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FEATURE ARTICLE

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Discovery of indirect parasite life cycles at deep-sea hydrothermal vents

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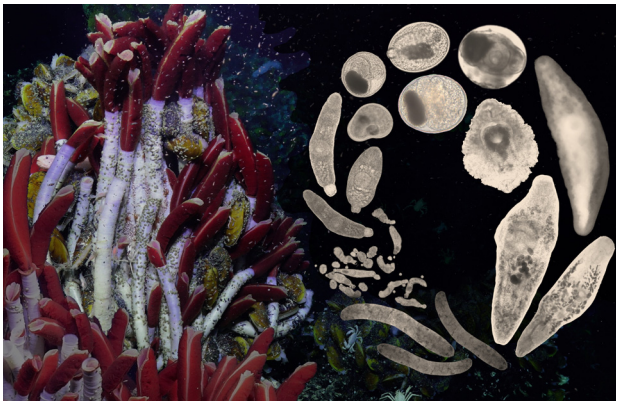
ABSTRACT: Deep-sea hydrothermal vents are extreme, isolated, and ephemeral ecosystems populated by unique endemic fauna, making them a potentially challenging setting for the survival of parasites with indirect (multi-host or 'complex') life cycles. We examined the animal communities at a frequently disturbed hydrothermal vent field (9° 50' N, East Pacific Rise) to explore whether parasites with indirect life cycles complete their life cycles within this island-like, ephemeral marine habitat, which has been previously hypothesized but is not known. Our dissections of individuals from 51 vent species revealed 7 morphogroups of parasitic flatworms (trematodes) including the genera *Biospeedotrema*, *Caudotestis*, and *Neolebouria*, and 4 life stages in the multi-host trematode life cycle. Adult trematode life stages lived in the vent fishes *Thermarces cerberus* and *Thermichthys hollisi*, while the intermediate life stages were found in a variety of crustacean, gastropod, and polychaete species. Genetic barcoding of the 18S, 28S, and ITS2 regions linked sequential life stages for some of the morphogroups, revealing the phylogenetic position and routes of transmission between hosts for vent trematodes. This study provides the most direct evidence yet that parasites very likely complete indirect life cycles in deep-sea hydrothermal vent ecosystems, with important implications for the persistence and diversity of parasites in disturbed environments.

KEY WORDS: Digenea · Hydrothermal vents · Life cycles · Parasite diversity · Phylogenetics · *Thermarces cerberus* · *Thermichthys hollisi* · Trematode



Multiple life stages of diverse parasitic flatworms discovered at deep-sea vents in the eastern Pacific.

Images: Woods Hole Oceanographic Institution; Lauren N. Dykman

1. INTRODUCTION

Parasites rely on hosts, and thus parasite diversity and abundance can provide critical information about ecosystem functioning and relationships between host species (Combes 1996, Lafferty & Kuris 2005, Lafferty 2012, Bitters et al. 2022). Parasites are known to be sensitive to environmental disturbances that alter host diversity, density, or trophic structure (Lafferty 1997, Wood et al. 2014) and some are directly harmed by environmental toxins such as heavy metals (Cross et al. 2001). For this reason, parasites have increasingly been used as indicators of environmental degradation (Lafferty 1997, Marcogliese 2005) or successful ecosystem restoration (Huspeni & Lafferty 2004, Moore et al. 2020).

Parasites with indirect life cycles (ILCs) are of particular interest in the context of ecosystem function because they must pass through multiple hosts — usually of very different taxa — to reproduce. For ILC parasites, transmission between hosts is a critical, risky step that occurs multiple times in one generation and may require transmission as free-swimming larvae or by one host feeding on another (trophic transmission) (Combes et al. 2002) (Fig. 1). All these transitions are made more efficient by high host den-

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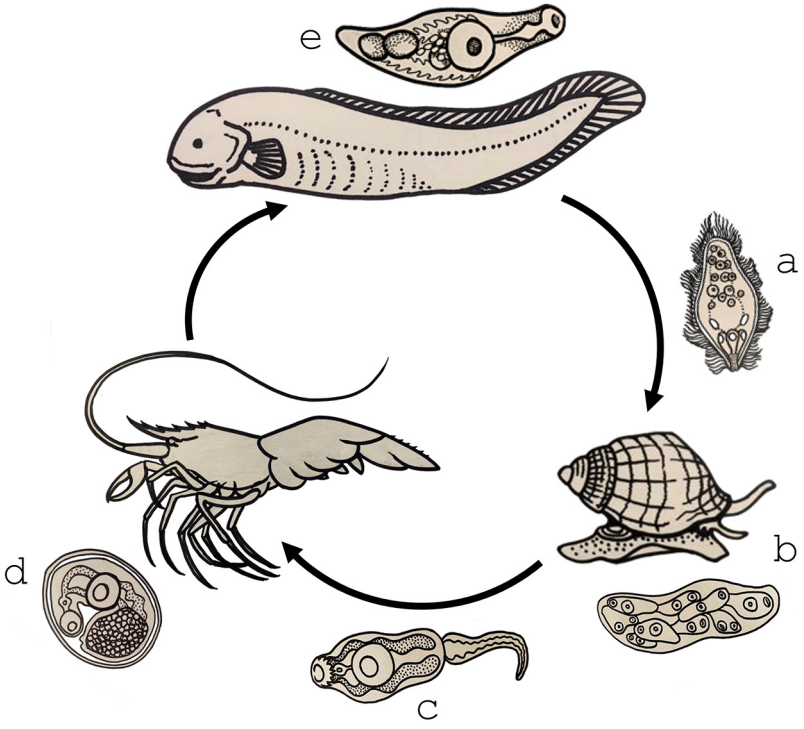

Fig. 1. An example of an indirect life cycle for a theoretical digenean species. Eggs are shed from the vertebrate definitive host and hatch a ciliated larva called a miracidium (a). The miracidium penetrates a molluscan first intermediate host, in this case, a snail, and forms asexually reproducing clones called sporocysts and rediae (b) in the host gonad or digestive gland. Sporocysts shed free-living larvae called cercariae (c). Cercariae find a second intermediate host which can be from a wide range of taxa, including mollusks, fish, polychaetes, and crustaceans. The cercaria enters the second intermediate host and encysts as a metacercaria (d). The metacercaria infects the definitive host when the latter feeds on the second intermediate host. The adult trematode (e) develops in the vertebrate definitive host (in this example, a fish) and sexually reproduces, shedding eggs to begin the cycle anew

sity or intact food chains, both of which are often altered by environmental disturbance (McCauley et al. 2012). Due to their diversity of hosts and life cycle transitions, ILC parasites are expected to face greater difficulty in completing their life cycles in disturbed habitats and be less likely to establish in isolated habitats (Dobson & May 1986). These expectations, however, have rarely been tested in remote systems with extreme rates of natural disturbance (Dykman et al. 2023a). Naturally disturbed or ephemeral settings allow us to understand which parasite life strategies are successful in disturbed environments when given the chance to invade and adapt to the system on evolutionary timescales.

