First documentation and molecular confirmation of three trematode species (Platyhelminthes: Trematoda) infecting the polychaete Marenzelleria viridis (Annelida: Spionidae)

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First documentation and molecular confirmation of three trematode species (Platyhelminthes: Trematoda) infecting the polychaete *Marenzelleria viridis* (Annelida: Spionidae)

Krystin Phelan¹ · April M. H. Blakeslee² · Maureen Krause¹ · Jason D. Williams¹

Received: 22 June 2015 / Accepted: 4 September 2015 / Published online: 18 September 2015
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**Abstract** Polychaete worms are hosts to a wide range of marine parasites; yet, studies on trematodes using these ecologically important species as intermediate hosts are lacking. During examination of the spionid polychaete *Marenzelleria viridis* collected on the north shore of Long Island, New York, putative trematode cysts were discovered in the body cavity of these polychaetes. In order to verify these cysts as metacercariae of trematodes, specimens of the eastern mudsnail *Ilyanassa obsoleta* (a very common first intermediate host of trematodes in the region) were collected for molecular comparison. DNA barcoding using cytochrome C oxidase I regions confirmed the presence of three species of trematodes (*Himasthla quissetensis*, *Lepocreadium setiferoides*, and *Zoogonus lasius*) in both *M. viridis* and *I. obsoleta* hosts. Brown bodies were also recovered from polychaetes, and molecular testing confirmed the presence of *L. setiferoides* and *Z. lasius*, indicating an immune response of the polychaete leading to encapsulation of the cysts. From the 125 specimens of *M. viridis* collected in 2014, 95 (76.8 %) were infected with trematodes; of these 95 infected polychaetes, 86 (90.5 %) contained brown bodies. This is the first confirmation that trematodes use *M. viridis* as a second intermediate host and that this intermediate host demonstrates a clear immune response to metacercarial infection. Future research should explore the role of these polychaetes in trematode life cycles, the effectiveness of the immune response, and transmission pathways to vertebrate definitive hosts.

**Keywords** Digenean · DNA barcoding · *Ilyanassa obsoleta* · Marine · Northwest Atlantic · Parasite

**Introduction**

Polychaete worms are ecologically important members of many benthic marine habitats that form critical links in food webs (Cosson et al. 1997; Marcogliese 2002). In addition, they can be important hosts for a range of marine parasites (Margolis 1971, 1973). Polychaetes have been documented as second intermediate hosts in the life cycles of a variety of trematode species (Peoples 2013); however, compared to the rich literature on other host groups, research on trematode/polychaete interactions and host specificity is lacking (but see Peoples and Poulin 2011; Peoples et al. 2012). Trematodes infect polychaete worms after being released from their first intermediate hosts (typically gastropod molluscs). Within gastropod hosts, trematodes replace host tissue (especially in the gonad region, resulting in castration) and may occupy a significant portion of the shell volume (Leung et al. 2009a). After developing as sporocysts and/or rediae and multiplying asexually, they emerge from the first intermediate host as free-swimming cercariae and seek a second intermediate host, which may be polychaetes or other invertebrate or vertebrate taxa. Within second intermediate hosts, they encyst as metacercariae until they are ingested by a definitive host (some trematodes lack a second intermediate host and form metacercarial cysts on substrates, or emerging cercariae can directly penetrate definitive hosts). Once in the
definitive host, trematodes complete their life cycle by reproducing sexually and releasing eggs through the host’s feces (Cribb 2005). Trematodes typically do not kill their hosts but can affect their behavior, overall fitness, and, scaled up to the community level, the structure of coastal systems (e.g., Mouritsen and Poulin 2005; Wood et al. 2007; Kuris et al. 2008).

Although some trematodes exhibit an abbreviated life cycle and reach sexual maturity within a single molluscan host (Poulin and Cribb 2002), most have a complex life cycle consisting of at least two hosts. They parasitize a diverse range of invertebrates (arthropods, molluscs, and annelids) and vertebrates across many ecosystems (Smith et al. 2007). A variety of vertebrates (e.g., fish, birds, and mammals) serve as definitive hosts for trematode species. Although trematodes exhibit an amazing diversity of life cycles, one nearly consistent characteristic of them all is the use of molluscs as a first intermediate host (Esch et al. 2002). Polychaetes worms represent the only other non-molluscan group documented to act as a first intermediate host to trematodes (see Køie 1982 and references therein). The trematode family Sanguinicolidae is the only digenean family known to infect non-molluscan first intermediate hosts such as marine polychaetes (Cribb et al. 2001).

