (Linnaeus, 1758)), *Koellikeria* sp. *renalis* and *Platocystis alalongae* Yamaguti, 1938 (0.329) (from *T. thynnus*), and *D. wedli* and *P. alalongae* (0.324–0.329) (from *T. orientalis* and *T. thynnus*) (Mladineo et al. 2010). In the present study we report the genetic distances between groups Didymozoidae sp. 1, sp. 2 y sp. 3 with values of 0.15 to 0.0.43 (K2P model. This information can be used to comparison in didymozoid larvae or adult DNA future studies. Combined observations of alive parasites to characterize internal morphology without the effect of preservatives and molecular analyses provided new evidence and opportunities to understand new insights in the didymozoids life cycle and how these common types of parasites interact with marine zooplankton species as the base of the marine pelagic food web.

Declarations

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Author contribution

HLC Original idea, sampling and specimen transportation, morphological identification of parasites and hosts, preparation of manuscript and edition of figures, text and videos; CAS-S Molecular analysis and text edition; AO-F Molecular analysis and text edition; CJR Sampling and specimens transportation, text edition and project administrator; JG-G Original idea, sampling and specimen transportation, preparation of manuscript and text and figure edition, and project administrator.

Conflict of interest

The authors declare that they have not conflict of interest.

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Figures

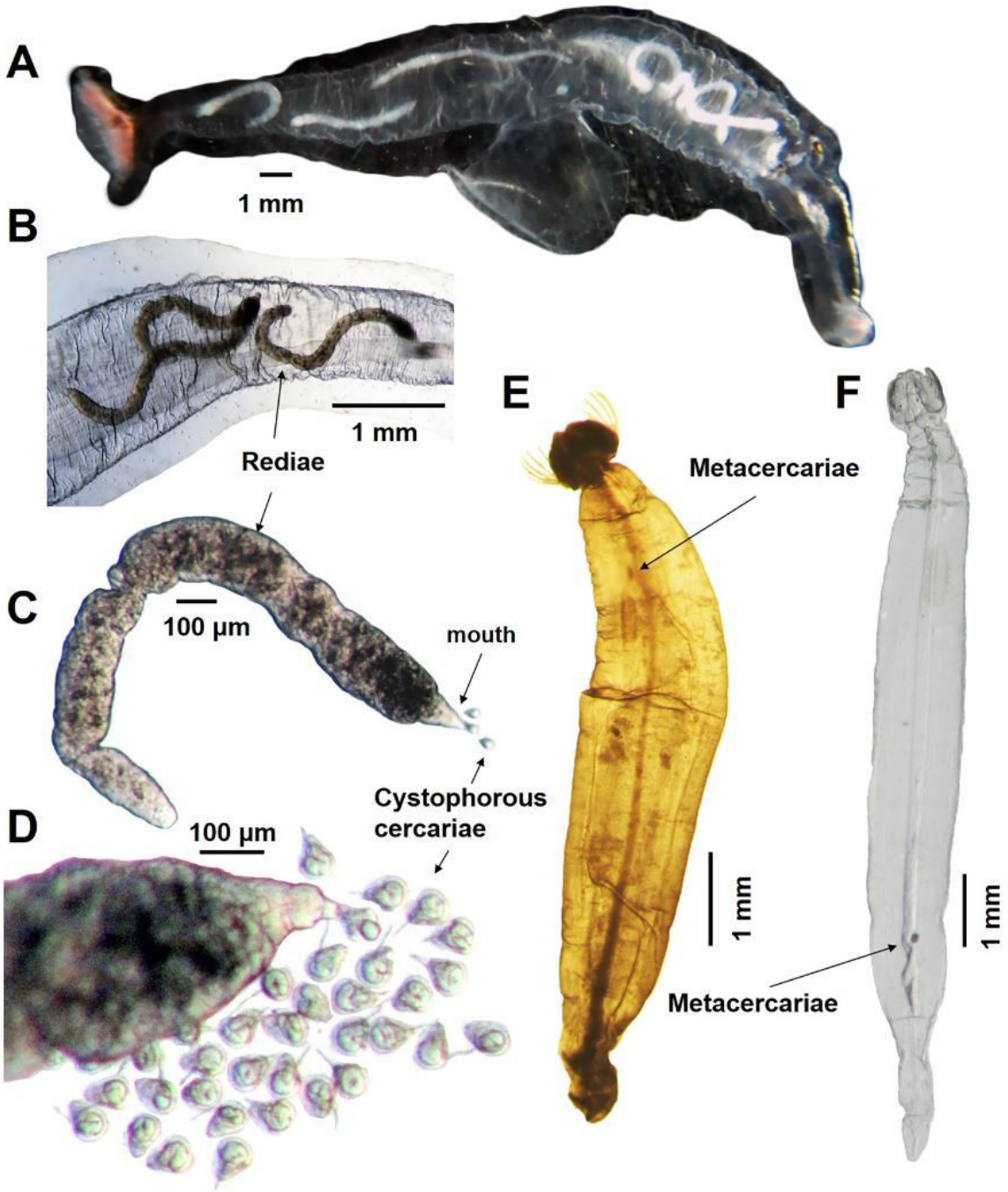

Figure 1

Marine zooplankton parasitized with didymozoid digenean larvae collected during 2014–2016 in the Gulf of California: (A-D) holoplanktonic heteropod mollusk *Firoloida desmarestia* parasitized with redia and cercaria, (B) redia in the coelom of *F. desmarestia*, (C-D) redia extracted from the hemocoel of *F. desmarestia* showing the mouth and redia releasing hundredths of cystophorous cercariae, (E) chaetognath *Flaccisagitta enflata* parasitized with metacercariae in the anterior region of the trunk coelom, (F) chaetognath *Flaccisagitta hexaptera* parasitized with metacercariae in the posterior region of the trunk coelom. Scale bars = 100 µm (C-D) and 1 mm (A-B, E-F).