Deep-sea hydrothermal vents are island-like, often ephemeral habitats, making them a compelling system in which to explore the boundaries of parasite persistence. Vent ecosystems have a well-constrained area set by geochemical outflow along oceanic plate spreading centers and back-arc basins (Tunnicliffe & Fowler 1996). These systems host unique endemic fauna fueled by chemosynthetic microbial production rather than by sunlight (Van Dover 2000). Most vent animals spend their adult lives close to centers of active venting and disperse as planktonic larvae (Adams et al. 2012), maintaining connectivity between separate vent populations (Mullineaux et al. 2010). Some vent systems, like the vent field at 9° 50′ N on the East Pacific Rise (EPR 9N), experience large-scale seafloor eruptions every 10–20 yr (Fornari et al. 2012). Natural disturbance on this scale requires all vent species to recolonize from afar (Mullineaux et al. 2010), while ILC parasites not only need to colonize from afar but also require 2 or more host species to colonize first and reach high enough densities to sustain transmission. If ILC parasites manage to persist in such a harsh environment, it is likely that they will complete their entire life cycles in the vent environments, since the interspecific interactions and feeding spheres of vent species are constrained to a small area.

Recent investigation into parasite diversity at EPR 9N revealed 10 morphogroups of 3 ILC parasite taxa exploiting vent hosts (Dykman et al. 2023a). These taxa (acanthocephalans, digeneans, and nematodes) live as adults in the 2 vent-endemic fish species at this vent field: the zoarcid eelpout *Thermarces cerberus* and the bythitid *Thermichthys hollisi*. Five digenean species (parasitic flatworms in Class Trematoda, Subclass Digenea) in the adult stage have previously been described from the vent fish *T. hollisi* from the Southern EPR (Bray et al. 2014). All these parasite taxa reproduce sexually as adults in the fish host but also have life stages in one or more invertebrate hosts, with the fish host being infected by feeding on invertebrate prey. Neither fish species was found to act as a paratenic host. Previously, one larval acanthocephalan and one larval nematode have been reported in vent hosts worldwide (de Buron et al. 2000, Desbruyères et al. 2006) but life stages have not yet been definitively linked. Observation and dietary analysis indicate that vent fish live and feed almost exclusively in vent habitat (Buckman 2009), making it likely that their parasites use vent invertebrates at other life

stages. The high incidence of endemic species at vents (Chapman et al. 2019) further suggests that vent parasites might be endemic as well, evolved to specialize in unique hosts in an extreme environment.

Here, we report the discovery of 4 life stages in the digenean life cycle (sporocyst, cercaria, metacercaria, and adult) of multiple species from the EPR 9N hydrothermal vent field. This study has 2 objectives: (1) to morphologically and genetically match life stages to determine whether the recently discovered digenean species complete their life cycles entirely within the vent environment; and (2) to place these species in the phylogenetic context of other deep-sea digenean species to investigate potential origins of introduction into the vent environment. Given that digeneans, acanthocephalans, and nematodes are common in deep-sea fishes (Campbell et al. 1980), it is impossible to determine whether the parasite species found in *T. cerberus* and *T. hollisi* might be endemic to vents without demonstrating that they complete all life cycle stages in vent hosts.

Knowledge of the hosts that are required to complete parasite life cycles is necessary to understand the conditions that a parasite needs to survive in an ecosystem, and can provide glimpses into its evolutionary history. Despite compelling reasons for resolving life cycles, most parasites are described only by the morphology of the adult (Blasco-Costa & Poulin 2017). Life cycle studies were a central focus of parasitology in the 1950s and 1960s, but the marked decrease in life cycle work in the decades since has inspired a call to 'resurrect an old tradition' (Blasco-Costa & Poulin 2017). The completion of life cycles, which requires the linking of all life stages, is usually achieved through a combination of morphological description, experimental infections, and, more recently, matching of genetic sequences by DNA barcoding. Life cycle description is particularly difficult in large, open systems like the deep sea (Bray 2020), where host species can be highly mobile and patchy, species occurrences and ranges are poorly known, and animals generally cannot be kept alive in culture. Few studies have sampled whole multi-trophic host communities and linked the life stages of many parasite species using genetic techniques (but see Bennett et al. 2023). Unsurprisingly, a similar effort has not been undertaken in the deep sea, and the life cycle of a multi-host, deep-sea parasite has yet to be resolved. We aim to fill this knowledge gap by attempting to describe the life cycles of several digenean parasite species at deep-sea hydrothermal vents. In addition to contributing to our basic knowledge of parasite–host interactions and parasite larval life stages at vents, the results of this study demonstrate that parasites with ILCs survive and thrive in extreme, disturbed, and isolated settings.

2. MATERIALS AND METHODS

2.1. Biological collections and dissections

Vent animals were collected from a range of environmental zones at 12 vent sites in the vent field at EPR 9N, as described previously (Dykman et al. 2023a). Specimens were collected using the human-occupied vehicle 'Alvin' and remotely operated vehicle 'Jason II' during 4 research cruises: AT15-15 (February 2007), AT37-12 (April 2017), AT42-21 (December 2019), and RR2102 (March 2021). Collection sites included Biovent, M Vent, Q Vent, Zeta Garden, East Wall, Teddy Bear, Tica Vent, Crab Spa, Riftia Mound, P Vent, V Vent, and L Vent (Fig. 2). Specimens were brought to the surface in sealed, insulated boxes and either dissected fresh aboard ship or frozen at −80°C for later dissection onshore (see supplementary material in Dykman et al. 2023a). All tissues of each host specimen were thoroughly examined under a dissecting scope by pressing the tissue between 2 glass slides and illuminating with transmitted light. Cercariae were collected on a 124 µm mesh from a sealed collection container that had housed settlement surface covered in small invertebrates. Dissection data are available in the publicly accessible BCO-DMO database (Dykman et al. 2023b).

2.2. Morphological comparison of trematode life stages

At the time of host dissection, digenean life stages were sorted into morphogroups based on gross morphological characteristics visible through a light dissecting microscope, including shape, size, relative dimensions of the oral sucker, pharynx, and ventral sucker, and the position and shape of the testes and eggs. Dimensions of key features were measured with a compound light microscope with an ocular micrometer while parasites were under cover slip pressure. For metacercariae, cyst length and width were also recorded, and the worm was removed from its cyst to measure dimensions. Dimensions were taken for both sporocysts and the developing cercariae within. Because specimens were collected during different cruises by different methods, some were frozen prior to fixation, others were fixed fresh at room temperature, and some had already been fixed in 95% ethanol