Within the Polychaeta, 18 families are known to act as second intermediate hosts for trematodes (Peoples 2013); however, limited sampling of polychaetes for trematode infection suggests that there may be many undocumented potential hosts (Peoples et al. 2012). Within the family Spionidae, only five species, Polydora ciliata (Johnston, 1838), Polydora cornuta (=ligni) Bosc, 1802, Pygospio elegans Claparède, 1863, Spio sp., and Streblospio benedicti Webster, 1879 (McCurdy et al. 2000; Peoples 2013), have been shown to host trematodes. All of these species are host to the trematode Lepocreadium setiferoides (Miller & Northup, 1926), which is native to the North Atlantic and uses the eastern mudsnail Ilyanassa obsoleta (Say, 1822) as its first host and fish such as the flounder Pseudopleuronectes americanus (Walbaum, 1792) as its definitive host (Stunkard 1972). At least nine other polychaetes along the east coast of the USA are second intermediate hosts for trematodes after release of cercariae from I. obsoleta (Curtis 1997, 2009; Blakeslee et al., 2012) (Table 1). The present work reports on new records of trematodes in the spionid polychaete Marenzelleria viridis (Verrill, 1873) collected on the north shore of Long Island, New York.

The species of focus, M. viridis, is an infaunal polychaete that burrows in sandy substrates of brackish to estuarine waters along the east coast of North America from Nova Scotia to Georgia but has also been introduced to the Baltic Sea, North Sea, and Danish Waters (e.g., Maciolek 1984; Zettler 1997a, 1997b; Zander and Reimer 2002; Sikorski and Bick 2004; Blank et al. 2008; Delefosse et al. 2012). M. viridis is deposit-feeding polychaete (Miller et al. 1992) that can reach high densities and have important ecological impacts, particularly in reducing other infaunal macroinvertebrates, possibly through competition for food and space (Delefosse et al. 2012). M. viridis also contributes greatly to the ecology of benthic communities by accelerating nitrogen remineralization, biodeposition processes, bioturbation, and ventilation in the sediment (Kotta et al. 2001; Quintana et al. 2011; Renz and Forster 2013; Jovanovic et al. 2014).

While studying M. viridis on the coast of Long Island, New York, preliminary investigations discovered clear to light green cysts resembling those of trematodes inside the polychaetes. Also observed were brown, opaque cyst-like structures. We hypothesized that these structures could represent an immune response of the annelids caused by the encapsulation of foreign objects too large to be phagocytized (Sima 1994). Although best studied in oligochaetes, cellular immunity has also been found in several polychaetes that are known to encapsulate living and non-living material (Cuvillier-Hot et al. 2014). This encapsulation forms a granuloma, producing “brown bodies” or “granulomata” (Dales 1983; Porchet-Henneré et al. 1987, 1990; Porchet-Henneré and M’Berri 1987; Porchet-Henneré and Vernet 1992; Valembois et al. 1992, 1994; Beschin et al. 1999; Dhainaut and Scaps 2001; Reinhart and Dollahon 2003; Field et al. 2004; Procházková et al. 2006; Cuvillier-Hot et al. 2014). While metacercariae have been documented in a wide range of polychaetes, encapsulation and brown body formation have not been studied in detail and few records exist from polychaetes collected in the field (Shaw 1933). Polychaetes, particularly tube-dwelling species, are often infected by trematodes in anterior segments, most likely due to the branchial currents bringing cercariae into contact with this region first (Shaw 1933; Stunkard 1938; Brown and Prezant 1986; Rangel and Santos 2009; Peoples and Poulin 2011).

The purpose of this research was to investigate the cysts and brown bodies found in M. viridis. Specifically, we used DNA barcoding (as previously completed for flatworms: Vilas et al. 2005; Leung et al. 2009a, 2009b; Moszczyńska et al. 2009; Sanna et al. 2009) and light microscopy to determine which species of trematode metacercariae were present in the polychaetes. We suspected that the first intermediate host for the trematodes infecting M. viridis could be the abundant eastern mudsnail (I. obsoleta), a common host to several trematode species in the region. Thus, DNA barcoding was also performed on trematode species identified in the snail and then compared to the DNA from the polychaetes. Finally, the prevalence and distribution of metacercarial cysts and brown bodies within specimens of M. viridis were quantified. Altogether, our work describing this previously undocumented host-parasite relationship can help inform future ecological studies in the region, as well as potential impacts on the bird and fish predators that act as definitive hosts for the trematodes.
### Table 1

Nine trematode species that use *Ilyanassa obsoleta* as a first intermediate host. Their second intermediate hosts, definitive hosts, larval types, and cercarial morphology are provided. All hosts known (including experimental) from the northwestern Atlantic are listed.