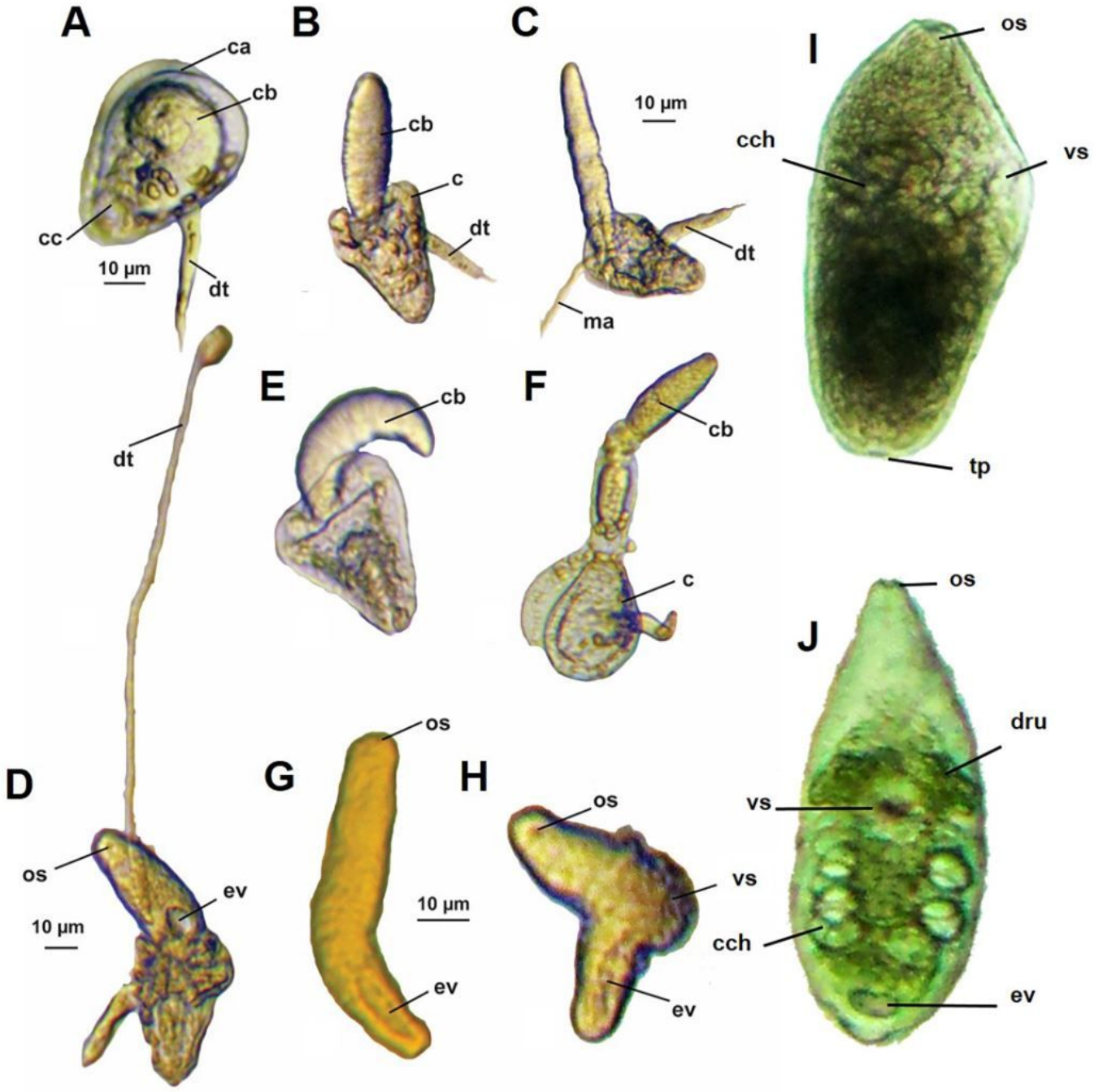

Figure 2

Morphologic development of didymozoid cercaria and metacercaria larval stages observed with light microscopy: (A-C) just released cystophorous cercariae (< 1 h), (D-F) cystophorous cercariae between 6–24 h old, (G-H) young metacercariae recently released of the cyst (>24 h), (I) lateral view of metacercariae Didymozoidae sp. 1 parasitizing the chaetognath *Flaccisagitta enflata*, (J) ventral view of metacercariae Didymozoidae sp. 2 parasitizing the chaetognath *Flaccisagitta hexaptera*. a = acetabulum; c = cyst; ca = cyst aperture; cb = cercarial body; cc = cyst caudal; cch = caecal chambers; dt = delivery tube; dru = ‘Drusenmagen’; ev = excretor vesicle; ma = motile appendage; os = oral sucker; tp = terminal pore. Scale bar = 10 µm.