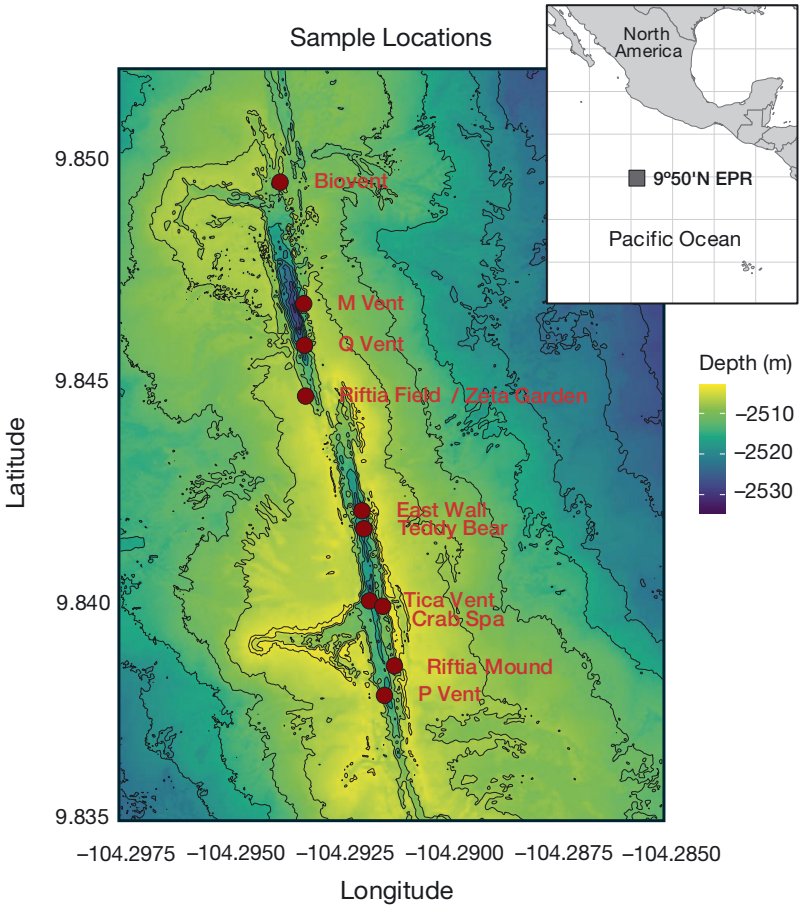

Fig. 2. Hydrothermal vent field at 9° 50′ N on the East Pacific Rise (EPR) showing vent sites where animals were collected. L Vent and V Vent (not shown in the figure) are on the same spreading center but are 7 km farther south

before sorting. Following sorting, specimens were preserved in 80% ethanol for morphological analysis, and some were preserved in 95% ethanol and frozen at −80°C after fixation for molecular analysis.

Following rough morphological sorting, at least 5 high-quality, adult specimens of each morphogroup were stained and mounted for examination of key features to distinguish species. First, specimens were keyed to genus; important features used for discerning species within genera were measured and compared, including the presence of a uterine seminal receptacle, genital pore position, cirrus pouch extent, vitelline field posterior extent, intestinal bifurcation position, testes position and texture, pharynx and prepharynx dimensions, etc. Although gross morphological features like shape and relative sucker dimensions are not enough to reliably distinguish species, our rough morphogroup sorting accurately separated species in most cases. Some specimens were preserved in sub-optimal ways, such as freezing before preservation or cold-fixing, making some species harder to distinguish definitively through morphology, yet the rarity of these specimens makes them worthy of inclusion and discussion within this study, and uncertainty will be discussed.

2.3. Molecular analysis

DNA was extracted from ethanol-preserved specimens using the DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's quick-start protocol for tissue, with the modification of using 35 µl Buffer AE for the elution step rather than 200 µl. In almost all cases for all life stages, individual parasites were extracted. In the case of one specimen (DIGE10 from *Thermichthys hollisi* 22), 10 individuals were pooled to increase tissue quantity. Effort was made to sequence the same parasite morphogroup from multiple host individuals, as is best practice. PCR was performed in 25 µl reaction mixtures containing 12.5 µl of GoTaq G2 Colorless Master Mix (Promega Corporation) (1.5 mM $MgCl^{2+}$ and 200 µM of each dNTP in the final reaction volume), 2.5 µl of each primer (1 µM in the final reaction volume), 6.5 µl nuclease-free water, and 1 µl of 0.03–10 ng μl^{-1} sample DNA. Partial 18S sequence (581–601 bp) was amplified with the primers 18S9modF (5′-GAT CCT GCC AGT AGT CAT ATG CTT G-3′) and 18S637modR (5′-TAC GCT WYT GGA GCT GGA GTT ACC G-3′) (Moszczynska et al. 2009, Van Steenkiste et al. 2015). Partial 28S sequence (1211–1247 bp) was amplified with the primers ZX-1 (5′-ACC CGC TGA ATT TAA GCA TAT-3′) or LSU5′ (5′-TAG GTC GAC CCG CTG AAY TTA AGC A-3′) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) (Tkach et al. 2003, Bray et al. 2009). The following touchdown cycling protocol was used for all reactions: 95°C for 3 min; 14 'touchdown' cycles of 95°C for 30 s, 65–52°C for 30 s (decreasing 1°C each cycle), and 72°C for 1 min; 20 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 1 min; and a final extension at 72°C for 7 min. The internal transcribed spacer 2 (ITS2) rRNA region was used to further confirm morphogroup boundaries and life stage matching (Nolan & Cribb 2005); results and protocol are presented in Text S1 in Supplement 1 at

www.int-res.com/articles/suppl/m755p001_supp1.pdf. Successful amplification was confirmed with gel electrophoresis, and PCR product was sent to Sequegen DNA Sequencing for Sanger sequencing in both directions. Raw sequence data were cleaned and consensus sequences for each sample were built from forward and reverse sequence reads using Sequencher (v.5.4.6) (Gene Codes Corporation). To find close matches to the new samples for inclusion in phylogenetic trees, the online basic local alignment search tool (BLAST; v.2.13.0) (Altschul et al. 1990) was searched using the megablast tool against the nr/nt database with an e-value cutoff of 0.05 and default settings.

2.4. Phylogenetic analysis

Reference sequences were gathered from NCBI, including the closest matches in the NCBI nr/nt database to the new samples, all sequences from known vent digeneans (Bray et al. 2014), and other deep-sea digenean genera in the same families (Sokolov et al. 2019, 2020, Bray 2020). Due to the low number of available deep-sea digenean sequences, the 18S and 28S genes were analyzed separately to allow for the inclusion of more taxonomic diversity in each analysis. When possible, the same species were used in both analyses. The digenean *Echinostoma revolutum* was used as an outgroup for consistency with previous studies (Sokolov et al. 2019). Sequences were aligned using the software clustalo (v.1.2.2) (Goujon et al. 2010, Sievers & Higgins 2021) and manually trimmed to remove all gaps. After alignment and trimming, a 447 bp segment was used for 18S analysis and a 1081 bp segment was used for 28S analysis with full coverage across all species. Trees were generated using the software IQ-TREE (v.2.2.0) (Nguyen et al. 2015). Branch supports were estimated using the ultrafast bootstrap with 1000 replicates (Hoang et al. 2018) as in Sokolov et al. (2019), and the best-fit model was selected based on the Bayesian information criterion (BIC) using the program ModelFinder (v.2.2.0) (Kalyaanamoorthy et al. 2017). The best model for the 18S tree was TNe+R2 (BIC = 3285.26) and the best model for the 28S tree was GTR+F+R3 (BIC = 17 835.170) based on the BIC. The next-best models were TIM2e+R2 (BIC = 3287.08) and GTR+F+G4 (BIC = 17 841.320) for the 18S and 28S trees, respectively. When possible, 2–3 individuals from each morphogroup were sequenced and included in preliminary analyses. If all individuals in a morphogroup were genetically identical, only one individual in the morphogroup was retained in the final tree.