<table>
<thead>
<tr>
<th>Trematode species</th>
<th>Taxonomic authority</th>
<th>Family</th>
<th>Second intermediate host(s)</th>
<th>Definitive host(s)</th>
<th>Larval type in snails</th>
<th>Cercarial morphological type</th>
<th>References for host data</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Diplostomum nassa</em></td>
<td>(Martin, 1945)</td>
<td>Diplostomatidae</td>
<td><em>Fundulus heteroclitus</em>, <em>Mugil cephalus</em></td>
<td><em>Bird species</em></td>
<td>Sporocysts</td>
<td>Strigea cercaria (forked-tailed, eyes absent)</td>
<td>Blakeslee et al. 2012</td>
</tr>
<tr>
<td><em>Paragonimus malaclemys</em></td>
<td>(Hunter, 1961)</td>
<td>Pronocephalidae</td>
<td><em>Chelonia mydas</em>, <em>Malaclemys terrapin</em></td>
<td><em>Chelonia mydas</em>, <em>Malaclemys terrapin</em></td>
<td>Redia</td>
<td>Monostomate cercaria (unforked tail with eyes present)</td>
<td>Blakeslee et al. 2012</td>
</tr>
<tr>
<td><em>Phanocercoides malaclemys</em></td>
<td>(Linton, 1900)</td>
<td>Acanthocolpidae</td>
<td><em>Menidia menidia</em>, <em>Paralichthys dentatus</em>, <em>Sphoeroides maculatus</em></td>
<td><em>Menidia menidia</em>, <em>Paralichthys dentatus</em>, <em>Sphoeroides maculatus</em></td>
<td>Redia</td>
<td>Parapleurolophocercous cercaria (unforked tail with lateral finfolds, eyes present)</td>
<td>Blakeslee et al. 2012</td>
</tr>
<tr>
<td>Trematode species</td>
<td>Taxonomic authority</td>
<td>Family</td>
<td>Second intermediate host(s)a</td>
<td>Definitive host(s)</td>
<td>Larval type in snails</td>
<td>Cercarial morphological typeb (key features used to identify)</td>
<td>References for host data</td>
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</tr>
<tr>
<td><em>Stephanostomum tenue</em>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(Linton, 1898)</td>
<td>Acanthocolpidae</td>
<td><em>Anguilla rostrata</em>, <em>Fundulus heteroclitus</em>, <em>Menidia menidia</em></td>
<td><em>Ammodytes americanus</em>, <em>Hemitripterus americanus</em>, <em>Menticirrhus saxatilis</em>, <em>Mormone saxatilis</em>, <em>Opsanus tau</em>, <em>Spheroideida maculatus</em></td>
<td>Redia</td>
<td>Oculate gymnocephalous cercaria (unforked tail, eyes present)</td>
<td>Curtis 2009</td>
</tr>
<tr>
<td><em>Zoogonus lasius</em>&lt;sup&gt;d&lt;/sup&gt;</td>
<td>(Leidy, 1891)</td>
<td>Zoogonidae</td>
<td><em>Alitta virens</em>, <em>Arabella iricolor</em>, <em>Arctica punctulata</em>, <em>Bdelloura candida</em>, <em>Hydroides sp.</em>, <em>Leonereis culveri</em>, <em>Lumbrineris hebes</em>, <em>Scalakites rubera</em>, <em>Testudinalia testudinalis</em></td>
<td><em>Anguilla rostrata</em>, <em>Liopsetta putnami</em>, <em>Leiostomus xanthurus</em>, <em>Trinectes maculatus</em>, <em>Menidia menidia</em>, <em>Parachirys dentatus</em>, <em>Opsanus tau</em>, <em>Tautoga onitis</em></td>
<td>Sporocysts</td>
<td>Cercariaeum cercaria (tail absent, eyes absent)</td>
<td>Bray 1987; Shaw 1933</td>
</tr>
</tbody>
</table>

<sup>a</sup> Many of these represent experimental infections

<sup>b</sup> From McDermott 1951; Schell 1970; Yamaguti 1975; Stunkard 1970, 1983

