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 (light microscopy) and molecular (mitochondrial cytochrome c oxidase subunit I gene, cox1) evidence. Didymozoid rediae parasitizing F. desmarestia were observed, just after field collection of the host, containing hundredths of completely developed cystophorous cercariae, releasing them through the birth pore at approximately one cercaria every 12 s. Cercariae lost their tails developing into a ‘young metacercaria’ in 1 d at 22 °C without need of an intermediate host. 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 cercariae, and the chaetognaths are the second intermediate hosts where non-encysted metacercariae were found. The definitive host is still unknown because cox1 sequences of present study did not genetically match with any available cox1 sequence of adult didymozoid. Our results demonstrate a potential overlap in the distribution of two carnivorous zooplankton taxonomic groups that are intermediate hosts of didymozoids in the pelagic habitat. 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 baseline information for the future morphological and molecular understanding of the Didymozoidae larvae that has been previously based on the recognition of the 12 known morphotypes.

Keywords Rediae · Cystophorous cercariae · Metacercariae · Carnivorous zooplankton · Cox1 · Gulf of California

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Introduction

The family Didymozoidae Monticelli, 1888 (Trematoda: Digenea) is one of the 13 families included in the Super-family Hemiuroidae Loss, 1899 that parasitize marine animals and share, in a broad sense, similar life cycles (Pozdnyakov and Gibson 2008). Didymozoid life cycle has not been fully investigated in natural or experimental conditions, and therefore, still poorly understood. However, recently, the first evidence, using molecular data matching a didymozoid life cycle with three hosts as follows: didymozoid species use a bivalve as first intermediate host, a benthivorous fish as intermediate host (apparently the third host), and large piscivorous fish as its definitive host (Louvard et al. 2022). Thus, Louvard et al. (2022) study provides new evidence of an alternative life pathway in the life cycle of didymozoids because previously was conceptualized as follows: the first intermediate hosts are gastropods, the second intermediate hosts are small crustaceans, possibly copepods (Madhavi 1968), 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 mesentery, intestine, and kidney 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; Schrøndt et al. 2016; Bárcenas de los Santos et al. 2021). Didymozoid metacercariae have been recorded in several marine invertebrates, such as cephalopods (Overstreet and Hochberg 1975), copepods, cnidarians, ctenophores, polychaetes, chaetognaths (Madhavi 1968; Reimer et al. 1971, 1975; Shimazu 1978; Yip 1984; Gómez del Prado-Rosas et al. 1999, 2007; Øresland and Bray 2005; Lozano-Cobo et al. 2017, 2018), and fish larvae (Køie and Lester 1985; Cribb et al. 2000; Tolonen and Karlsbak 2003; Felizardo et al. 2011), as well as, infecting elasmobranch fishes as paratenic hosts (Rodriguez-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. 2017, 2018) and a lack of molecular studies at early development stages to match with sequences of adult life stages of didymozoid species.

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 Hemiuroidae, that also parasitize marine invertebrate and vertebrate hosts (Køie and Lester 1985; Køie 1995). Based on the information obtained from other digeneans, such as Hemiuroidae, it has been proposed that sporocysts, rediae (both asexual larvae), cercariae, and metacercariae (sexual larvae) of didymozoids would parasitize marine bentonic and holoplanktonic mollusks (Shameem et al. 1990; Morales-Ávila et al. 2018); however, to date, no records of the intermediate hosts have been documented.

Lester and Newman (1986) did the first report of digenetic trematode rediae infecting Firoloidea desmarestia Lesueur, 1817 collected in the Gulf of Carpentaria, Australia (12°S, 139°E, 12°S, 141°E, November 1975) proposing that those rediae were didymozoids. Later, Newman (1990) described morphologically these same rediae and cercariae specimens of hemiuroid trematode infecting F. desmarestia at the west coast of Baja California Peninsula, Mexico (25°59′ 35′′N, 114° 27′ 101′′ W, March 2012). They proposed a conceptual model of the role of the holoplanktonic mollusk F. desmarestia as host for digenetic trematodes including a synthesis of helminths parasites that infect holoplanktonic mollusks (see Table 1 of Morales-Ávila et al. 2018).

Several studies were able to distinguish between species of Didymozoon and Helicodidymozoon helicis (Lester, 1979) Anderson and Cribb, 1994 parasitizing marine fishes from Australia using nuclear DNA sequences (ITS 1, ITS 2, 5.8S, 28S) (Anderson and Barker 1993, 1998; Barker et al. 1993). Other previous 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); therefore, it is expected a low number of available cox1 sequences in public databases, like GenBank and BOLD Systems.