3. RESULTS

3.1. Digenean species and life stages from hydrothermal vents at EPR 9N

Dissections of potential hosts of 51 species from the EPR 9N vent communities revealed 7 morphologically distinct adult trematodes, 7 metacercariae, and one sporocyst that contained developing decaudate cercariae (Table 1) (Dykman et al. 2023a). Examination of the wash from recovered settlement surfaces also revealed one cercarial morphogroup that was very similar to those inside the sporocysts, also lacking a tail. The adult trematodes were found in the stomach, intestine, and gall bladder of the vent fishes *Thermarces cerberus* and *Thermichthys hollisi*. Metacercariae were found in the abdomen of the shrimp *Alvinocaris lusca*, the gills of the crab *Bythograea thermydron*, the head, foot, and visceral mass of the gastropods *Bathymargarites symplector* and *Lepetodrilus ovalis*, and the muscle, gut, pharynx, and parapodia of the polychaetes *Amphisamytha galapagensis*, *Archinome rosacea*, *Branchinotogluma sandersi*, *Branchipolynoe symmytilida*, *Nereis sandersi*, *Nicomache* sp., *Thermiphione risensis*, and an unidentified polychaete species. Sporocysts were found in the gonad of the gastropod *Eulepetopsis vitrea*.

Of the parasite morphogroups and life stages collected, high-quality consensus sequences were obtained from 5 adult, 2 metacercarial, 1 sporocyst, and 1 cercarial morphogroup (Table 1). For each parasite morphogroup, successful sequences were obtained from individuals living in 1–6 separate host individuals. A summary of the collection localities, hosts, and GenBank accession numbers for the sequenced specimens are provided in Table S2 in Supplement 2 at www.int-res.com/articles/suppl/m755p001_supp2.xlsx, and sequence data from this study can be found in NCBI GenBank. Accession numbers of museum-deposited specimens will be included in future publications in which we formally describe each morphogroup as a new taxon. One of the adult trematode morphogroups has been formally described at this time (DIGE08: *Neolebouria mullineauxae*; Dykman et al. 2025). Specimens are deposited at the Harold W. Manter Laboratory of Parasitology (accession numbers HWML 217927–217932) and the Smithsonian National Museum of Natural History (accession numbers USNM 1741983–1742034).

Table 1. Prevalence and intensity of the digenean morphogroups and their life cycle stages encountered during dissections of vent hosts. Prevalence is the proportion of examined host individuals infected by a parasite of a particular species, and intensity (range, with mean in parentheses) is the number of individual parasites found in an infected host, omitting hosts without infection (Bush et al. 1997). The parasite morphogroups are arranged in order of life stage, with the putative genus, when known, listed after the morphogroup name. The number of hosts dissected is shown (n), as well as the number of 18S, 28S, and ITS2 sequences obtained. The superscript next to the sequence number shows the number of host individuals from which parasite sequences were obtained. For early life stages, morphological identification is not perfect, so prevalence and intensity data are not exactly accurate but presented to give a rough idea of the occurrence of these life stages in hosts. NA: not available

Parasite morphogroup	Host species	n	Prev.	Intensity	Sequence
Adult					
DIGE09 (*Biospeedotrema*)	*Thermarces cerberus* (Vertebrata)	11	0.91	1–24 (6.6)	
DIGE10 (*Biospeedotrema*)	*Thermichthys hollisi* (Vertebrata)	24	0.67	1–443 (63.0)	18S (4)[2], 28S (5)[2], ITS2 (5)[2]
DIGE13 (*Biospeedotrema*)	*Thermichthys hollisi* (Vertebrata)	24	0.79	1–113 (30.6)	18S (3)[1], 28S (1)[1], ITS2 (3)[1]
DIGE20 (*Biospeedotrema*)	*Thermarces cerberus* (Vertebrata)	11	0.18	10–11 (10.5)	
	Thermichthys hollisi (Vertebrata)	24	0.38	1–49 (19.1)	18S (2)[1], ITS2 (2)[1]
DIGE01 (*Caudotestis*)	*Thermarces cerberus* (Vertebrata)	11	0.73	1–51 (13.3)	18S (5)[3], 28S (1)[1], ITS2 (6)[3]
DIGE08 (*Neolebouria*)	*Thermarces cerberus* (Vertebrata)	11	0.91	1–107 (34.9)	18S (9)[4], 28S (2)[2], ITS2 (10)[6]
DIGE11	*Thermichthys hollisi* (Vertebrata)	24	0.17	1–8 (3.5)	
Metacercaria					
META01 (*Biospeedotrema*)	*Alvinocaris lusca* (Decapoda)	10	0.90	1–270 (107.0)	18S (2)[1], 28S (3)[2], ITS2 (4)[2]
META04	*Branchipolynoe symmytilida* (Polychaeta)	23	0.04	1 (1.0)	
META05	*Amphisamytha galapagensis* (Polychaeta)	94	0.18	1–7 (2.1)	
	Archinome rosacea (Polychaeta)	71	0.04	4–10 (6.3)	
	Bathymargarites symplector (Gastropoda)	99	0.04	1–4 (2.0)	
	Bythograea thermydron (Decapoda)	36	0.03	1 (2.0)	
	Nicomache sp. (Polychaeta)	1	1.00	1 (1.0)	
	Thermiphione risensis (Polychaeta)	6	0.33	1 (1.0)	
META06	*Branchinotogluma sandersi* (Polychaeta)	14	0.07	6 (3.0)	
	Lepetodrilus ovalis (Gastropoda)	38	0.03	8 (3.0)	
	Polychaete sp. (Polychaeta)	3	0.67	4–5 (4.5)	
	Thermiphione risensis (Polychaeta)	6	0.17	1 (1.0)	
META08 (*Caudotestis*)	*Branchipolynoe symmytilida* (Polychaeta)	23	0.04	1 (1.0)	18S (1)[1], ITS2 (1)[1]
META11	*Nereis sandersi* (Polychaeta)	8	0.13	1 (1.0)	
META14	*Bathymargarites symplector* (Gastropoda)	99	0.02	1–2 (1.5)	
	Nicomache sp. (Polychaeta)	1	1.00	1 (1.0)	
Cercariae					
CERC01	NA	NA	NA	NA	18S (1), ITS2 (1)[1]
Sporocyst					
SPOR01 (*Neolebouria*)	*Eulepetopsis vitrea* (Gastropoda)	100	0.05	NA	18S (5)[5], 28S (4)[4], ITS2 (1)[1]