<sup>c</sup> Martin (1939) indicated that this species had cercaria with a stylet and reported the metacercaria of the trematode from the Atlantic silverside *Menidia menidia* and the American eel *Anguilla rostrata*; the species has a wide range of fish definitive hosts including the striped bass *Roccus saxatilis*. Later, McDermott (1951) found differences between the sizes and morphology of his specimens and those from New England, including lack of a stylet and setae in the cercaria. As indicated by McDermott (1951), the stylet may be missing in those naturally emerging from snails or perhaps overlooked. Positive PCR amplification of Long Island samples dissected from *I. obsoleta* in the present study also suggests the presence of *S. tenue*, the stylet and setae appeared to be absent in these samples. These findings indicate that there may be two species under the name *S. tenue*, and future researchers should investigate this possibility with molecular data, including material from the type locality of *S. tenue* (Woods Hole, MA) and compare it with material from New York and New Jersey. The species has also been reported from the Pacific Coast of Mexico, and this record should be reexamined (Bravo 1956)

<sup>d</sup> Many previous authors considered this species a synonym of *Zoogonus rubellus* (Olsson, 1868), but as shown by Bray and Gibson (1986) and Bray (1987); these are distinct species. *Zoogonus rubellus* is found in the northeastern Atlantic, the Mediterranean Sea, the Red Sea, and the west coast of Africa, whereas *Z. lasius* is restricted to the northwestern Atlantic
Materials and methods

Sample collection and storage

*M. viridis* polychaetes and *L. obsoleta* mudsnails were collected from Hempstead Harbor located in Sea Cliff, New York (40° 50′ 27.33″ N, 73° 39′ 11.65″ W) from May 2013 to July 2014. Polychaetes (*n=33* in 2013, *n=125* in 2014) were collected with a shovel and sieve (0.5 mm mesh size) and placed in buckets containing unfiltered seawater. The 2013 collection of polychaetes was non-random; we specifically isolated polychaetes that appeared to contain cysts in order to document the presence of trematodes and refine amplification and sequencing protocols necessary for that work. The 2014 polychaetes were collected randomly to assess prevalence and incidence of trematode infection. Mudsnails (*n=274* in 2013, *n=100* in 2014) were collected haphazardly by hand at the same locality. After transport to the lab, mudsnails were stored in a refrigerator at −14 °C with aeration until time of dissection and analysis. Specimens of *M. viridis* were dissected within 24 h of collection.

Sample dissection

Shells of live *L. obsoleta* were cracked, and confirmation of trematodes was made by the presence of cercariae and sporocysts and/or rediae in the tissue dissected from the digestive and/or reproductive glands using a compound light microscope. Larval trematodes were identified based on sporo- or rediae in the tissue dissected from the digestive and/or reproductive glands using a compound light microscope. Larval trematodes were identified based on sporocysts and/or rediae in the tissue dissected from the digestive tract of polychaetes and/or mudsnails, respectively. After transport to the lab, mudsnails were stored in a refrigerator at −14 °C with aeration until time of dissection and analysis. Specimens of *M. viridis* were dissected within 24 h of collection.

In order to immobilize *M. viridis* for dissection, the polychaetes were submerged in 3 % MgCl~2~ (~50:50 mixture of 7 % MgCl~2~ and seawater) and placed in the freezer for approximately 20 min. Specimens of *M. viridis* often had sand and organic particles in the posterior end of their digestive tract, making it difficult to observe/certify cysts in this region; for this reason, only the anterior segments (~30–60 segments) were analyzed. After cutting off the posterior end, the anterior end of the polychaete was squashed between two microscope slides and observed under an Olympus SZX12 stereomicroscope and Olympus CX31 compound microscope. The total number of segments of these anterior ends, their length (in mm), and total number of cysts and brown bodies found throughout the region were quantified. The first (anterior-most) segment containing cysts or brown bodies was recorded; when polychaetes had cysts or brown bodies in >1 segment, the last (anterior-most) segment infected was also recorded.

When small ovoid clear or brown cysts were observed during analysis, the individual cyst or a small section of the polychaete was cut out with a micro-scalpel and preserved in 70 % ethanol for molecular testing. In order to provide enough template material for molecular studies, in some instances, more than one cyst and/or brown body per polychaete were preserved and analyzed together. During the observation of polychaete samples, the presence of both clear to light green cysts and brown opaque cysts, or brown bodies, were recorded. Pictures of each type of cyst were taken with an Olympus CX31 with Olympus DP11 camera; ImageJ was used to make length and width measurements from these images.

