Parasitology Research
https://doi.org/10.1007/s00436-020-07027-1

HELMINTHOLOGY - ORIGINAL PAPER

Insights on foodborne zoonotic trematodes in freshwater snails in North and Central Vietnam

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Received: 7 August 2020 / Accepted: 20 December 2020

Abstract

Foodborne zoonotic trematode (FZT) infections are common neglected tropical diseases in Southeast Asia. Their complicated life cycles involve freshwater snails as intermediate hosts. A cross-sectional study was conducted in Yen Bai and Thanh Hoa provinces in North and Central Vietnam, to investigate the diversity of cercariae of potential FZT and to construct the phylogenetic relationship of trematode cercariae based on the Internal Transcribed Spacer 2 (ITS2) region. Among 17 snail species collected from various habitats, 13 were infected by 10 cercarial groups among which parapleurolophocercous, pleurolophocercous, and echinostome cercariae were of zoonotic importance. The monophyletic tree separated cercarial sequences into different groups following the description of the cercariae families in which Haplorchidae, Opisthorchiidae, Echinochasmidae, and Echinostomatidae are important families of FZT. The overall prevalence was different among snail species and habitats and showed a seasonal trend. Parapleurolophocercous and echinostome cercariae emerged as the most common cercariae in snails in Yen Bai, while monostome, echinostome, and megalura cercariae were most common in Thanh Hoa. Using a molecular approach, we identified *Parafossarulus striatulus* as the first intermediate snail host of *Clonorchis sinensis* in Thac Ba Lake. *Melanoides tuberculata* and *Bithynia fuchsiana* were we identified preferred intermediate snail hosts of a diverse range of trematode species including intestinal flukes (i.e., *Haplorchis pumilio* and *Echinochasmus japonicus*) in Yen Bai and Thanh Hoa, respectively.

Keywords Foodborne zoonotic trematode · Cercaria · *Clonorchis sinensis* · Intestinal fluke · Freshwater snails · Vietnam

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Section Editor: David Bruce Conn

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Introduction

Foodborne zoonotic trematodes (FZT), belonging to the subclass Digenea, include the liver flukes (*Clonorchis sinensis*, *Opisthorchis* spp., *Fasciola* spp.), intestinal flukes (*Echinostoma* spp., *Fasciolopsis buski*, Heterophyids), and lung flukes (*Paragonimus* spp.). Humans can acquire FZT infection by eating raw or undercooked vegetables, crustaceans, or fishes carrying the infective metacercaria stages. The life cycles of FZT are complex and go through several larval stages within a first and a second intermediate host according to the genus. The cercarial stage develops in certain freshwater snail species as the obligatory first intermediate hosts (Esch et al. 2001; Littlewood and Bray 2000), and the presence of these snails in aquatic habitats is a crucial risk factor in the epidemiology. Several species of FZT cause economic losses to society through infection of domestic animals or due to their public health impact. Currently, an estimated 200 million people are infected with FZT, and almost a billion people are at risk, mainly in low- and middle-income countries of Southeast Asia (Engels and Savioli 2006; Keiser and Utzinger 2005; Nyindo and Lukambagire 2015; Wang et al. 2015); FZT are among the most neglected tropical diseases. In Vietnam, FZT infections are common in humans and considered as an important public health problem, especially the giant liver fluke (*Fasciola gigantica*) and the fishborne trematodes, such as small liver (*Clonorchis sinensis* and *Opisthorchis viverrini*) and intestinal flukes (Bui TD et al. 2016; Do et al. 2007; Nguyen et al. 2003; Phan et al. 2011). In recent years, studies on trematode larval stages in snail hosts have received increasing attention because of the importance of data on cercarial infection in snails for epidemiological modeling and integrated control of FZT. The traditional method used to identify trematode cercariae considered only the morphological characteristics. However, this method is fragrantly difficult due to morphological similarity, the paucity of fully reliable diagnostic morphological characters, and inadequate historical descriptions (Graczyk 1991; Kostadinova et al. 2003). As an alternative to the morphological determination, molecular information is expected to provide a better resolution on trematode identification at the larval stage (Anucherngchai et al. 2016; Kim et al. 2009; Kostadinova et al. 2003). Particularly, the internal transcribed spacer 2 (ITS2) has proven to be an efficient target to identify trematodes at different developmental stages and for use in phylogenetic relationship analysis (Chontananarth et al. 2017; Prasad et al. 2011; Thaenkham et al. 2010). In Vietnam, studies using molecular methods have mainly focused on metacercarial and adult stages of trematodes in fish, cattle, and humans because of the direct and obvious effects on public health and the economic losses they cause (Le et al. 2007, 2008; Nguyen et al. 2009; Nguyen and Le 2011; Pham et al. 2007; 2016), although a study by Kim et al. (2009) reported the use of ITS2 sequences of cercariae of intestinal trematodes (*Haplorchis pumilio*). The objective of this study was to investigate the diversity and the differences in prevalence of trematode infections in different snail species collected in different habitats, with an emphasis on FZT in Yen Bai and Thanh Hoa provinces. Moreover, the phylogenetic relationship of trematode cercariae based on the ITS2 region was explored.

Materials and methods

The study areas and sampling strategy

The study was conducted in two provinces located in the northern and north central region of Vietnam, Yen Bai and Thanh Hoa, respectively. These provinces were selected due to their known high infection rate of FZT, i.e., small liver fluke and intestinal flukes, in humans and fish (Bui NT et al. 2016; Clausen et al. 2012; Ngo 2016; Nguyen 2004; Nguyen et al. 2018; Nguyen 2019). In each province, two communes were studied including Vu Linh and Phuc An (Yen Binh District in Yen Bai province) and Ha Vinh and Ha Duong (Ha Trung District in Thanh Hoa province) (Fig. 1). A cross-sectional study was performed with stratified random sampling by hamlets and available habitats (Parsons 2017) during the cool dry season (March–April) and the hot rainy season (July–August) in 2018. Sampling sites were selected so that all hamlets and habitats were represented by one or more sites. In Yen Bai, a total of 133 sampling sites in 22 hamlets represented 5 habitats, including lake, pond, stream, drainage ditch, and rice field. In Thanh Hoa province, a total of 130 sampling sites in 16 hamlets represented 5 habitats including, pond, drainage ditch, canal/river, and rice field, was established.

In order to understand how environmental parameters vary in the study area, some physicochemical parameters in the water such as temperature, salinity, pH, dissolved oxygen (DO), and total dissolved solids (TDS) were measured at each site during the time of sampling using the WQC-22A DKK TOA water quality checker. There were significant increases in temperature in different habitats in rainy season in both provinces (Student's t test, $P < 0.0001$). The average monthly precipitation (in millimeters per month) in Yen Bai and Thanh Hoa provinces in 2018 was recorded by the National Centre for Hydro-Meteorological Forecasting. The data showed significant differences in rainfall between studied months.