3.2. Phylogenetic matching of life stages and relationship to known species

The digenean parasite species from vents at EPR 9N form 2 distinct taxonomic clusters, and several of the intermediate life stages match very closely to adult life stages both morphologically and phylogenetically. The first cluster falls within Family Opecoelidae, a common marine digenean family with many species in the deep sea (Bray et al. 1999). The first cluster's position relative to other species in the family is poorly supported in the 18S tree (bootstrap support value: 24) (Fig. 3) but well supported in the 28S tree (bootstrap support value: 100) (Fig. 4). It contains the adult morphogroup DIGE08 (Fig. 5a) from the gut of the eelpout *T. cerberus* and the sporocyst SPOR01 (Fig. 5b) from the gonad of the glass limpet *E. vitrea*. The adult and sporocyst have identical sequences in the 18S and 28S regions examined (447 out of 447 and 1081 out of 1081 bp, respectively). Based on a BLASTn search of the partial 28S gene, DIGE08 showed 99.9% (1080 out of 1081 bp) similarity to *Neolebouria georgiensis* (28S: MH892478.1) (bootstrap support value: 100) from the icefish *Trematomus pennellii* in the Weddell Sea, Antarctica (Faltýnková et al. 2017). Adult DIGE08 was consistent with *Neolebouria* in having a well-developed cirrus pouch, canalicular seminal receptacle, and clearly submedian genital pore, among many other features, yet differed from *N. georgiensis* in having a cirrus pouch that does not extend beyond the posterior margin of the ventral sucker. Therefore, we place this new species in the genus *Neolebouria* with high confidence (Dykman et

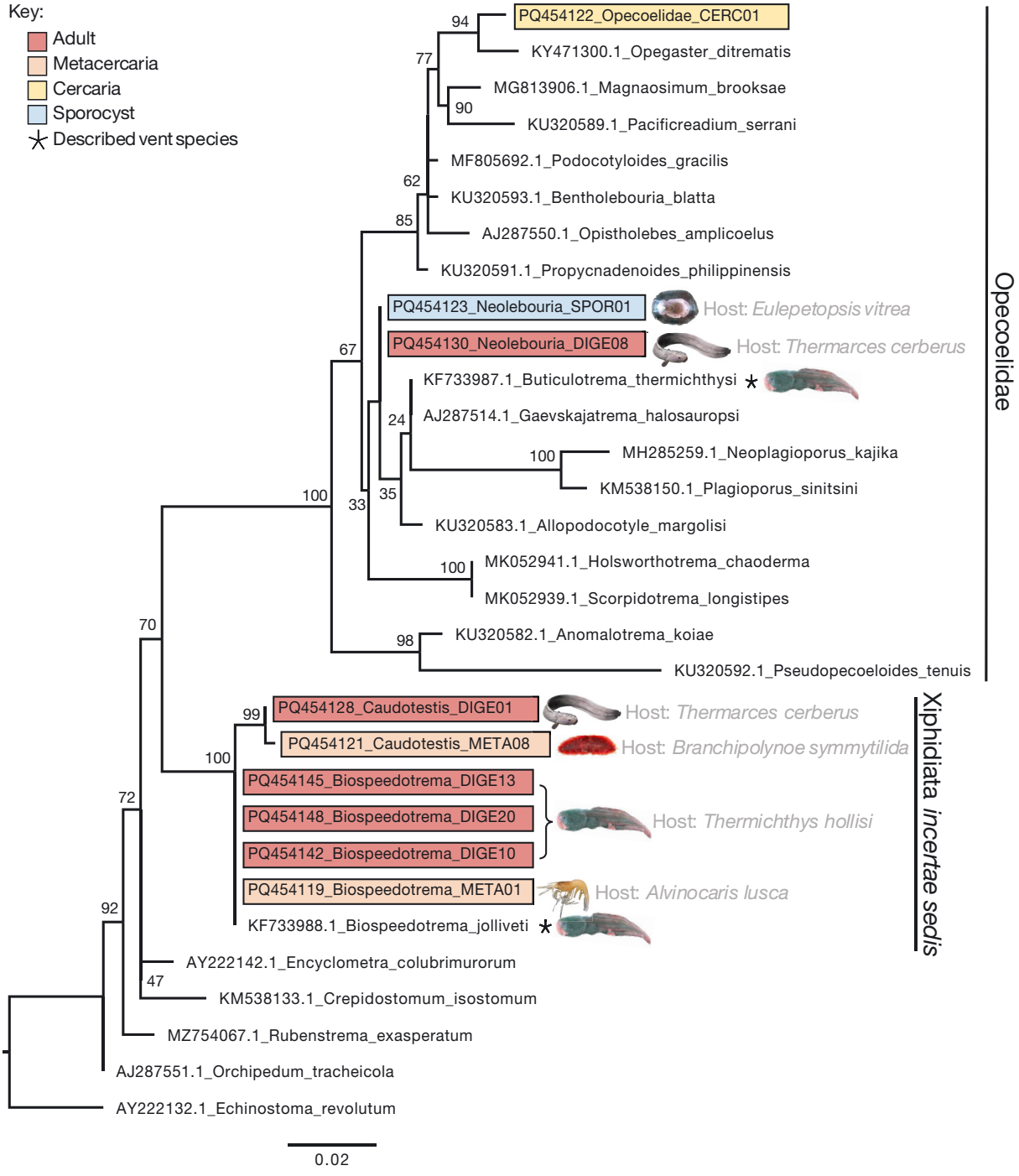

Fig. 3. Phylogenetic tree resulting from maximum likelihood analysis of the 447 bp partial 18S rDNA alignment. This analysis includes species closely related to the newly found vent morphogroups, other known deep-sea genera, and *Echinostoma revolutum* as an outgroup. Support values were generated with 1000 bootstraps. The newly found vent parasite morphogroups are shown in boxes with the life stages color coded and the host species shown to the right. The scale bar indicates the expected number of substitutions per site. GenBank accession numbers are shown before the species names

al. 2025). Interestingly, DIGE08 lacks the large, robust body and denser and more numerous vitelline follicles and eggs seen in other *Neolebouria* species, while sharing some features associated with the genus *Mesobathylebouria* established by Martin et al. (2019). However, DIGE08 only shared 92% (998 out of 1081 bp) similarity with *M. lanceolata* (28S: KJ001210) (bootstrap support value: 100).

