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Prevalence of fish-borne zoonotic trematode infection in Jilin Province, China

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A B S T R A C T

Fish-borne zoonotic trematodes (FZTs) are the most serious food-borne parasites in Asia and have become a burden to public health and a new challenge in food safety. In Jilin Province, China, the prevalence of FZTs in intermediate and definitive hosts has not been extensively explored. In the present study, we investigated the prevalence of FZTs in Jilin Province, China. From July to November 2020, a total of 132 freshwater snails (the first intermediate host of FZTs), 4122 wild freshwater fishes (the second intermediate host of FZTs) and 143 fecal samples from canines, ducks and swine (the definitive host of FZTs) were collected from the Yitong River basin of Jilin Province. FZT species were identified by morphological observation combined with internal transcribed spacer (ITS) sequence analysis. The prevalence of FZTs was then calculated. The results showed that the prevailing species of FZTs in Jilin Province, China, were *Clonorchis sinensis, Metorchis orientalis* and *Echinochasmus japonicus.* The total prevalence of FZTs was 29.74% (1226/4122) in fish, the total infection rates were 2.27% (3/132) in snails, 75.00% (21/28) in canines and 37.18% (29/78) in ducks. The coinfection rates of the two trematodes were 13.39% (552/4122) in fish, 35.71% (10/28) in canines and 7.69% (6/78) in ducks. The co-infection rate of the three flukes was 2.60% (107/4122) in fish. Nine of the 12 fish species examined were infected with FZT metacercariae.

1. Introduction

Fish-borne zoonotic trematodes (FZTs) are an important group of zoonotic parasites transmitted by fish and fish products (Hop et al., 2007). A total of 59 species of FZTs have been found to parasitize humans. FZTs' life cycle involve a first intermediate host (freshwater snails), a second intermediate host (freshwater fish) and a definitive host (human or other animals). These trematodes can be grouped into two main categories, the liver fluke group (*Opisthorchiidae*: 12 species) and the intestinal fluke group (*Heterophyidae*: 36 species, *Echinostomatidae*: 10 species and *Nanophyetidae*: 1 species) (Qiu et al., 2017). They are primarily related to liver and biliary disorders and intestinal diseases, causing severe disease and economic burden (Tang et al., 2016; Yu and Mott, 1994).

FZTs are primarily distributed in Asia, including China, South Korea, Japan, Laos, Vietnam, Thailand and the Far East of Russia (Qian et al., 2016; Stefan et al., 2013; WHO, 1995). More than 26 million people have been infected with liver flukes, including 15 million infected with *C. sinensis* (Qian et al., 2016; Sripa et al., 2007). In cases of intestinal FZTs, an estimated 40 to 50 million people are infected with one or several species (Hung et al., 2015; Keiser and Utzinger, 2009). The estimates of at-risk populations for FZTs are more than 1.1 billion (Santos and Howgate, 2011; Sripa et al., 2007, 2010). The World Health Organization recently added FZT infections to its list of emerging infectious diseases (Clausen et al., 2012). The prevalence of *C. sinensis* in freshwater fish is 67.5% in Korea and 12.5% in Vietnam (Sohn et al., 2018). In China, the prevalence of *C. sinensis* metacercariae in fish is 35.1% (in 19 provinces/municipalities) (Zhang et al., 2020).

FZT species that have been reported in China include *C. sinensis*, *E. japonicus*, *M. orientalis*, *Isthmiophora hortensis*, *Procerovum varium*,

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Metagonimus yokogawai, *Haplorchis pumilio*, *Stellantchasmus falcatus*, *Haplorchis taichui*, and *Centrocestus formosanus* (Choi et al., 2006; Qiu et al., 2017). Jilin Province, located in the northeast region of China, has a high infection rate of *C. sinensis* in humans (Li et al., 2011) because the local people have a habit of eating raw and undercooked fish (Lun et al., 2005). The prevalence of *C. sinensis* in the Songhua River, Fuyu County, Western Jilin Province, is 31.96% (Zhang et al., 2014, 2020). However, data on the FZT species and prevalence in Jilin Province have not been explored extensively, which is very detrimental to the prevention and control of FZTs in Jilin Province. Therefore, this study conducted an epidemiological investigation on FZTs in Jilin Province from July to November 2020.