Snail sampling and identification

Semi-quantitative snail sampling was conducted in the selected sites. In each site, snails were searched and manually collected using scooping and handpicking depending on habitat conditions, by two persons for 30 min during morning hours, because of the expected higher snail activity (Madsen et al. 1987; Olivier and Schneiderman 1956). Snails were kept out of direct sunlight and placed separately in labeled vials containing water for further identification. Snails were identified following descriptions by Dang and Ho (2017) and Bouchet (2005).

Trematode identification

Morphological identification

Snail specimens were first inspected for cercariae under a ZEISS Stemi DV4 stereo microscope using two different methods: crushing and cutting (Bui et al. 2010). After that, the living cercarial specimens were identified under a KRÜSS MBL2000 microscope and photographed with a Cannon IXUS digital camera. The cercarial classification was based on the cercaria keys of Schell (1970) and Christensen and Frandsen (1984). Morphological details of each specimen

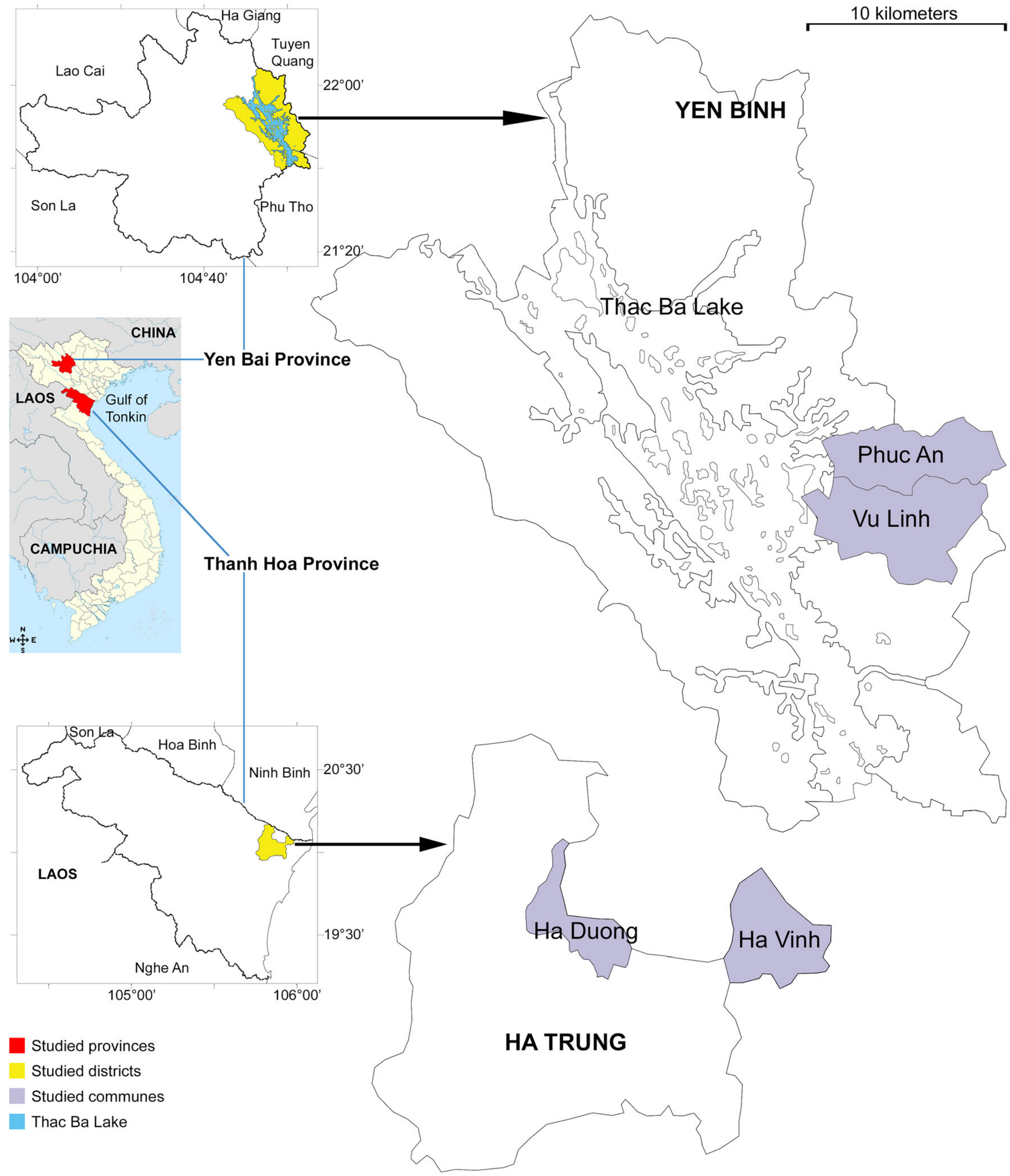

Fig. 1 Map of studied communes in Yen Bai (upper maps) and Thanh Hoa (lower maps) provinces (communes in purple, districts in yellow, and provinces in red)

were photographed at different magnifications to allow subsequent identification and measurement. Cercariae were preserved in ethanol 70% solution for further molecular identification.

Molecular analyses

About 1% of the samples of each cercarial group was randomly selected and used for molecular analysis. Total genomic DNA was isolated from ethanol-fixed cercariae using proteinase K (10 mg/ml) (Derycke et al. 2012) and Worm Lysis Buffer (WLB) (Williams et al. 1992) and stored at − 20 °C. The forward primer ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and the reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) targeting the cercarial ITS2 region were used following Barber et al. (2000). The amplicons were visualized on 1% agarose gel (1 g agarose per 100 ml TAE buffer 0.5X) stained with 4 μl RedSafe™ Nucleic Acid Staining Solution (iNtRon Biotechnology). The PCR products were purified, and cycle sequencing reactions based on Sanger method were performed (in both directions) by BigDye® Terminator v3.1 at 1st BASE company (Malaysia).

Data analysis

Differences between habitats and seasons for snail abundance were analyzed using one-way ANOVA. Significant differences of the overall infection rate between snail species, habitats, and seasons were tested using the Chi-square test. Logistic regression was used to analyze the infection prevalence of each cercarial type depending on habitat and snail species as predictors. Differences in salinity, pH, DO, temperature, and TDS in each habitat between the two seasons of each province were compared by independent samples *t* test. All statistical tests with p value < 0.05 were considered statistically significant. Statistical analyses were conducted using R software (https://www.r-project.org/). Non-metric Multi-Dimensional Scaling (MDS) analysis based on Bray-Curtis similarity matrix was used to visualize the patterns and structures of larval trematode communities in different snail species using the software PRIMER 6 (Clarke and Gorley 2006).