In the present study, we showed the morphology and behavior of the early larval stages of didymozoids using light microscopy and obtained mitochondrial DNA sequences (cox1) of each of the larval stages to infer how many didymozoid biological species infect gelatinous zooplankton (holoplanktonic mollusks and chaetognaths). This taxonomical and molecular information represents the first integrative characterization of larval stages of didymozoids parasitizing marine gelatinous zooplankton 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, and 71-cm length, to observe live zooplankton (Live net, Table S1). This zooplankton net was hauled typically between 10 and 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 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.

| Accession code    | Morphological/molecular identification of larval parasite (r/m) | TL (mm) | W (mm) | Host species | GDS | TL (mm) | W (mm) | Intensity of infection | Microhabitat inside the host |
|-------------------|---------------------------------------------------------------|---------|--------|--------------|-----|---------|--------|------------------------|-----------------------------|
| OM925480/ZPCR_170 | Didymozoidae/Didymozoidae sp. 1 (r)                          | -       | -      | *F. desmarestia* | -   | 13.5    | 2.1    | 17                     | Hemocoel                    |
| OM925482/ZPCR_171 | Didymozoidae/Didymozoidae sp. 1 (r)                          | -       | -      | *F. desmarestia* | -   | 13.5    | 2.1    | 17                     | Hemocoel                    |
| OM925483/ZPCR_218 | Didymozoidae/Didymozoidae sp. 1 (r)                          | -       | -      | *F. desmarestia* | -   | 13.2    | 2.0    | 21                     | Hemocoel                    |
| OM925481/ZPCR_216 | Monilicaecum/Didymozoidae sp. 1 (m)                          | 0.25    | 0.10   | *F. enflata*    | 0   | 6.9     | 0.7    | 1                      | Trunk coelom                |
| OM992279/ZPCR_217 | Didymozoidae/Didymozoidae sp. 2 (r)                          | -       | -      | *F. desmarestia* | -   | 13.2    | 2.0    | 21                     | Hemocoel                    |
| ON007166/ZPCR_111 | Monilicaecum/Didymozoidae sp. 2 (m)                          | 0.18    | 0.08   | *F. enflata*    | 0   | 7.7     | 0.5    | 1                      | Trunk coelom                |
| ON007165/ZPCR_062 | Monilicaecum/Didymozoidae sp. 2 (m)                          | 0.225   | 0.06   | *F. enflata*    | I   | 7.6     | 0.9    | 1                      | Trunk coelom                |
| OM992277/ZPCR_212 | Monilicaecum/Didymozoidae sp. 2 (m)                          | 0.165   | 0.06   | *F. hexaperta*  | IV  | 11.2    | 1.0    | 1                      | Trunk coelom                |
| OM992278/ZPCR_215 | Monilicaecum/Didymozoidae sp. 2 (m)                          | 0.15    | 0.05   | *F. hexaperta*  | I   | 5.8     | 1.0    | 2                      | Trunk coelom                |
| ON007167/ZPCR_112 | Monilicaecum/Didymozoidae sp. 3 (m)                          | 0.15    | 0.08   | *F. enflata*    | I   | 7.3     | 1.2    | 1                      | Trunk coelom                |
|                   | 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                |
Mexico. Zooplankton samples at Punta Lobos and Cabo Pulmo National Park were collected between surface and 10 m depth during 10 min in both coastal regions using a 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 – 6.6X 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 photographic and video documentation. Cercariae 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 (Canon G11, 10 MP resolution) fitted to a light microscope (Carl Zeiss, 10–40X magnification). Rediae and cercariae 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.

Didymozoid metacercariae parasitizing the coelom of the trunk of chaetognaths were observed and photographed and videotaped 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 photographs 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 species identification keys are currently available for the first larval stages of digeneans (sporocysts, rediae, and cercariae). Therefore, our specimens were only identified to superfamly 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; Koie 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; Koie and Lester 1985; Gómez del Prado-Rosas et al. 1999, 2007; Felizardo et al. 2011; Rodríguez-Ibarra et al. 2011; Lozano-Cobo et al. 2017, 2018; Morales-Ávila et al. 2018).