2. Materials and methods

2.1. Description of sampling sites and sampling time

The Yitong River basin runs through the central area of Jilin Province, which is located in multiple important economic locations and is an important supply source for freshwater fish in Jilin Province. It is the second largest tributary of the Songhua River, with a basin area of 8440.00 square kilometers and a total length of 342.50 km. Freshwater fish and snail samples were collected in the upstream, midstream and downstream areas of the Yitong River basin, with 13 sampling points covering reservoirs, ponds, river mainstems, river tributaries, streams and wetlands. The fish samples were continuously harvested from July to November 2020. Fecal samples were collected from the definitive hosts living in villages or wildlife living around these 13 collection sites (Fig. 1). The definitive host feces and wild freshwater snails were harvested from July to November 2020.

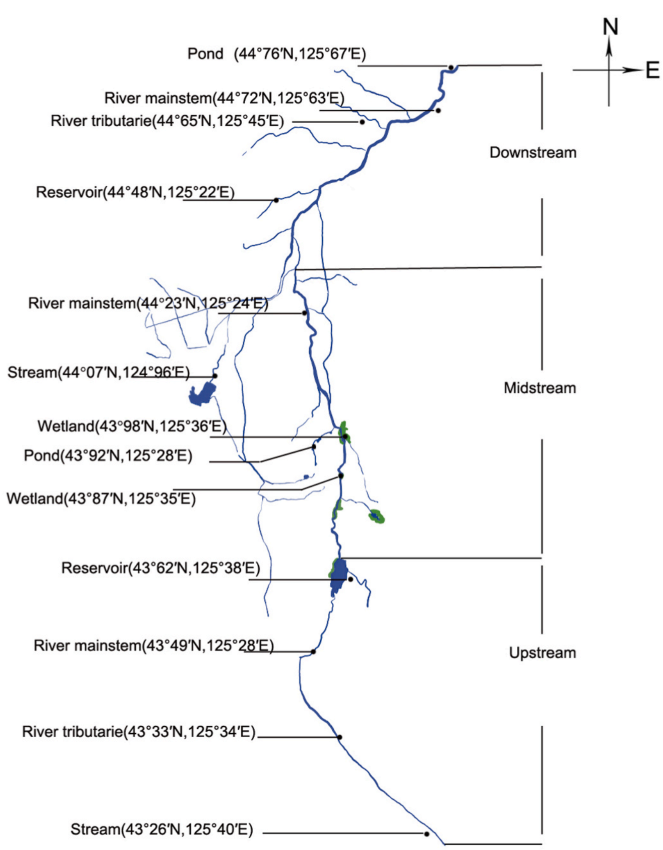

Fig. 1. Sampling sites of freshwater fish, freshwater snails and the definitive hosts' feces.

2.2. Sample collection

2.2.1. Freshwater fishes

A total of 4122 freshwater fishes from 12 species (*Pseudorasbora parva*, *Hemiculter leucisculus*, *Perccottus glehnii*, *Rhodeus sinensis*, *Ctenogobius giurinus*, *Carassius auratus*, *Saurogobio dabryi*, *Misgurnus anguillicaudatus*, *Cyprinus carpio*, *Silurus asotus*, *Ophiocephalus argus Cantor* and *Hemisalanx prognathus Regan*) were randomly collected from the Yitong River basin. We collected freshwater fish using fishing nets or purchased them from fishermen, and the collected fish are commonly and popularly consumed species in the area. The sampling information for the freshwater fish is provided in Additional File Table S1.