Sequence quality control

DNA sequences were checked for ambiguities and errors using DNASTAR Lasergene SeqMan Pro v7.1.0. The sequences were submitted to GenBank (submit.ncbi.nlm.nih.gov) (Benson et al. 2017) and subsequently aligned in MEGA v6.0 (Tamura et al. 2013). A neighbor-joining (NJ) tree was constructed with thirty-seven studied sequences in MEGA v6.0 using the pairwise distance method. The NJ trees were validated with bootstrap analysis of 1000 replicates. Reference sequences were acquired from the NCBI data base.

Results

Characterization of the environment

In Yen Bai and Thanh Hoa, the water temperature was recorded with a statistically significant difference between the two seasons in all habitats with an increase in rainy season ($P < 0.05$). The average monthly precipitation (in millimeters per month) also showed significant differences between studied months (March–April vs. July–August) (supplementary files 2 and 3).

Snail fauna

A total of 87,415 snail specimens representing 17 species was collected during the dry and rainy seasons in Yen Bai and Thanh Hoa provinces (Tables 1 and 2). Two species, *Bithynia fuchsiana* and *B. goniomphalus* were only found in Thanh Hoa. Snail species composition varied greatly among habitats and seasons. Rice fields were found to harbor the highest number of snail species in both provinces. The abundance of different snail species greatly differed between habitats and seasons.

Cercariae diversity

Morphological identification

A total of 10 cercarial groups were identified, 7 and 9 in Yen Bai and Thanh Hoa, respectively (Fig. 2; Tables 1 and 2). With reference to FZT, five morphological types of parapleurolophocercous cercariae and two types of echinostome cercariae were recorded. Type 1 of parapleurolophocercous cercariae was found only in *Parrafossarulus striatulus* in Thac Ba Lake. Types 2, 3, and 5 were found only in *Melanoides tuberculata* in various habitats, while type 4 was mainly found in *M. tuberculata* and occasionally in Lymnaeidae snails. Pleurolophocercous cercariae were found only in *M. tuberculata.* Types 1 and 2 of echinostome cercariae were both found in *M. tuberculata* and Lymnaeidae snails, but type 2 was also found in *B. fuchsiana* and *B. goniomphalus*.

Molecular identification and phylogenetic relationship

BLAST searches on GenBank and pairwise p-distance comparisons of the ITS2 region demonstrated that the cercarial groups found in the study comprised of several species. Regarding FZT, *Clonorchis sinensis*, *Haplorchis pumilio*, and *Procerovum* sp. were identified from different types of parapleurolophocercous cercariae (types 1, 3, and 5, respectively). The specimen of pleurolophocercous cercaria was identified as *Centrocestus formosanus*. Echinostome type 2 was highly identical to *Echinochasmus japonicus*. All cercarial sequences appeared in a monophyletic tree with *Angiostrongylus cantonensis* (Nematoda) used as the outgroup taxon.

The NJ tree was almost identical and separated into five distinct lineages, which covered three orders, namely, Opisthorchiida (clade 1), Echinostomatida (clade 2), and Plagiorchiida (clades 3, 4, and 5) (Fig. 3). The phylogenetic tree also clearly recovered different families of based on referenced sequences. Especially, the clade 1 was comprised of two families of intestinal flukes and small liver flukes, Heterophyidae (parapleurolophocercus cercariae types 2, 3, and 5 and pleurolophocercous cercariae) and Opisthorchiidae (parapleurophocercous cercariae types 1 and 4), respectively. These data sequences revealed that the clade 5, which consisted of the family Notocotilydae, was separated from other groups.

Table 1 Prevalence (%) of cercarial infection and relative abundance (% of all infections in snails) of cercarial groups in different snail hosts in Yen Bai

Dry season snail species	No. examined	No. infected	Overall prevalence	Amphistome	Armatae	Echinostome	Megalura	Monostome	Parapleurolo-phocercous	Ornatae
Allocinma longicornis	1406	4	0.28	–	–	–	–	0.28	–	–
Angulyagra polyzonata	927	0	–	–	–	–	–	–	–	–
Austropeplea viridis	24,601	455	1.85	–	0.02	1.80	–	–	0.03	–
Filopaludina sumatrensis	527	0	–	–	–	–	–	–	–	–
Gyraulus convexiusculus	1040	20	1.92	0.38	–	1.54	–	–	–	–
Hippeutis umbilicalis	166	14	8.43	4.82	–	3.61	–	–	–	–
Melanoides tuberculata	1960	253	12.91	–	–	0.61	0.05	–	12.19	0.05
Parafossarulus striatulus	744	4	0.54	–	0.13	–	–	0.13	0.27	–
Pomacea canaliculata	245	0	–	–	–	–	–	–	–	–
Radix auricularia	1198	4	0.33	–	–	0.25	–	–	0.08	–
Sinotoia aeruginosa	465	0	–	–	–	–	–	–	–	–
Stenothyra messageri	410	0	–	–	–	–	–	–	–	–
Tarebia granifera	9	0	–	–	–	–	–	–	–	–
Thiara scabra	196	1	0.51	0.51	–	–	–	–	–	–
	33,894	*755*	*2.23*	$\chi2$=1035.9, df=7, *p* value[a] <2.2×10^{-16}						
p value[b]				<0.001	ns	<0.001	ns	ns	<0.001	ns
Relative abundance (% of all infection in snails)				*1.7*	*0.7*	*63.7*	*0.1*	*0.7*	*33.0*	*0.1*
Rainy season snail species	No. examined	No. infected	Overall prevalence	Amphistome	Armatae	Echinostome	Megalura	Monostome	Parapleurolo -phocercous	Ornatae
Allocinma longicornis	23	2	8.70	–	–	–	–	8.70	–	–
Angulyagra polyzonata	370	–	–	–	–	–	–	–	–	–
Austropeplea viridis	105	–	–	–	–	–	–	–	–	–
Filopaludina sumatrensis	130	–	–	–	–	–	–	–	–	–
Gyraulus convexiusculus	412	1	0.24	–	–	0.24	–	–	–	–
Hippeutis umbilicalis	31	1	3.23	3.23	–	–	–	–	–	–
Melanoides tuberculata	2096	66	3.15	–	0.33	–	–	–	2.81	–
Parafossarulus striatulus	27	13	48.15	–	–	–	–	–	48.15	–
Pomacea canaliculata	74	–	–	–	–	–	–	–	–	–
Radix auricularia	3042	5	0.16	0.03	–	0.10	–	–	0.03	–
Sinotoia aeruginosa	61	–	–	–	–	–	–	–	–	–
Stenothyra messageri	1253	2	0.16	–	–	–	–	0.16	–	–
Tarebia granifera	117	–	–	–	–	–	–	–	–	–
Thiara scabra	12	–	–	–	–	–	–	–	–	–
	7753	*90*	*1.16*	$\chi2$=576.2, df=6, *p* value[a] <2.2×10^{-16}						
p value[b]				ns	ns	ns	–	–	<0.001	–
Relative abundance (% of all infection in snails)				*2.2*	*7.8*	*4.4*	*0.0*	*4.4*	*81.1*	*0.0*