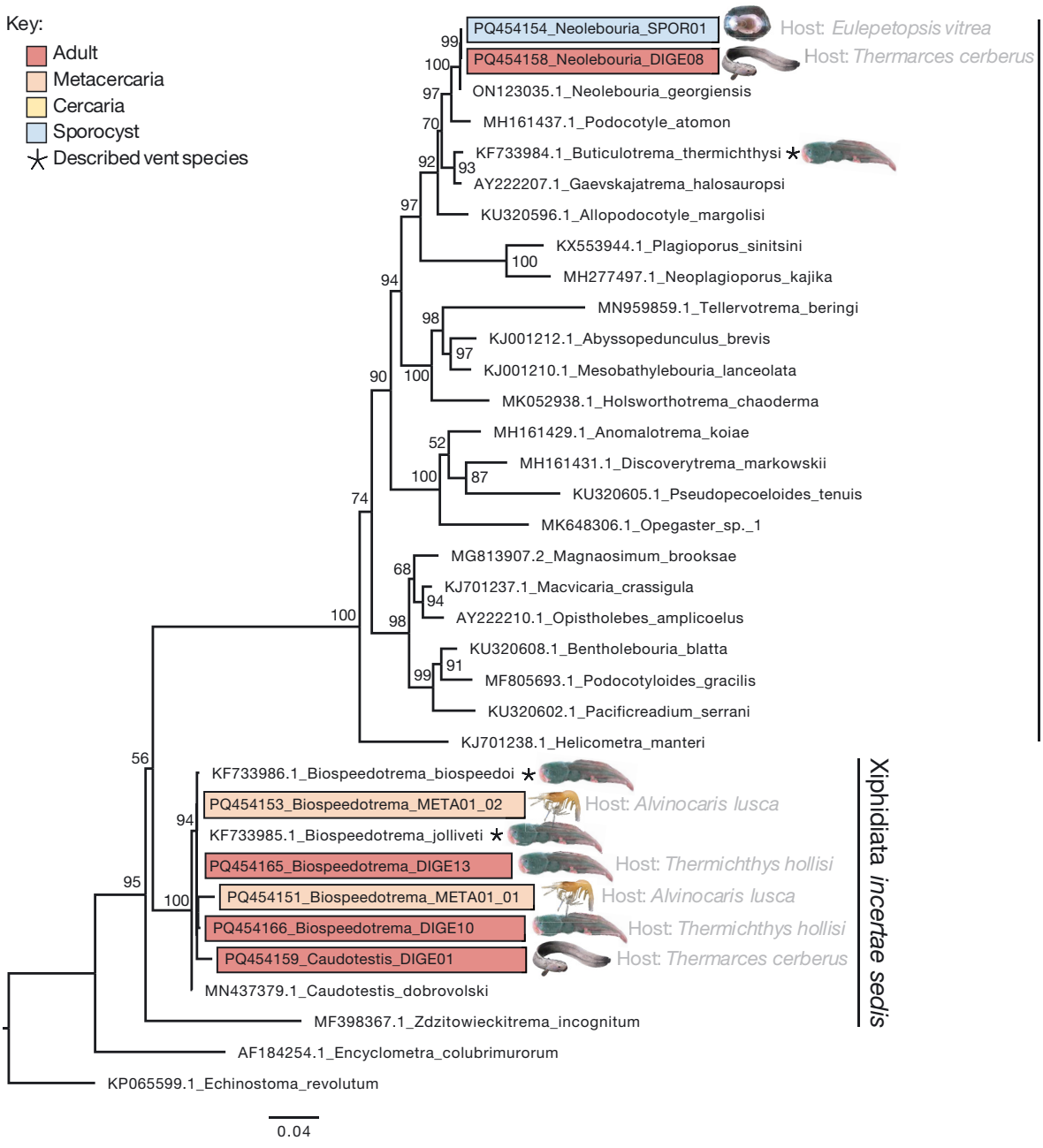

Fig. 4. Same as Fig. 3, but for maximum likelihood analysis of the 1081 bp partial 28S rDNA alignment

The rest of the adult and metacercarial morphogroups cluster within a sister group to the Opecoelidae, Xiphidiata *incertae sedis* (Sokolov et al. 2019) (Figs. 3 & 4). This group contains species morphologically and genetically consistent with genera *Biospeedotrema* and *Caudotestis*, which were described from vents 3000 km farther south on the EPR (Bray et al. 2014). The 3 adult morphogroups DIGE10 (Fig. 5c), DIGE13 (Fig. 5d), and DIGE20 (Fig. 5e) from the fish *T. hollisi*, and the metacercaria META01 (Fig. 5f) from the shrimp *A. lusca* are all genetically identical for the 18S fragment (447 out of 447 bp) (Fig. 3) and up to 99.7% (1078 out of 1081 bp) similar for the 28S fragment (Fig. 4), despite having morphological differences. However, these morphogroups were genetically distinct in the ITS2 rDNA region (Fig. S1, Table S1 in Supplement 1). The 3 adult morphogroups share most features with *Biospeedotrema* originally described from vent specimens collected from the Southern EPR (Bray et al. 2014), however none share all the features consistent with the previously described species. While DIGE10 and DIGE13 were abundant in the stomach

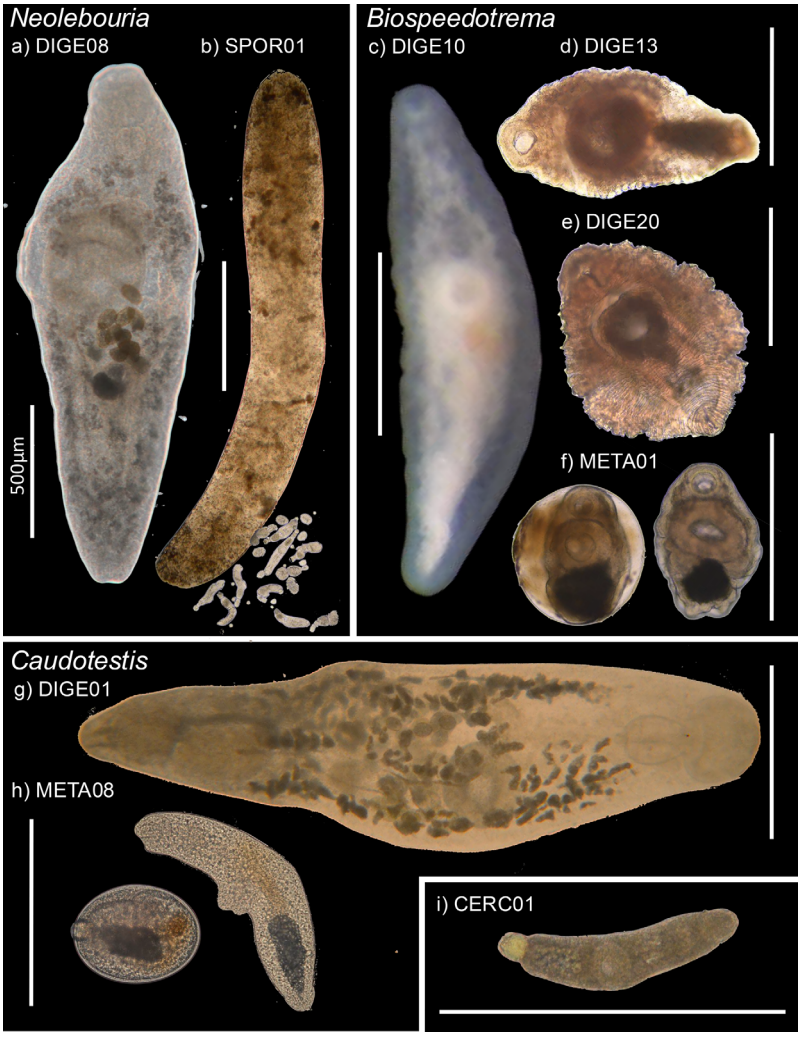

Fig. 5. Images of digenean life stage morphogroups found at the hydrothermal vents at 9° 50' N on the East Pacific Rise. Morphogroups are grouped taxonomically in the separate boxes based on their phylogenetic and morphological similarities. The boxes are labeled by the group's genus at the top left, where relevant. The sporocyst SPOR01 (b) is shown with emerged cercariae. The metacercariae META01 (f) and META08 (h) are shown both encysted (left) and excysted (right). All scale bars: 500 µm