2.2.2. Freshwater snails

Freshwater snails were collected during the morning for 20 min per site by fully randomly handpicking or catching them with a net. In 9 of 13 sampling collection sites, a total 132 snails from 4 species (46 *Parafossarulus striatulus*, 11 *Bithynia. sp.,* 68 *Viviparidae gen. sp.* and 7 *Radix auricularia*) were obtained for trematode detection.

2.2.3. Fecal samples of canines, swine and ducks

A total of 143 fecal samples from canines (28 samples), ducks (78 samples) and swine (37 samples) were randomly collected. The majority of animals were domestic and feral (12 ducks) and more than 12 months old (100% canines, 100% swine, 46.15% ducks). Feces from canines, ducks and swine were directly sampled immediately after defecation. The duck breeds were Partridge duck and *Anas platyrhynchos*. The dogs were primarily Chinese field dogs, and the swine were Landrace and Large Yorkshire. The sampling information from the definitive hosts is provided in Additional File Table S2.

2.3. Morphological observation of FZTs in the samples

2.3.1. FZT metacercariae observation in freshwater fishes

FZT metacercariae were detected using a combination of compression and artificial digestion methods as previously described (WHO, 1995). Briefly, a compression process was used for small-sized fish ($\leq$10 g): each fish was compressed using two glass slides and observed under a microscope. The fish were repeatedly compressed and observed three times. Then, FZT-positive fish were used to isolate metacercariae. Artificial digestion was used for large-sized fish or to isolate metacercariae. Each whole fish was cut into small pieces and ground using a homogenizer, which was placed in a beaker with digestion solution (10 g pepsin, 6 g NaCl and 10 mL concentrated HCl in 1 L distilled water) at a ratio of 1 g tissue per 10 mL solution. The mixture was thoroughly stirred at 37 °C for 3 h. After digestion, the large particles were removed by filtering through a 250 μm/aperture copper sieve. The filtered solution was transferred to a triangle measuring cup, diluted in tap water and kept still for 2 h. The supernatant was discarded, and the sediment was retained. This procedure was repeated until the supernatant became clear. The sediment was transferred to a cell culture dish containing 5 mL sterile PBS, and the metacercariae were morphologically identified and classified under a microscope and counted.

The prevalence, metacercariae abundance and calculated intensity/100 g fish of FZTs were calculated as shown (Bush et al., 1997).

$$\text{Prevalence} = \frac{\text{The number of metacercariae} - \text{infected fish}}{\text{The number of fish examined}} \times 100\%$$

$$\text{Calculate intensity}/100\text{ g fish} = \frac{\text{The total number of metacercariae}}{\text{The total weight of each fish species (g)}} \times 100$$

Metacercariae abundance: The number of metacercariae in each fish.

2.3.2. FZT examination in snails

Small snails were crushed using two glass slides, and FZT larvae were observed under a microscope. The large hard-shelled snails were opened, and visceral tissue and hemolymph were transferred onto glass slides for microscopic observation (Hung et al., 2015). The infection rate of FZTs was calculated (Bush et al., 1997).

2.3.3. FZT egg detection in fecal samples

Fecal samples were examined for FZT eggs using direct smears and microscopic observation. The smear was repeated three times for each sample to improve the detection rate. The infection rate of FZTs was calculated in definitive hosts (Bush et al., 1997).

2.4. ITS sequence amplification and analysis for FZTs

2.4.1. Genomic DNA extraction

All of the fecal samples and freshwater snails were stored at 4 °C until DNA extraction. Different kinds of metacercariae species from three independent experiments were stored in Alsever's solution at 4 °C prior to DNA extraction. Genomic DNA from different metacercariae species and freshwater snail samples was extracted using a genomic DNA Kit (TIANGEN BIOTECH, Beijing, China). DNA from the definitive host fecal samples was performed strictly according to the instructions of the Fecal Genomic DNA Kit (TIANGEN BIOTECH, Beijing, China). All DNA precipitates were stored at −80 °C.