[a] *p* values are from logistic regression analysis with snail species as predictors

[b] Comparison between snail species

Table 2 Prevalence (%) of cercarial infection and relative abundance (% of all infections in snails) of cercarial groups in different snail hosts in Thanh Hoa

Dry season snail species	No. examined	No. infected	Overall prevalence	Amphistome	Armatae	Brevifurcate-pharyngeate	Echinostome	Megalura	Monostome	Parapleurolophocercous	Pleurolophocercous	Vivax
Allocinma longicornis	1687	81	4.80	–	–	–	0.24	–	4.56	–	–	–
Angulyagra polyzonata	3734	0	–	–	–	–	–	–	–	–	–	–
Austropeplea viridis	3362	29	0.86	–	–	–	0.83	0.03	–	–	–	–
Bithynia fuchsiana	7838	307	3.92	0.08	0.03	–	0.73	0.04	2.99	0.01	–	0.05
Bithynia goniomphalus	0	0	–	–	–	–	–	–	–	–	–	–
Gyraulus convexiusculus	95	0	–	–	–	–	–	–	–	–	–	–
Hippeutis umbilicalis	677	6	0.89	–	–	–	0.74	–	0.15	–	–	–
Melanoides tuberculata	1062	130	12.24	0.19	–	–	0.38	11.30	0.09	0.28	–	–
Parafossarulus striatulus	3239	132	4.08	0.03	0.37	–	1.30	–	2.28	–	–	0.09
Pomacea canaliculata	997	0	–	–	–	–	–	–	–	–	–	–
Radix auricularia	1814	80	4.41	–	–	0.17	4.19	0.06	–	–	–	–
Sermyla tornatella	25	0	–	–	–	–	–	–	–	–	–	–
Sinotoia aeruginosa	79	0	–	–	–	–	–	–	–	–	–	–
Stenothyra messageri	727	0	–	–	–	–	–	–	–	–	–	–
Tarebia granifera	174	7	4.02	–	–	–	–	4.02	–	–	–	–
Thiara scabra	66	2	3.03	–	–	–	–	3.03	–	–	–	–
	25,576	*774*	*3.03*	$\chi2$=303.3, df=8, *p* value[a] <2.2×10^{-16}								
p value[b]				ns	<0.001	–	<0.001	<0.001	<0.001	ns	–	ns
Relative abundance (% of all infection in snails)				*1.2*	*1.8*	*0.4*	*27.9*	*17.3*	*50.0*	*0.5*	*0.0*	*0.9*
Rainy season snail species	**No. examined**	**No. infected**	**Overall prevalence**	**Amphistome**	**Armatae**	**Brevifurcate-pharyngeate**	**Echinostome**	**Megalura**	**Monostome**	**Parapleurolophocercous**	**Pleurolophocercous**	**Vivax**
Allocinma longicornis	552	19	3.44	–	–	0.18	–	–	3.08	–	–	0.18
Angulyagra polyzonata	3050	0	–	–	–	–	–	–	–	–	–	–
Austropeplea viridis	366	9	2.46		–	–	2.19	–	0.27	–	–	–
Bithynia fuchsiana	8177	153	1.87	0.20	–	–	0.48	0.02	1.09	0.09	–	–
Bithynia goniomphalus	979	99	10.11	–	–	–	7.76	–	2.35	–	–	–
Gyraulus convexiusculus	630	2	0.32	–	–	0.16	–	0.16	–	–	–	–
Hippeutis umbilicalis	547	12	2.19	–	–	0.18	1.46	–	0.55	–	–	–
Melanoides tuberculata	731	77	10.53	–	–	–	0.82	8.76	0.68	0.14	0.14	–
Parafossarulus striatulus	1791	51	2.85	–	–	–	0.67	–	2.01	–	–	0.17
Pomacea canaliculata	1441	0	–	–	–	–	–	–	–	–	–	–
Radix auricularia	74	0	–	–	–	–	–	–	–	–	–	–
Sermyla tornatella	37	5	13.51	–	–	–	–	13.51	–	–	–	–
Sinotoia aeruginosa	77	0	–	–	–	–	–	–	–	–	–	–
Stenothyra messageri	1646	1	0.06	–	–	–	–	0.06		–	–	–
Tarebia granifera	73	0	–	–	–	–	–	–	–	–	–	–
Thiara scabra	21	0	–	–	–	–	–	–	–	–	–	–
	20,192	*428*	*2.12*	$\chi2$=459.81, df=9, *p* value <2.2×10^{-16}								
p value[b]				<0.05	–	ns	<0.001	<0.001	<0.001	ns	ns	ns
Relative abundance (% of all infection in snails)				*3.7*	*0.0*	*0.7*	*34.8*	*17.1*	*40.7*	*1.9*	*0.2*	*0.9*

[a] *p* values are from logistic regression analysis with snail species as predictors

[b] comparison between snail species

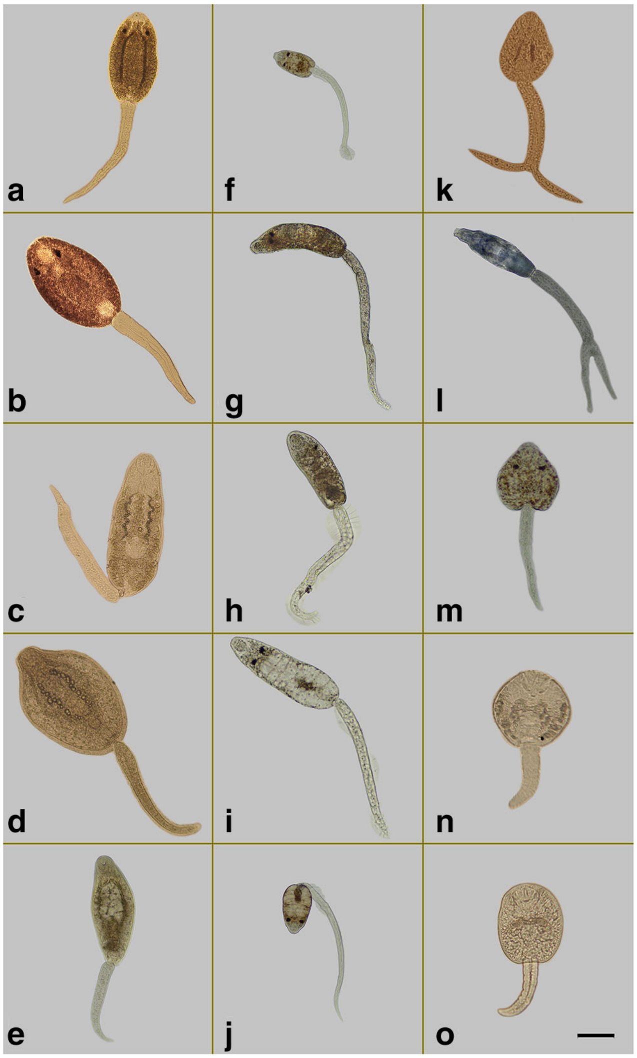

Fig. 2 Cercariae found in freshwater snails collected in Thanh Hoa and Yen Bai provinces, i.e., monostome (**a**), amphistome (**b**), echinostome (types 1–2: **c**–**d**, respectively), megalura (**e**), parapleurolophocercous (types 1–5: **f**–**j**, respectively), vivax (**k**), brevifurcate-pharyngeate (**l**), pleurolophocercous (**m**), armate (**n**), and ornatae (**o**) (**a**–**b** scale bar = 100 µm, **c**–**l** scale bar = 50 µm, **m**–**o** scale bar = 25 µm)