Molecular analyses

In order to excise the parasite tissue from polychaete samples, mechanical homogenization was required before DNA extraction. Fifty microliters of 5–10 % Chelex®-100 (Bio-Rad) slurry and approximately 40–50 mg of 0.5-mm BioSpec glass beads were added to the samples. Samples were vortexed for approximately 10 min. Afterwards, a simple Chelex® extrac- tion protocol was followed (Walsh et al. 1991).

Because DNA extracted by Chelex did not always result in PCR amplicons in preliminary samples, a MOBIO Laboratories, Inc. Ultraclean™ PCR Clean-up™ Kit was used before performing PCR in an attempt to remove additional PCR inhibitors following the manufacturer’s protocol. Approximately 50 μL of Chelex-purified sample was processed. A final volume of approximately 50 μL was obtained using the elution buffer (10 mM Tris).

To determine which trematode species were present in the collected samples, multiple primers amplifying either the 18S or cytochrome c oxidase I (COI) barcoding regions were used (Supplemental Table 1). All primers were ordered from Invitrogen. “18S1” and “18S2” are universal 18S primers designed for metazoan species (Machida and Knowlton 2012). The “Hq_COIF”/“Hq_COIR,” “Ls_COIF”/“Ls_COIR,” and “Zl_F”/“Zl_R” primers are forward and reverse primers specific to the cytochrome C oxidase I region in the trematode species *Himasthla quissetensis* (Hq), *L. setiferoides* (Ls), and *Zoogonus lasius* (Zl); note that previous records of this species from the east coast of the USA as *Zoogonus rubellus* are actually *Z. lasius*; see Table 1), respectively (Blakeslee and Fowler 2012). Each PCR reaction consisted of a total volume of 30 μL and contained a final concentration of 100 μM of both forward and reverse primers along with 1× OneTaq Master Mix (New England Biolabs) containing 0.2 mM dNTPs, 20 mM Tris HCl, 22 mM KCl, 22 mM NH₄HCl, and 1.8 mM MgCl~2~, approximately 300 ng of template DNA. Using the 18S1/18S2 and Hq_COIF/Hq_COIR primers, the degenerate 18S and *H. quissetensis* cytochrome oxidase fragments were amplified using a program which consisted of denaturing at 95 °C for 1 min, followed by 45 cycles of denaturing at 94 °C for 20 s, annealing at 50 °C for 20 s, and extending at 68 °C for 2 min with a final extension at 68 °C for 10 min. The Ls_COIF/Ls_COIR and Zl_F/Zl_R primers were used to
amplify the *L. setiferoides* cytochrome oxidase fragment using a program which consisted of 96 °C for 1 min, followed by 45 cycles of 94 °C for 20 s, 52 °C for 30 s, 72 °C for 1 min, and a final DNA extension at 72 °C for 10 min.

For select samples that did not amplify with any COI-specific primers and amplified mostly or all host DNA using the 18S1/18S2 primers, the “worm A” and “worm B” 18S primers were used. These primers flank variable domains 1–6 of the 18S ribosomal DNA (Machida and Knowlton 2012), a smaller region than the entire small subunit amplified by the 18S1/18S2 primer pair. They were amplified under the same conditions as the Hq_COIF/Hq_COIR and 18S/18S2 primers. If host DNA was detected in the 18S sequences using the worm A and worm B primers, this primer set served as a shorter sequencing alternative to try to obtain parasitic-specific DNA.

Samples were sent out for Sanger sequencing to Macrogen Corporation (Rockville, MD, USA). In order to compare sequences, a contiguous sequence of the forward and reverse amplified DNA for each sample was made using BioEdit’s CAP contig assembly program using a minimum base overlap of 20 bases and an 85 % match minimum. The ClustalW multiple nucleotide sequence alignment tool was used to align these sequences (Li 2003). Both the distance-based neighbor-joining and character-based maximum likelihood analysis algorithms were used for phylogenetic estimation (Hall 2011). Sequences of *H. quissetensis*, *L. setiferoides*, and *Z. lasius* were used as in-groups for these estimations while a reference sequence for the liver fluke *Fasciola hepatica* (Linnaeus, 1758) was used as an out-group. For each analysis, the best evolutionary model was chosen in MEGA after the data was grouped by gene, either 18S or COI. The genetic pairwise distance, using the simultaneous estimation method in MEGA 6, within each species was calculated (Tamura et al. 2004). A bootstrap method of 2000 replicates with the maximum composite likelihood methods were used for these calculations (Efron 1982). COI sequences for three specimens of *H. quissetensis* (two samples from *I. obsoleta* and one sample from *M. viridis*), *L. setiferoides* (one sample from *I. obsoleta*), and *Z. lasius* (four samples from *M. viridis*), as well as 18S sequences for *H. quissetensis* previously analyzed by Blakeslee (unpub.) from infected *I. obsoleta*, were used for comparison with our cyst samples from the polychaetes. Consensus COI and 18S sequences for the trematode species are shown in Supplemental Figs. 1 and 2, respectively.