and intestines of the vent fish *T. hollisi*, DIGE20 was found only in the gall bladder. This apparent gall bladder specialist had a distinctive morphology, being pear-shaped to circular, with often ruffled margins. DIGE10 is spindle-shaped with small ventral and oral suckers, its uterus extends to the level of the testes and not into the post-testicular space, and 2 bands of vitelline follicles run longitudinally along the lateral margins with a lateral band across the anterior end. DIGE13 is stouter and less elongate, has larger oral and ventral suckers, its uterus extends further posterior and into the post-testicular space, and often there was a break in the vitelline follicles lateral to the ventral sucker. The metacercaria META01 from the vent shrimp matches most closely to the adult DIGE13 both genetically (100% similarity, 1081 out of 1081 bp) and morphologically (Fig. 5d,f). Some specimens in the morphogroup META01 were genetically distinct from other META01 individuals and all other sequenced morphogroups in our dataset (98.6% similarity, 1066 out of 1081 bp) (Fig. 4), confirming the difficulty of distinguishing metacercariae morphologically, and indicating that we did not successfully sequence or encounter other life stages to match this metacercarial lineage.

Branching from the *Biospeedotrema* cluster in Xiphidiata *incertae sedis* within the 18S tree is a subcluster containing one adult morphogroup and one metacercarial morphogroup with morphology suggesting *Caudotestis* (see Cribb 2005) (Fig. 3). DIGE01 (Fig. 5g) is an adult from the vent fish *T. cerberus* and META08 (Fig. 5h) is a metacercaria from the polychaete *B. symmytilida*. The adults have an elongate-oval body, a relatively large oral sucker that is terminal to slightly subterminal, a large pharynx, and a small sessile ventral sucker. Eggs are moderate in size and oval, and the uterus extends from the level of the ovary to the genital pore, which is pre-acetabular. Testes are oval to spherical and located far posteriorly. We also confirmed the presence of a uterine seminal receptacle, an entire ovary, and a median to submedian genital pore, which is consistent with the genus (Cribb 2005). The 28S sequences from the newly found vent *Caudotestis* specimens do not group closely with the only *Caudotestis* sequence available in GenBank, *Caudotestis dobrovolski* (28S: MN437379.1) (Fig. 4), from a scorpaeniform fish off Simushir Island, Northwestern Pacific Ocean (Sokolov et al. 2020) (98% agreement, 1063 out of 1081 bp; bootstrap support value: 94). The inconsistency with new molecular evidence suggests that the genus may require revision or that the newly found vent morphogroup may instead be an undescribed taxon. One *Caudotestis* species, *C. ventichthysi*, has been described from a vent fish on the southern EPR (Bray et

al. 2014), but no sequence is available for this species. DIGE01 differs from *C. ventichthysi* in having a ventral sucker smaller than or equal in size to the oral sucker, a larger pharynx, a very short or absent prepharynx, a significantly longer cecum, a uterus not reaching the level of the testes, and a larger egg among other distinct features. The metacercaria META08 was closest to DIGE01 morphologically, with its elongate body, relatively large terminal to subterminal oral sucker, and relatively small ventral sucker. It had 99.8% genetic similarity to the adult DIGE01 sequences in both the 18S gene (446 out of 447 bp; bootstrap support value: 99) (Fig. 3) and the ITS2 fragment (Fig. S1).

The single cercarial morphogroup (Fig. 5i) fell within the Opecoelidae for the 18S gene, but was genetically distant from the other vent samples in our study (97% agreement with DIGE08, 434 out of 447 bp; Fig. 3), although it was morphologically similar to the decaudate cercariae found in the sporocyst SPOR01 (Fig. 5b,i). It was most closely related to *Opegaster ditrematis* (18S: KY471300.1) (99% agreement, 441 out of 447 bp; bootstrap support value: 94) from the South China Sea, Malaysia (R. M. Elshawesh et al. unpubl. data). These formed a clade with *Magnaosimum brooksae* (MG813906.1) and *Pacificreadium serrani* (KU320589.1) (bootstrap support value: 77). This distant relationship to other vent species indicates that additional digenean diversity at vents remains undiscovered.

4. DISCUSSION

4.1. Parasite life cycles at deep-sea hydrothermal vents

By genetically matching parasite life stages, we demonstrate that parasites with ILCs reproduce and thrive within hydrothermal vent ecosystems. Both our genetic and morphological data, based on a relatively small number of samples, suggest a surprising diversity of trematode parasites in this system. This finding demonstrates the ubiquity and resilience of parasites, even those with ILCs, in one of the most extreme settings on the planet.

The transmission of parasites through the vent food web points towards ecological relationships between vent-endemic species. The distinct digenean parasite fauna within 2 vent fish species in this region may reflect 2 separate phenomena. First this separation may arise from distinct feeding and habitat niches of the 2 fishes. The metacercaria morphogroup (META01) found encysted in high densities in vent shrimp *Alvinocaris lusca* was genetically identical to the adult *Biospeedotrema* morphogroup DIGE13 found in the fish *Thermichthys hollisi*. This fish species lives and feeds in the periphery of active venting habitat (Buckman 2009), and it likely obtains its heavy infections by eating shrimp and other crustaceans, which are mobile scavengers that may stray farther from active venting centers. The other sequenced metacercarial morphogroup (META08) was found in the scaleworm *Branchipolynoe symmytilida*, which lives commensally inside vent mussels. This metacercaria was similar to an adult stage of *Caudotestis* (DIGE01) in the vent zoarcid fish *Thermarces cerberus*, indicating that this fish species, which lives mainly in intermediate to high-flux zones, may be infected by eating small invertebrates associated with active venting. A second possibility is that the parasites are more specific for their definitive host but less so for their second intermediate host — infecting a wide range of invertebrates in the vent field, some of which become 'dead ends' when they are eaten by fish in which the parasite cannot survive. Intermediate hosts can also be 'dead ends' if their living habits make them unlikely prey. For example, commensal scaleworms are protected by the mussel host and thus difficult for predators to access, so this host may not be the primary route of transmission to fish for this species. It should be noted that 49 of the highly abundant *Bathymodiolus* mussels were dissected and were not found to host digeneans.