All specimens of the family Philophthalmidae, Echinostomatidae, Encyclometridae, Paramphistomatidae, Cyathocotylidae, Prosthogonimidae, and Notocotylidae formed single clades with high support values (100%).

FZT in freshwater snails

FZT infection in different snail species

Among 14 snail species collected in Yen Bai, 8 were found infected with trematodes (Table 1). The overall prevalences of trematode infection in different snail species were significantly different.

Melanoides tuberculata was found to harbor the highest number of trematodes in Yen Bai (6 cercarial groups) (Table 1). The most common cercariae, constituting more than 96% in the dry season and 85% in the rainy season of all cercariae recorded in Yen Bai, were FZT and those that use fish as second intermediate host, i.e., parapleurolophocercous and echinostomes. In the dry season, echinostome accounted for the highest percentage (63.8%) of all cercariae, while in the rainy season, parapleurolophocercous accounted for more than 80% of trematode infection occurrences (Table 1). Parapleurolophocercous cercariae were found in four snail species, including *A. viridis*, *M.s tuberculata*, *P. striatulus*, and *R. auricularia.* The prevalence of cercarial infection in. *M. tuberculata* and *P. striatulus* was highest in the dry and rainy season, respectively. Echinostome cercariae were found in five snail species including *Radix auricularia*, *Autropeplea viridis*, *Gyraulus convexiusculus*, *Hippeutis umbilicalis*, and *M. tuberculata*.

In Thanh Hoa, 12 infected snail species were found among 16 sampled snail species (Table 2) in which *B. fuchsiana* was found to harbor the most diverse trematodes (7 cercarial groups). In both seasons, monostome cercariae were the most common, accounting for about 50% of trematode infections in the dry season and more than 40% in the rainy season, followed by echinostome cercariae. Parapleurolophocercous cercariae and pleurolophocercous cercariae appeared with very low frequency. Echinostome cercariae were mainly found in *R. auricularia* (dry season) and *B. goniomphalus* (rainy season) and less in other snail hosts, such as *M. tuberculata*, *P. striatulus*, *B. fuchsiana*, *A. viridis*, *G. convexiusculus*, and *H. umbilicalis* (Table 2).

The 2D-MDS graphs depicted in Figs. 4 and 5 display the (dis)similarities of cercariae composition based on cercarial infections between different snail species in the dry and rainy seasons in Yen Bai and Thanh Hoa. At a similarity level of about 10%, the cercariae assemblages in Bithyniidae snail (e.g., *A. longicornis* and *P. striatulus*) were in a same group, which is separated from the group of other snail species in both seasons and provinces. At a similarity level of about 30%, the cercariae composition of the snail *M. tuberculata* was separated from other groups in both seasons and provinces. The cercariae assemblages in *Autropeplea viridis*, *Gyraulus convexiusculus*, and *Hippeutis umbilicalis* were always in the same group at about 50% of similarity.

FZT infection in snails from different habitats

The overall prevalence of cercarial infections in snails from different habitats in Yen Bai varied from 1.3 (stream) to 3.2% (ditch) in the dry season and from 0 (pond) to 3.7% (ditch) in the rainy season (Table 3). Except for ditches, the prevalence of trematode infection in snails from different habitats tended to decrease from dry to rainy season. The overall prevalence in snails from different habitats in Thanh Hoa ranged from 0.4 (pond) to 5.5% (ditch) in the dry season and from 1 (pond) to 3.6% (ditch) in the rainy season (Table 4). In the rainy season, the prevalence of trematode infection in snails collected in ditch, rice field, and river decreased,