**Results**

*M. viridis* dissected during this study were found with encysted metacercariae (Fig. 1a, b) and/or brown bodies (Fig. 1c, d) that contained trematode DNA. Though difficult to distinguish based on light microscopy unless cysts are removed from hosts, the metacercariae of each species have characteristic traits that distinguish them. Metacercariae of *H. quissetensis* have a collar of small spines surrounding the anterior end (Fig. 1e), whereas *Z. lasius* metacercariae have a single stylet positioned in the center of the oral sucker (Fig. 1f). Free-swimming trematode cercariae released from the first intermediate host also have species-specific traits (Table 1). The cercariae of *H. quissetensis* are released from sporocysts and lack eyespots and have an unforked tail and a spinous cuticle. The cercariae of *L. setiferoides* are released from rediae and have eyespots and a setose tail. The cercariae of *Z. lasius* are released from sporocysts and lack eyespots and a tail.

Among the 33 polychaete samples found with cysts in 2013, 17 contained clear cysts, nine contained isolated brown body cysts, and seven contained a mixture of both clear and brown cysts. Fourteen of these samples were successfully sequenced: One was confirmed to contain only *H. quissetensis*, 11 contained only *Z. lasius*, and two samples contained both *H. quissetensis* and *Z. lasius*. No polychaete samples collected in 2013 tested positive for *L. setiferoides*. The remaining 19 samples did not amplify with any available primers, which may be because of DNA degradation, too little template material, or because the cysts represent other trematode species we did not have primers for. Of the 14 samples that amplified, four were from isolated brown body cysts, confirming that they contain trematode parasite DNA.

In 2013, 274 mudsnails were analyzed and 13 (4.7 %) were parasitized by trematodes. Based on light microscopy examination of the cercariae, these were provisionally identified as *Diplostomum nassa* (one specimen), *H. quissetensis* (three specimens), *L. setiferoides* (two specimens), *Stephanostomum tenue* (five specimens), and *Z. lasius* (two specimens). In subsequent molecular analyses, four of these samples were omitted because they were sequenced before the universal 18S1/18S2 primers for *H. quissetensis*, *L. setiferoides*, and *Z. lasius* were available. Of the nine samples with positive PCR amplification, four were confirmed (three as *H. quissetensis* and one as *L. setiferoides*), four (one *L. setiferoides* and three *Z. lasius*) did not match the morphological identification, suggesting the possibility of co-infection, and one sample did not amplify.

In 2014, 95 (76.8 %) of the 125 *M. viridis* dissected were found to be parasitized based on light microscopy. Of the 95 infected polychaetes, 35 (36.8 %) contained cysts and brown bodies, whereas 10 (10.5 %) contained cysts only and 50 (52.6 %) contained brown bodies only. The cysts and brown bodies overlapped in distribution within the polychaetes: they were found from segments 1 to 50 and 2 to 53, respectively (Table 2). There was no significant difference in the first and last segments of the cysts and brown bodies within *M. viridis* (first segment $t_{130}=0.94$, $P=0.35$; last segment $t_{52}=1.23$, $P=0.22$). There were significantly more brown bodies than clear cysts identified within specimens of *M. viridis* across all our
samples ($t_{106}=-9.1, \ P<0.001$). On average, cysts ($n=47$) were $0.16\pm0.06$ mm long and $0.18\pm0.06$ mm wide whereas brown bodies ($n=54$) were $0.12\pm0.03$ mm long and $0.14\pm0.11$ mm wide. The cysts were significantly larger than brown bodies in both length ($t_{68}=4.31, \ P<0.001$) and width ($t_{82}=2.25, \ P=0.027$).

Table 2  The average length and number of segments of the anterior ends of Marenzellaria viridis dissected and examined for trematode metacercaria. Number of cysts and brown bodies in each worm and the range of segments they were found in are reported. Mean ± standard deviations are presented; sample size is 125 for all measures.