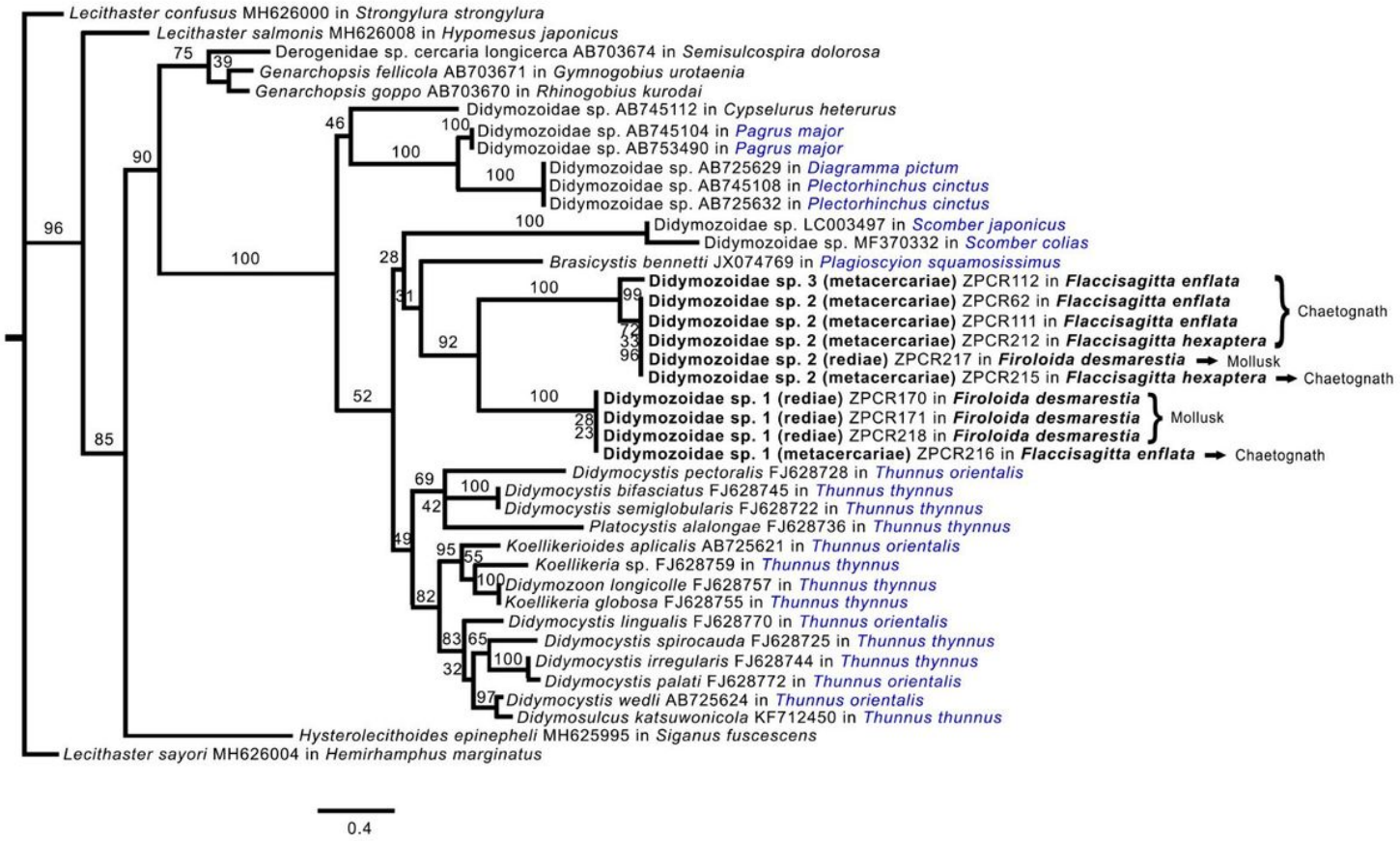

Figure 3

Neighbor joining tree based on the analyses of the partial mitochondrial cytochrome oxidase subunit I (*cox*1) gene of Didymozoidae parasitizing marine fishes (downloads from GenBank/Bold Systems), the holoplanktonic heteropod *Firoloida desmarestia* and the chaetognaths *Flaccisagitta enflata* and *Flaccisagitta hexaptera* of this study. Values next to nodes indicate bootstrap values above 50%. For Didymozoid specimens and species with a single representative, taxon names are followed by GenBank accession numbers. Sequences generated in the present study are showed in bold. The genus and species of the host is shown for each sequence *cox*1 gene trees. Fish Perciformes hosts are showed in blue.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 03LozanoCoboetal.DidymozoidsSupplmat.docx
- OnlineResource1RediainfectingheteropodFiroloidadesmarestia1.mp4
- OnlineResource2RediainfectingF.desmarestiareleasingcercariae.mp4
- OnlineResource3Youngmetacercariaeoutsidethecystofcercariae.mp4
- OnlineResource4DidymozoidaeinfectingChaetognaths.mp4