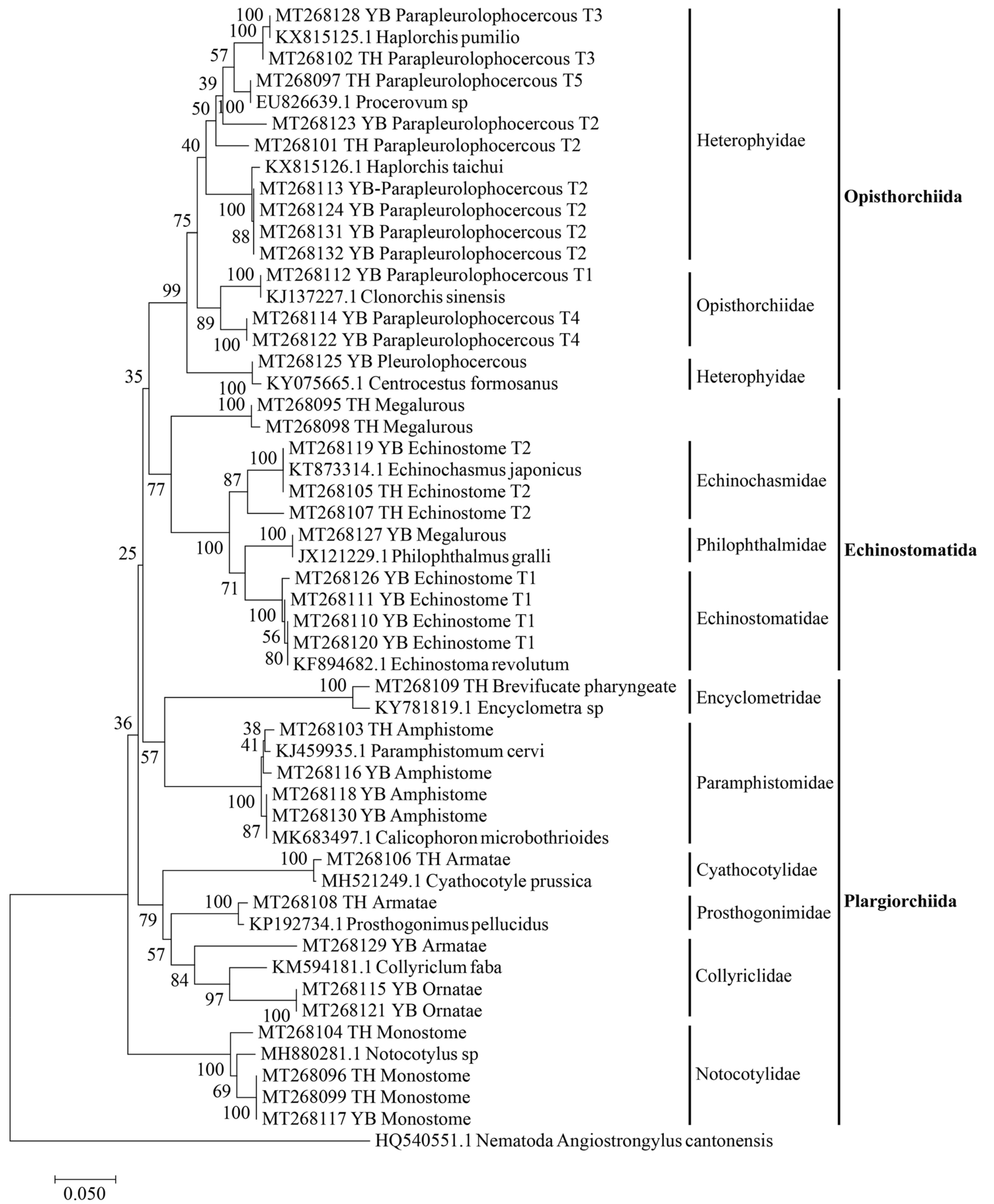

Fig. 3 Phylogenetic tree included thirty-seven studied sequences of trematode cercariae from Yen Bai and Thanh Hoa provinces (GenBank accession numbers started with MT followed by six digits) and sixteen referenced sequences from GenBank based on partial ITS2 region. The evolutionary history was inferred using the neighbor-joining (NJ) method, and evolution distance was computed using p-distance method and number of base differences per site. All positions with less than 95% site coverage were eliminated. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *Angiostrongylus cantonensis* (Nematoda) was used as the outgroup taxon (YB, Yen Bai Province; TH, Thanh Hoa Province; T, Type)

while it increased in pond and canal. In Yen Bai, snails collected in rice fields were found to harbor the most diverse cercarial groups (7 groups), while in Thanh Hoa, river and rice field were preferred snail habitats to a higher number of cercarial groups (9 groups) in comparison with other habitats. The logistic regression result showed that the infection of some cercarial groups was associated with snail habitat (Tables 3 and 4).

More specifically to FZT, parapleurophocercous cercariae in Yen Bai were recorded from snails in all types of habitats among which the ditch was found the most favorable in both

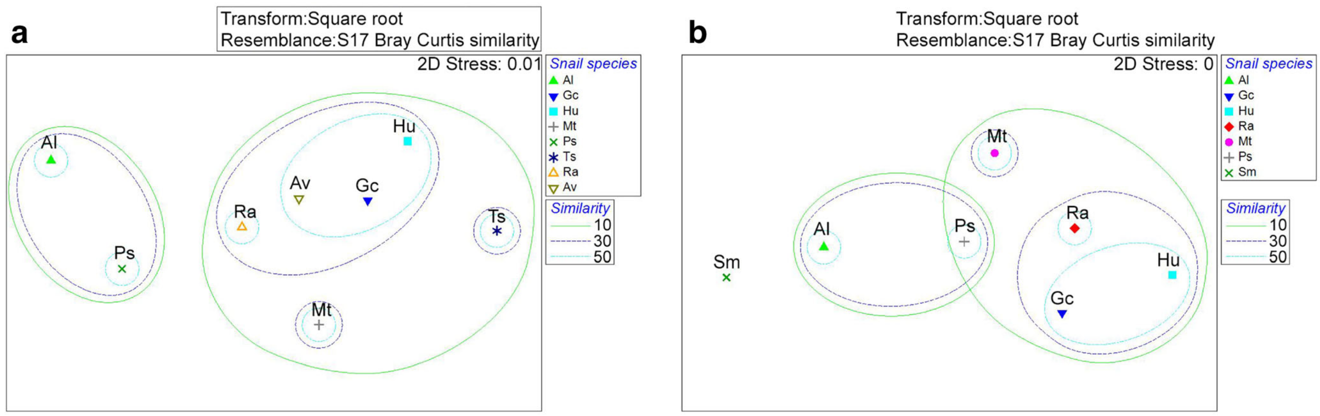

Fig. 4 The 2D-MDS plot for the prevalence infection in different snail species in dry (**a**) and rainy (**b**) seasons in Yen Bai with overlying clusters at three similarity levels (Al, *Allocinma longicornis*; Av, *Autropeplea viridis*; Gc, *Gyraulus convexiusculus*; Hu, *Hippeutis umbilicalis*; Mt, *Melanoides tuberculata*; Ps, *Parafossarulus striatulus*; Ra, *Radix auricularia*; Sm, *Stenothyra messageri*; Ts, *Thiara scabra*)

seasons ($p < 0.01$) (Table 3). Echinostome cercariae were not found in lake habitats. In Thanh Hoa, parapleurophocercous cercariae were recorded in snails collected in rice field and river habitats, while pleurophocercous cercariae were only recorded in rice fields. Echinostome cercariae were found with highest infection rates in snails from ditches during the dry season and in canals in the rainy season (Table 4).

Discussion

Diversity of FZT in freshwater snail hosts

Based on morphological criteria, the present study recorded 15 different cercarial types within 10 cercarial groups, including parapleurolophocercous, pleurolophocercous, and echinostome cercariae, which belong to the families of liver and intestinal flukes. These records can be compared with findings in Nam Dinh Province, where Madsen et al. (2015) and Bui et al. (2010) found 12 and 10 cercariae types, respectively, including the groups found in this study.