DNA analysis

Total DNA from four rediae recovered from the heteropod F. desmarestia, four metacercariae from the chaetognath Flaccisagitta enflata (Grassi, 1881), and two metacercariae from Flaccisagitta hexaperta (d’Orbigny, 1836) was extracted using the automated glass fiber protocol (Ivanova et al. 2006). DNA extraction was done at the 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´ TTTTGTGGCATCCT GAGGTTTTAT 3´ (Bowles et al. 1992) and CO1-R-Trema 5´ CAACAAATCATGATGCAAAGG 3´ (Miura et al. 2005). Amplification reactions were performed in a thermo-cycler (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 ddH2O. 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 Biosysystem 3500×1 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 (BLAST) analysis (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* (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 another 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 the present study. All sequences were aligned, and the genetic distances among pairs of sequences were calculated using the K2P model in the software PAUP* v.4.0 (Swoford 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 10,000 replicates using RaxML v. 8.2 program. Additionally, genetic distances (K2P model) 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 in the Gulf of California (Fig. 1A–B, Table 1). The heteropod hosts were identified as *F. desmarestia* 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. demarestia* specimens followed a slow zigzag 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 birth pore, located in the anterior end of the body (Fig. 1C, D; Online Resource 2). Released cystophorous cercariae were incubated at 22 °C (Figs. 1D and 2A–F) and transformed into a young metacercaria (or a cercaria without cyst) after 24 h and cercariae died 2 h later (Fig. 2G, H;

![Fig. 2](image-url) 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 and 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 = “Drusenma- gen”; ev = excretor vesicle; ma = motile appendage; os = oral sucker; tp = terminal pore. Scale bar = 10 μm.
Online Resource 3). Four of these rediae were characterized on the molecular basis obtaining three cox1 sequences (Table 1, Tables S1 and S2).

**Redia (Fig. 1A–D and Table 1)**

Alive rediae brown opaque (observed with a light stereoscope illuminated with bottom transmitted light) or pale whitish appearance inside the transparent heteropod host scope illuminated with bottom transmitted light) or pale whitish appearance inside the transparent heteropod host (observed with multispectral white light and black background) (Fig. 1A–D). Rediae with cylindrical and elongated body (3.05–3.18 mm length and 0.18–0.24 mm width, n = 3) (Fig. 1C). Pharynx with rounded muscular walls (Fig. 1D). Recovered redia had slow wriggling movements (Online Resources 1 and 2). Each alive redia (observed, immediately after field collection inside F. desmarestia hemocoel) contained hundreds of completely developed cystophorous cercariae (Online Resource 2). Thus, under experimental conditions those hundredths of cystophorous cercariae were expelled through the birth pore of the rediae at approximately one cercaria every 12 s swimming free in the hemocoel of their F. desmarestia host (Fig. 1D; Online Resource 2).

**Cercaria (Fig. 2A–F)**

Cystophorous cercaria has a caudal cyst enclosing the cercarial body with a delivery tube and bears an external motile appendage (Fig. 2A–C). Cercarial body oval (0.075 mm length and 0.030 mm in maximum width) just anterior to slight constriction between the body and tail. Body tegument with transverse circumferential folds (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 length and 0.008 mm width (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)**

Young metacercaria outside their cyst crawled at the bottom of the Petri dish transforming into metacercaria after 24 h at 22 °C (Online Resource 3). Metacercaria body oval, thick, and with transverse circumferential folds (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).

In short, we observed that the redia release numerous encysted cercariae, then they excyst showing their typical tail. However, we observed also numerous cercariae losing their tail almost at the same incubation time that we interpreted as “young metacercaria” that develop without need of an intermediate host. This alternative abbreviated development would be particularly advantageous when cercariae do not readily find an intermediate host (chaetognaths). However, we are aware that this abbreviated development was observed only under laboratory conditions and must be tested in field observations. The observation of metacercaria parasitizing chaetognaths from field collected specimens which never were observed vestiges of the cercaria cyst or tail inside the chaetognath hemocoel support this abbreviated development hypothesis.

**Parasites of chaetognaths**

A total of 51 out of 150 analyzed chaetognaths were found parasitized with at least one metacercariae (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 was based on six non-encysted Didymozoidae specimens parasitizing the chaetognath F. enflata and F. hexaperta (Table 1). Body oval to sub-cylindrical, elongate with rounded extremities, cuticle thick, without spines, transversely striated. Metacercariae body oval (0.148 mm mean length and 0.055 mm mean width, n = 10). 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 ventral sucker. Intestinal caecum at each side occupies lateral regions of body, moniliform type, and sinuous, and each one composed with 4–8 inflated chambers oriented towards the posterior end of 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.