<table>
<thead>
<tr>
<th>No. of segments</th>
<th>Length</th>
<th>Cyst presence</th>
<th>Brown body presence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of cysts</td>
<td>First seg mean</td>
</tr>
<tr>
<td>44.73±6.77</td>
<td>15.39±2.66</td>
<td>0.5±0.75</td>
<td>20.18±12.87</td>
</tr>
</tbody>
</table>
Of the 77 cysts and/or brown body samples tested with PCR, 46 samples had positive primer species-specific results, 35 with Z. lasius COI-specific primers, and 6 with the L. setiferoides COI-specific primers. Five samples positively amplified with both sets of primers. There were no positive identifications of H. quissetensis DNA in these samples. Although we did not have positive amplification in polychaetes, H. quissetensis was present snails (see below).

Overall, nine (9 %) of the gastropod samples from 2014 were infected with trematodes. Based on light microscopy examination of the cercariae, these were provisionally identified as H. quissetensis (one specimen), L. setiferoides (three specimens), S. tenue (one specimen), Z. lasius (two specimens), and unidentified (two specimens). Molecular data confirmed four of these as trematodes (two H. quissetensis and two Z. lasius) and confirmed one of the unidentified trematodes as Z. lasius. Positive PCR amplicons suggested the possibility of co-infection in two samples and did not support the morphological identification of one of the L. setiferoides samples. One sample did not amplify.

Phylogenetic trees were constructed using both the neighbor-joining and maximum likelihood methods with the COI and 18S sequences (Fig. 2). COI data produced monophyletic groupings of H. quissetensis and Z. lasius, whereas three samples of L. setiferoides were monophyletic and a fourth sample came out as basal to all in-group taxa (Fig. 2a, b). In the 18S analysis, a monophyletic grouping of H. quissetensis was also found. However, L. setiferoides grouped with Z. lasius, likely due to the fact that there was no L. setiferoides 18S sequence for reference (Fig. 2c, d).

Discussion

This study is the first confirmation of the polychaete M. viridis acting as a second intermediate host for any trematode species. A high prevalence of metacercarial cysts was found in M. viridis (~77 % of polychaetes in 2014), similar to a study by McCurdy et al. (2000), which found high prevalence of trematodes in the spionids P. elegans (75 %) and S. benedicti (50 %). In total, molecular data confirmed three species of trematodes (H. quissetensis, L. setiferoides, and Z. lasius) from metacercariae in the polychaetes, all of which utilize I. obsoleta as their first intermediate host. Thus, it appears as if exposure to infected I. obsoleta from the same, or a nearby site, can result in high levels of infection in specimens of M. viridis. Even so, it remains unclear whether these trematodes would then be tropically transmitted from the polychaetes to suitable definitive hosts. For example, one of the trematode species (H. quissetensis) found infecting M. viridis use birds (seagulls and others) as definitive hosts (Table 1), but these types of birds have not been documented to feed on M. viridis. However, these birds do feed on infaunal invertebrates, like other species of polychaetes (Ambrose 1986; Heard 1982; Leopold and van Damme 2003), so it remains possible that trophic transmission could be occurring for this trematode species. In contrast, both L. setiferoides and Z. lasius use fish as their definitive hosts (Table 1), and both have also been documented to infect other polychaete species. M. viridis has been found in the guts of fish (Essink and Kleef 1991; Winkler and Debus 1996; Derrick and Kennedy 1997; Sardá et al. 1998) and thus may be an appropriate intermediate host for these trematodes. Detailed examination of these potential definitive hosts is required to determine whether trophic transmission is successfully occurring or if M. viridis instead represents a sink for these trematode species, acting as a low competency host or part of a “dilution effect” preventing transmission (Johnson and Thieltges 2010; Koppel et al. 2011).

Another noteworthy result of our work is that we determined the brown bodies detected in M. viridis contain trematode DNA, confirming that they are encapsulated cysts. Thus, ours is the first study to reveal an immune response in this polychaete as a result of trematode infection. However, the time course of encapsulation and viability of brown bodies remain unknown and should be tested in the future. In prior work, several polychaetes (Hydroides sp., Lumbrineris hebes, Scoloplos robusta, and Arabella iricolor) have been shown to encapsulate the metacercaria of Z. lasius, which appeared to be dead within the capsule; alternatively, in another polychaete (Alitta virens), the metacercaria remained viable in the parapodia for over 3 weeks (Shaw 1933). Moreover, Koie (2000) noted encapsulation of a nematode parasite (Cucullanus heterochrous) during experimental infections of Hediste diversicolor and other polychaetes and showed that the nematode larvae were uninfective to fish definitive hosts after 3 weeks. Encapsulation of nema-
todes has also been found in oligochaete hosts, which similarly appeared to be non-inflective (Poinar and Hess 1977), and in some cases was eliminated by autonomy from the oligochaete (Bilej 1994).