The present study is the first to report molecular identification of cercariae in Yen Bai and Thanh Hoa provinces. In comparison to morphological identification, which allows distinguishing cercariae only up to group or family levels, the ITS2 data in this study allowed identification up to species level. A number of FZT was revealed from different cercarial types, including Opisthorchiidae, Heterophyidae, and Echinostomatidae. *Clonorchis sinensis*, the most prevalent human liver fluke in Asia, was found in Thac Ba Lake, Yen Bai. Notably, in a previous study, *C. sinensis* was also reported in various wild fish species in Thac Ba Lake (Bui NT et al. 2016). Since Thac Ba Lake is a major source of fish for the local population that has the habit of eating raw or undercooked fish (Bui NT et al. 2016; Phi 2018), there is a high risk of infection by the small liver fluke. Trematode species of the family Heterophyidae in the study included *Haplorchis pumilio* and *H. taichui.* Infection with these intestinal flukes is an important and continuing public health problem in many Southeast Asian countries, including Vietnam

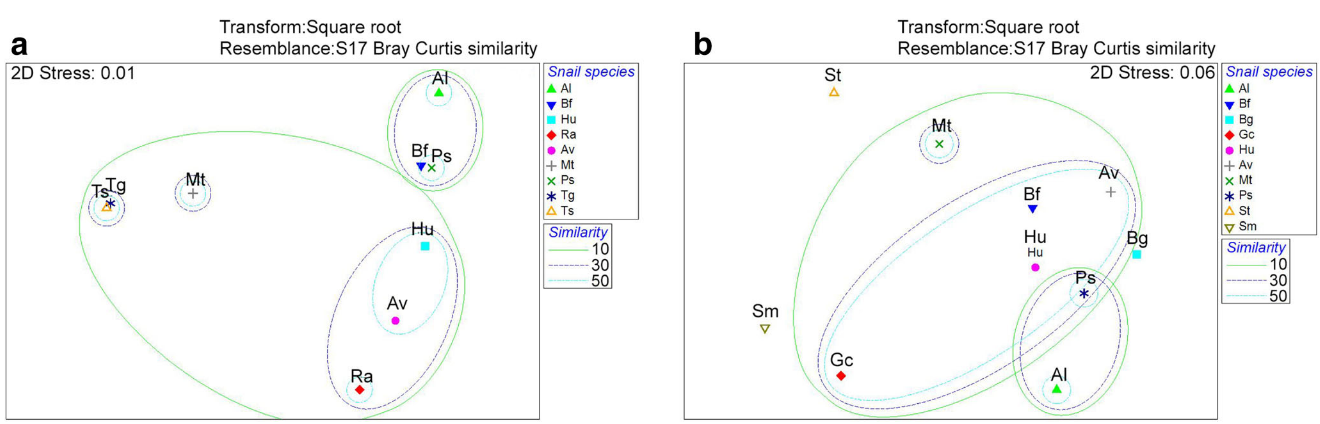

Fig. 5 The 2D-MDS plot for the prevalence infection in different snail species in dry (**a**) and rainy (**b**) seasons in Thanh Hoa with overlying clusters at three similarity levels (Al, *Allocinma longicornis*; Av, *Autropeplea viridis*; Bf, *Bithynia fuchsiana*; Bg, *Bithynia goniomphalus*; Gc, *Gyraulus* convexiusculus; Hu, *Hippeutis umbilicalis*; Mt, *Melanoides tuberculata*; Ps, *Parafossarulus striatulus*; Ra, *Radix auricularia*; Sm, *Stenothyra messageri*; St, *Sermyla tornatella*; Tg, *Tarebia granifera*; Ts, *Thiara scabra*)

Table 3 Prevalence (%) of cercarial infection in different habitats in Yen Bai (ditch, 11; lake, 24; pond, 7; rice field, 64; stream, 27)

Dry season habitats	Amphistome	Armatae	Echinostome	Megalura	Monostome	Parapleurolophocercous	Ornatae	Overall prevalence
Ditch	–	–	0.72	–	–	2.49	–	3.21
Lake	0.09	–	–	–	0.18	1.12	–	1.39
Pond	–	–	1.09	–	–	0.48	–	1.58
Rice field	0.01	0.02	1.76	0.004	0.004	0.40	0.004	2.20
Stream	0.46	–	0.76	–	–	0.05	–	1.27
p value	ns	–	<0.001	–	ns	<0.001	–	
Rainy season habitats	Amphistome	Armatae	Echinostome	Megalura	Monostome	Parapleurolophocercous	Ornatae	Overall prevalence
Ditch	–	0.22	–	–	–	3.47	–	3.69
Lake	–	0.19	–	–	–	0.57	–	0.76
Pond	–	–	–	–	–	–	–	–
Rice field	0.05	0.02	0.05	–	0.10	0.98	–	1.19
Stream	–	0.16	0.08	–	–	0.53	–	0.77
p value	–	ns	ns	–	–	<0.001	–	

p values are from logistic regression analysis with habitat as predictors

(Chai et al. 2005, 2009; Do et al. 2007). Moreover, the common finding in the two provinces of *Melanoides tuberculata*, a suitable snail host for these *Haplorchis* spp., together with fish and reservoir hosts and the common habit of eating raw fish are important risk factors for haplorchiasis in communities in these areas. Echinostome cercariae in the study were found to belong to different intestinal fluke species. *Echinostoma revolutum* is known to be a medically important zoonotic intestinal parasite in humans and commonly occurs in Southeast Asia (Saijuntha et al. 2011). *Echinochasmus japonicus* is a trematode of birds and mammals, but human infections have also been reported in China, Korea, and Laos (Mao 1991; Seo et al. 1985; Sayasone et al. 2009).

The phylogenetic tree based on ITS2 generally discriminated all specimens in five major clades in accordance with different trematode families. However, a combination of morphological and molecular approaches is needed to resolve the systematic and taxonomic challenges of the super-family Opisthorchiidae.

FZT infection in different snail species

Seasonal variation in prevalence of larval trematodes in different snail species was recorded. Some snail species showed a higher prevalence of cercariae in a specific season, while others were observed with a lower rate. Also, the proportions of cercarial infections by each cercarial group showed differences between seasons and provinces. As discussed in previous studies, the distribution of cercarial stages of different trematode taxa closely relates to the seasonal occurrence of freshwater snails (Chung et al. 1980; Imani-Baran et al. 2013; Soldánová et al. 2012). Snail abundances were significantly different between seasons in both Yen Bai and Thanh Hoa. Especially, the snail counts in Yen Bai dramatically declined from dry season to rainy season, particularly in *A. viridis*, *A. longicornis*, and *P. striatulus.* Therefore, the decrease in overall prevalence from dry to rainy season in the study might relate to the decrease in snail abundance and change in snail composition. Poulin (2006) also pointed out that even a small change in air and water temperature can affect the distribution of some snail-borne diseases by regulating snail populations. Temperature is also an important factor for trematode transmission (Mouritsen 2002). The significant seasonal changes in environmental factors, such as water temperature (in all habitats) and rainfall observed in this study, might explain the temporal fluctuation of trematode prevalence in snail hosts. However, also other factors, such as the change in density of definitive hosts (cattle, dogs, cats, and waterfowl) between season and the degree to which they introduce egg-contaminated feces into the snail's environment, may affect the trematode prevalence in snails. Therefore, the relation between environmental factors and intermediate and definitive hosts distribution and FZT infection needs to be further investigated in a longitudinal study.