**Molecular identification and phylogenetic analyses**

Ten metacercariae specimens were characterized on the molecular basis obtaining three distinct haplotypes of cox1 sequences (Table 1, Tables S1 and S2). In total, 10
cox1 sequences were generated; four rediae from redia parasitizing the heteropod *F. desmarestia* (GenBank accession codes OM925480, OM925482, OM992279, and OM925483), four from metacercariae parasitizing the chaetognath *F. enflata* (ON007165, ON007166, ON007167, OM925481), and two metacercariae specimens parasitizing the chaetognath *F. hexaptera* (OM992277 and OM992278) (Table 1, Table S2). BLAST analysis (Altschul et al. 1990) 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* Thatcher, 1979, *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 GenBank/Bold Systems 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 −12,212.44 (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 *F. desmarestia* and one specimen obtained from a metacercaria parasitizing the chaetognath *F. 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 *F. hexaptera*. Finally, the single Didymozoidae sp. 3 cox1 sequence was obtained from a metacercaria parasitizing *F. enflata*. No genetic variation was found within 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 to 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.

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 cox1 gene trees. Fish Perciformes hosts are showed in blue.
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. 2I, 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 codes 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 *F. desmarestia* and the metacercariae found in two chaetognath species (*F. enflata* and *F. hexaptera*) correspond to the same didymozoid species. Louvard et al. (2022) reported the first study of the life cycle of didymozoids (*Saccularina magnacetabula* Louvard, Cutmore, Yong, Dang, Cribb, 2022 infecting an arcid bivalve) using molecular evidence from partial *cox1* 217–695 pb sequences that unfortunately, does not overlap with the *cox1* 775–1625 pb sequences reported in the present study preventing comparison and inclusion of those sequences in our neighbor joining tree (Fig. 3).

The present 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 zooplankton taxonomic groups (Heteropoda and Chaetognatha). Thus, we provide the first observational and molecular 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 Hemirhamphidae) 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. Our results demonstrate a potential overlap in the distribution of two carnivorous zooplankton taxonomic groups that are intermediate hosts of didymozoids in the pelagic habitat. We demonstrate that *F. desmarestia* is the first intermediate host, evidenced by the presence of rediae and that cercariae were released in the hemocoel of *F. desmarestia*, as it was mentioned in two previous studies (Lester and Newman 1986; Morales-Ávila et al. 2018).

The controversial part of the life cycle has been to infer the function of chaetognaths in the life cycle of didymozoids, usually reported as paratenic hosts thus, metacercariae of didymozoids can be found in a wide variety of marine zooplankton groups (Madhavi 1968; Reimer et al. 1971, 1975; Shimazu 1978; Yip 1984; Gómez del Prado-Rosas et al. 1999, 2007; Øresland and Bray 2005; Lozano-Cobo et al. 2017, 2018). However, the chaetognaths are the most frequent hosts of didymozoid metacercariae stage, and with the molecular evidence of the present study, we were able to confirm that chaetognaths are the second intermediate host of didymozoids in the pelagic habitat, where non-encysted metacercariae were found. The definitive host is still unknown because *cox1* sequence of present study did not genetically match with any available *cox1* sequences of adult didymozoid of previous studies (Louvard et al. 2022). 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 diagnostic 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 didymozoid 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 didymozoid allowed us to determine the development rate, ontogeny, and behavior of the redia, cercaria, and metacercaria 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 (Alvaríno 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 Cox1 sequences and reported as highest level of interspecific genetic distance between Didymocystis pectoralis (Yamaguti, 1970) Podznyakov, 1990, and Didymosulcus spirocauda Yamaguti, 1970 (0.087–0.086) from Thunnus orientalis (Temminck & Schlegel, 1844) and Thunnus thynnus (Linnaeus, 1758). Koellikeria sp. renalis and Platocystis alalongae Yamaguti, 1938 (0.329) (from T. thynnus), and D. wedli Ariola, 1902 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 0.043 (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.

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Author contribution Horacio Lozano Cobo, original idea, sampling and specimen transportation, morphological identification of parasites and hosts, preparation of manuscript and edition of figures, text and videos; Claudia Alicia Silva-Segundo, molecular analysis and text edition; Alejandro Oceguera-Figueroa, molecular analysis and text edition; Carlos Jorge Robinson, sampling and specimens transportation, text edition and project administrator; Jaime Gómez-Gutiérrez, original idea, sampling and specimen transportation, preparation of manuscript and text and figure and video edition, and project administrator.

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Data availability Data of the present manuscript are available upon request to the first author.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval The zooplankton sampling and specimen analysis protocols that were developed in the present study fully complied with the ethical principles of animal experimentation prepared by regulations of the Mexican government.

Consent to participate The authors declare that they provide consent to participate in the present manuscript.

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Conflict of interest The authors declare no competing interests.

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