We also found the distribution of live cysts and brown bodies to overlap within the anterior segments of the polychaetes. The prominence of cysts in the anterior region is probably the result of cercariae being drawn in by water currents created by the branchiae of M. viridis (Quintana et al., 2011). Such findings are similar to Rangel and Santos (2009) who showed the metacercariae of Gymnophallus choledochus almost exclusively in the branchial segments of Diopatra neapolitana and suggested that the cercariae may penetrate the branchiae directly. Peoples (2013) indicated that trematode cercariae may have evolved to penetrate the anterior region of polychaetes because they are more likely ingested by predators, thus increasing the likelihood for trophic transmission. In M. viridis, future studies should examine cercarial penetration of polychaete hosts, focusing on the factors that may influence their distributions within the polychaete.
method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. c Phylogenetic tree based on 18S rRNA using the neighbor-joining method. The optimal tree with the sum of branch length is shown. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. d Phylogenetic tree based on 18S rRNA using the maximum likelihood method based on the Tamura 3-parameter model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. e Phylogenetic tree based on 18S rRNA using the neighbor-joining method. The optimal tree with the sum of branch length is shown. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. Samples with the prefix “p” were from polychaetes; samples with the suffix “C” were from molluscs.

Fig. 2 Phylogenetic trees of trematodes based on the COI region and 18S rRNA. a Phylogenetic tree based on the COI region using the neighbor-joining method in MEGA6. The optimal tree with the sum of branch length is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. b Phylogenetic tree based on the COI region using maximum likelihood method based on the Tamura 3-parameter model using MEGA6. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood approach. c Phylogenetic tree based on 18S rRNA using the neighbor-joining method. The optimal tree with the sum of branch length is shown. The percentage of replicates in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. d Phylogenetic tree based on 18S rRNA using the maximum likelihood method based on the Tamura 3-parameter model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Samples with the prefix “p” were from polychaetes; samples with the suffix “C” were from molluscs.

Studies in other annelids have found trematodes to influence fragmentation and survival of hosts. For example, McCurdy (2001) found that when the spionid P. elegans was infected with the trematode L. setiferoides, it fragmented earlier than controls, suggesting that such early fragmentation could be a host response to minimize costs on the polychaete—–even so, negative impacts were still evident in that parasitized individuals were smaller and had lower survivorship (McCurdy 2001). Similarly, McCurdy and Moran (2004) found that Alitta virens experimentally infected with metacercariae had higher mortalities and foraged less than uninfected individuals. M. viridis is able to regenerate both posterior and anterior body segments (Williams, pers. obs.), so future studies could examine whether trematodes similarly influence fragmentation, regeneration, and survivorship in this species. Other trematodes have been documented to impact polychaete hosts, including G. choledochus Odhner, 1900 which can invade the parapodia and cause disruption of the polychaete setal sacs (Rangel and Santos 2009).

The ultimate fate of the trematodes we detected in M. viridis is unclear and requires further investigation, along with the immune response of the polychaetes as a result of trematode infection. Future research could examine the progression of brown body formation and be coupled with experimental studies testing cyst viability by feeding the encapsulated metacercariae at various stages to definitive hosts. Moreover, because M. viridis has been documented as a non-native species in the Baltic Sea, North Sea, and Danish Waters (Zettler 1997a, 1997b; Zander and Reimer 2002; Sikorski and Bick 2004; Blank et al. 2008; Delefosse et al. 2012), researchers should also explore its potential role as a second intermediate host in these regions. Zander and Reimer (2002) examined an introduced population of M. viridis in the Baltic Sea and did not find any parasites, although trematodes were confirmed in other polychaetes and molluscs in the area.

In conclusion, we report the first record of M. viridis as a host of trematode metacercariae, finding three trematode species in the polychaetes. Due to that fact that relatively few polychaetes have been investigated as second intermediate hosts, there are likely many additional species that harbor metacercariae remaining to be discovered. Our findings indicate that M. viridis could be an important player in the life cycle of trophically transmitted trematodes that infect a wide range of vertebrate
hosts. Future research should focus on elucidating the role of polychaetes in aiding or impeding the transmission of these ecologically important parasites.

Acknowledgments The support of Hofstra University is appreciated, particularly Faculty Research and Development Grants to MKK and JDW.

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