Snail hosts as risk factor of FZT

The distribution of cercarial stages of different trematode taxa highly adapts and closely relates to the occurrence of freshwater snails in a great variety of habitats (Imani-Baran et al. 2013). In general, cercarial infection was more prominent in *M. tuberculata* and snail species of Bithyniidae than in other snail hosts in the studied provinces. Similar results were found in Thailand and Vietnam (Anucherngchai et al. 2016; Bui et al. 2010; Madsen et al. 2015). The present study also revealed that some snail species are apparently capable of acting as primary hosts to a large number of cercarial groups. *Melanoides*

Table 4 Prevalence (%) of cercarial infection in different habitats in Thanh Hoa (canal, 15; ditch, 5; pond, 28; rice field, 60; river, 22)

Dry season habitats	Amphistome	Armatae	Brevifurcatepharyngeate	Echinostome	Megalura	Monostome	Parapleurolophocercous	Pleurolophocercous	Vivax	Overall Prevalence
Canal	–	–	–	0.65	0.06	1.35	–	–	0.18	2.23
Ditch	0.27	–	–	5.28	–	–	–	–	–	5.55
Pond	–	–	–	0.34	–	0.03	–	–	–	0.36
Rice field	0.04	0.04	–	0.75	0.90	1.56	0.01	–	0.01	3.31
River	0.02	0.06	0.06	0.53	0.13	2.78	0.04	–	0.06	3.61
p value	<0.05	<0.05	–	<0.001	<0.001	<0.005	ns	–	<0.01	
Rainy season habitats	Amphistome	Armatae	Brevifurcatepharyngeate	Echinostome	Megalura	Monostome	Parapleurolophocercous	Pleurolophocercous	Vivax	Overall Prevalence
Canal	0.27	–	–	1.82	–	1.09	–	–	–	3.19
Ditch	–	–	–	0.24	0.48	2.87	–	–	–	3.59
Pond	0.08	–	–	0.85	–	0.08	–	–	–	1.00
Rice field	0.09	–	0.01	0.69	0.50	0.69	0.05	0.01	–	2.04
River	–	–	0.03	0.62	0.05	1.48	0.03	–	0.11	2.31
p value	ns	–	ns	<0.001	<0.001	<0.001	ns	–	–	

p values are from logistic regression analysis with habitat as predictors

tuberculata was found to harbor four cercarial groups, while *P. striatulus* was capable of acting as a host to at least six groups of cercariae.

The results of MDS analysis indicate specific patterns of larval trematode assemblages in associating with different snail species. Therefore, the structure of the snail host population in the waterbodies might shape the cercariae community structure. While some snail species have not been recorded as hosts of any FZT cercariae in previous studies (Bui et al. 2010; Madsen et al. 2015) and in this study as well such as snail species of the families Viviparidae and Ampulariidae, the presence of other snail species can be a potential risk factor for predicting the likelihood of specific groups of FZT occurring in waterbodies. For instance, pleurolophocercous cercariae were only recorded from *M. tuberculata*, while parapleurolophocercous were recorded in snails belonging to three families, i.e., Bithyniidae, Thiaridae, and Lymnaeidae. *Melanoides tuberculata* and *P. striatulus* appeared with highest prevalence of parapleurolophocercous that make them a potential risk factor for spreading FZT infections, i.e., clonorchiasis and haplorchiasis. The finding of echinostome cercariae in snail species belonging to three families, i.e., Bithyniidae, Thiaridae, and Lymnaeidae, is comparable to some studies in Vietnam and other countries, such as Thailand, Bangladesh, Nepal, Iran, Germany, and Iceland (Anucherngchai et al. 2017; Bui et al. 2010; Dodangeh et al. 2019; Georgieva et al. 2013; Islam et al. 2012; Madsen et al. 2015).

Remarkably, regarding host specificity, cercariae of *C. sinensis* were only found in *P. striatulus* in Thac Ba Lake. Although many fish species can serve as the second intermediate hosts of *C. sinensis*, very few snail species have been found to serve as the first intermediate host. Most snail hosts of *C. sinensis* belong to the Bythinidae in which *Parafossalurus manchouricus* is an important host in China (Lai et al. 2016; Lun et al. 2005), Japan (Yoshida 2012), Korea (Choi and Chung 1975; Choi 1984; Joo 1980), and Russia (Petney et al. 2013). However, in a previous study in Vietnam, *M. tuberculata* was identified as the first intermediate host of *C. sinensis* (Le et al. 1996; Nguyen et al. 1996), but that result remains questionable since morphological identification of cercariae to species level is difficult (Pham and Nawa 2016). Therefore, our finding using a molecular approach indicating *P. striatulus* as the intermediate snail host of *C. sinensis* is very important to complete the knowledge of its life cycle in Vietnam and to develop intervention strategies.

FZT in different habitats

Our results pointed out that FZT infections in snails were found in all types of habitat surveyed, especially in rice fields, lakes, and ponds and that they showed a seasonal trend, i.e., a decrease from dry season to rainy season. Again, the seasonal variation of environmental factors might cause changes in prevalence of trematode infection by affecting snail abundance and distribution. Host demographic changes can cause influences on temporal variation in parasite prevalence (Mullowney et al. 2011). Consequently, a sharp decrease in snail abundance and species composition between seasons in the study might explain the decrease in trematode infection rate. Moreover, the significant change in environmental factors in the study, such as temperature (in all habitats), might have a direct effect on snail abundance. Therefore, the link between environmental factors, snail host distribution, and trematode infection in the study needs to be further investigated. Rice fields undergo the most pronounced seasonal changes due to agricultural practices (Madsen et al. 2015), and ponds also change their environment linked to aquaculture practice (Bui et al. 2010). Obviously in Thac Ba Lake, the water level dramatically increases during the rainy season, which may limit snail appearance, therefore affecting trematode infections. In rivers, snail density and diversity can be similarly affected by the rise of the water level together with duck raising (adult ducks), which was more common during rainy season in Thanh Hoa (Edan 2006; Nguyen 1993).

Conclusions

Foodborne zoonotic trematodes are widely distributed and continue to be an important public health problem in Southeast Asia, including Vietnam. The present study is part of the FOODTINC project, which aims at contributing to reducing the impact of FZT infections on the Vietnamese population and especially in developing an integrated control of FZT infection in Yen Bai and Thanh Hoa Provinces. The result of this study provides important information on the diversity and epidemiological situation of larval trematodes present in freshwater snail hosts in different habitats with an emphasis on FZT, which can be used for prevention, management, and epidemiological control programs.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00436-020-07027-1.

Acknowledgments We thank Prof. Dr. Frédéric Farnir, MSc, Nguyen Thi Bich Ngoc, MSc, and Huynh Chi Cuong (University of Liège, Belgium) for statistical analysis advice. We also wish to thank Mr. Hoang Tuan Anh for graphical design.

Funding The study was mainly supported by the international project "Foodborne Zoonotic Trematode infections and integrated control in Vietnam" (FOODTINC) and the Académie de recherche et d'enseignement supérieur (ARES-CCD), Belgium, and partly by the national project for molecular work under grant number 106.05-2017.26 funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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