While we were able to pair metacercariae with adults, we were unable to pair a sporocyst with a metacercaria, which would have provided useful information on species interactions and habitat suitability for parasite transmission. Transmission between the first and second intermediate host is by free-living cercariae, which have a very short planktonic duration (~24 h) and are sensitive to chemical and thermal stress (Cross et al. 2001, Tobler et al. 2007). The glass limpet that hosted sporocysts and the small invertebrate species found to host metacercariae mostly inhabit warm to cool diffuse zones of active vent habitat (Micheli et al. 2002, Mills et al. 2007), which may partly explain their suitability as hosts for parasitic stages that transmit as free-living larvae. In other ecosystems, high reduced sulfur and heavy metal concentrations damage free-living parasite stages (Cross et al. 2001, Tobler et al. 2007), although some parasites can tolerate such conditions (Sures 2001, Riesch et al. 2020). The harsh chemical environment in hot, high-flux zones in the vent field may therefore provide a refuge from parasitism for those species adapted to live there (i.e. tubeworms, alvinellid worms, some lepetodrilid

limpets), while transmission occurs between species living in the cooler zones where venting fluids are more diluted by ambient seawater. Further sampling to resolve the within-site distribution of parasite life stages is needed to clarify the role of thermal and chemical zonation in structuring areas of intensified parasite transmission within the vent field.

Although evidence indicates that several digenean parasite species likely complete their life cycles at vents, we could not unravel the complete life cycle of any species. We were able to successfully sequence only a subset of the morphogroups and life stages discovered. In some cases, this was due to small sample sizes and tissue volumes, especially for metacercariae. Some morphogroups did not amplify well with established PCR primers and protocols. It should also be noted that no or very little genetic variation was seen in the 18S and 28S barcoding regions among the morphogroups in *Biospeedotrema* (Figs. 3 & 4), although they had slight morphological differences. These morphogroups may be either several species very early in speciation or one highly variable species (Nolan & Cribb 2005). A lack of genetic variation in the 18S and 28S regions between different parasite species is unusual but not unheard of in digenean taxa (Martin et al. 2018), and different barcoding genes can tell different stories. The ITS2 gene is the 'gold standard' for distinguishing digenean species (Nolan & Cribb 2005) and was accordingly more effective at resolving differences between our *Biospeedotrema* morphogroups (Supplement 1), although those differences were still modest (often <1%). ITS should be the focus of future investigation, as well as the COI gene and genome-wide approaches, which would have more power to distinguish between species early in speciation. Finally, intermediate life stages are often found at low prevalences, so additional sampling at vents is encouraged to collect a greater number of individuals for sequencing, especially among the gastropods, which are key hosts for sporocysts. Despite the large and unprecedented depth of sampling in this study, we are still likely only scratching the surface of the parasite diversity and parasite–host relationships in vent ecosystems.

4.2. Phylogenetic context of vent parasites

Phylogenetic analysis suggests that the diversity of digenean trematodes at EPR 9N vents arose from multiple founder species with potential host-switching events. First, a clade of closely related species in the genera *Biospeedotrema* and *Caudotestis* span a geographic range of at least 3000 km along the EPR (Bray et al. 2014). The range of these genera along the EPR and the genetic similarity between species indicate the connectivity of mobile fish hosts between distant vent fields on evolutionary timescales. Ancestors of this clade may have been introduced to vents with ancestors of the bythitid *T. hollisi*, spread geographically, and radiated within the vent environment. The preference of *T. hollisi* for peripheral vent habitats (Buckman 2009) might indicate that it is more comfortable moving between vent fields than the zoarcid *T. cerberus*, which could explain the 3000 km known geographic range of the parasite genera of *T. hollisi*. *Biospeedotrema* species have so far only been found at vents, so it remains possible this parasite genus is endemic to this habitat. Collections of more deep-sea fish species from nearby, non-vent seafloor habitats are needed to clarify whether *Biospeedotrema* is endemic to vent ecosystems.

A second group of digenean vent species in the genus *Neolebouria* appear to have originated independently from the *Biospeedotrema/Caudotestis* clade, indicating at least 2 separate invasion events. Intriguingly, the vent species DIGE08 had a very similar 28S sequence to *Neolebouria georgiensis* from an icefish in Antarctic waters. The ecological and geographic distance between the vent species and *N. georgiensis* is noteworthy given their genetic similarity. This might suggest that the genus is globally distributed, and some species are known to be very generalist (e.g. the deep-sea digeneans *Derogenes varicus* and *Gonocerca phycidis*). However, this hypothesis is somewhat tentative given the scarcity of closely related deep-sea reference sequences. Introduction into the vent environment could have occurred with the ancestors of *T. cerberus* or from a more recent host-switching event from more generalist deep-sea fishes. The first intermediate host in this life cycle is the glass limpet *Eulepetopsis vitrea*, which leaves an open question as to when and how *Neolebouria* invaded chemosynthetic limpets. The genus *Eulepetopsis* (Family Neolepetopsidae) currently contains only 2 species, *E. vitrea* from the EPR, and *E. crystallina* from the Central Indian Ridge (Chen et al. 2022), both of which are endemic to active venting habitats. All 3 genera in the Family Neolepetopsidae (*Eulepetopsis*, *Paralepetopsis*, and *Neolepetopsis*) are endemic to chemosynthetic seafloor features, including active vents and inactive sulfide mounds (Chen et al. 2022). The possibility that the occurrence of *Neolebouria* at vents arose from cospeciation with an ancestral neolepetopsid species should be explored further. A host switch into a chemosynthetic limpet

remains possible (Araujo et al. 2015) but may present an evolutionary challenge: in species introductions and range expansions, ILC parasite species often cannot establish until a suitable first intermediate host is introduced, as reviewed in Bauer & Hoffman (1976). Investigation of different neolepetopsid species from other chemosynthetic habitats may provide greater insight into the route of establishment of *Neolebouria* in vent ecosystems.

Given that this study looks only at morphology and barcoding genes with a relatively small number of other deep-sea and hydrothermal vent species available for comparison, the natural history of parasite introduction into deep-sea hydrothermal vents remains speculative. In the future, a comparison of hosts and their parasites across a broader geographic range, including vent and nearby non-vent host species, would help clarify the host specificity, endemicity, and origins of vent parasites. Genomic techniques that can estimate the time of evolution into the vent environment of parasites and their hosts (e.g. Sun et al. 2019) would help clarify the relative times of transition between host species and the role of different hosts as agents for introduction into an extreme new environment.

5. CONCLUSIONS

The discovery of multiple stages in the trematode life cycles at vents provides an important example of parasite species with multi-host life cycles reproducing and persisting in a frequently disturbed, isolated ecosystem with endemic hosts. The matching of life stages points toward relationships between vent fish, invertebrate prey, and their parasites that facilitate parasite reproduction in this ecosystem. Phylogenetic comparison to closely related deep-sea species suggests that at least 2 distinct ancestral digenean clades were introduced to the vent environment. The large known geographic range and close genetic relatedness of species in one of these clades indicate considerable connectivity between vent fields on the EPR. Greater insight into the host requirements and evolutionary origins of vent parasites is limited by the amount of available data on deep-sea parasites. Similar sampling efforts should be conducted at vent fields along the EPR to better understand parasite species ranges, connectivity, and potential routes of introduction into vent ecosystems.

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