2.4.2. Detection of ITS sequences for FZTs

The internal transcribed spacer (ITS) sequences of FZTs from all the extracted DNA were amplified by PCR using ITS sequence primers (Additional file Table S3) as previously described (Qiu et al., 2019; Zhou et al., 2010). After amplification, 10 μL of PCR product was examined by agarose gel (1.5%) electrophoresis and ethidium bromide substitution (Applygen Technologies Inc. Beijing, China) staining. All positive products were sequenced by Life Technology Company (Comate Bioscience Co., Ltd., Jilin, China).

2.4.3. Phylogenetic analysis of ITS sequences

To study the phylogenetic relationships of the isolated and other FZTs, ITS sequences of liver flukes (*M. orientalis, M. ussuriensis, C. sinensis, Opisthorchis sudarikovi, Opisthorchis felineus*, *Erschoviorchis anuiensis* and *Euryhelmis costaricensis*) and intestinal flukes (*Echinostoma trivolvis, Echinoparyphium mordvilkowi, Stephanoprora uruguayense, Stephanoprora chasanensis, Stephanoprora pseudoechinata, E. japonicus, Echinochasmus milvi, Echinochasmus coaxatus*) from GenBank of the National Center of Biotechnology Information (NCBI) were used for phylogenetic analysis using *Aspidogaster chongqingens* as the outgroup. The phylogenetic tree for ITS genes of FZTs was reconstructed using MEGA X software. The methods of maximum likelihood (ML), maximum parsimony (MP) and neighbor joining (NJ) were used for phylogenetic construction, and the initial trees were automatically obtained by selecting the topology with a superior log likelihood value (Wang et al., 2020).

2.5. Data analysis

The overall prevalence of FZT metacercariae in freshwater fish and their respective prevalence with respect to different factors (fish species, sampling location, sampling water source, sampling time) were statistically analyzed using SPSS24.0 (IBM, Armonk, NY, USA), and the analysis shows the 95% confidence intervals (95% CI). Differences in data between groups and statistical graph plotting were performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). A p value < 0.05 was considered to indicate a significant difference.

3. Results

3.1. The prevalence of FZTs in wild freshwater fish

3.1.1. The prevalence of FZTs in wild freshwater fish in Jilin Province

Morphological observation of metacercariae combined with ITS sequence analysis revealed that the prevailing FZTs in Jilin Province were *C. sinensis*, *M. orientalis* and *E. japonicus* (Figs. 2 and 3). The metacercariae of *C. sinensis* were oblate or elliptical, 135.65 ± 27.65 μm × 93.23 ± 12.76 μm in size and contained an active larva with two layers of thin cyst walls. The excretory sac of the larva was dark brown, and the oral sucker and ventral sucker were similar in size (Fig. 2 A and D). The metacercariae of *M. orientalis* were oval or round, 113.37 ± 12.52 μm × 101.89 ± 13.87 μm in size, with two layers of thick cyst walls (10.05 ± 2.50 μm thick). The metacercariae contained a movable larva with a slower activity frequency than that of *C. sinensis*, with a brownish yellow excretory sac with a similar size between the oral and ventral suckers (Fig. 2 B and E). The metacercariae of *E. japonicus* were elliptical and very small, approximately 78.63 ± 12.18 μm × 43.66 ± 8.95 μm in size. The metacercariae were translucent, with various round particles distributed in the excretion tube (Fig. 2 C and F).

The ITS sequences of *C. sinensis*, *M. orientalis and E. japonicus* metacercariae from different fish species were analyzed by molecular biology and deposited in NCBI GenBank (Nos. **MW828640**, **MW828729** and **MW828605**), which were 100% homologous to the reported ITS sequences of the trematodes deposited in NCBI GenBank (Nos. **MF319654**, **MK482055** and **KT873314**) (Fig. 3) (Besprozvannykh et al., 2017; Na et al., 2016; Tatonova et al., 2017). The ITS sequence phylogenetic tree indicated that the sequences were classified as *C. sinensis*, *M. orientalis* and *E. japonicus* (marked with * in Fig. 3).

The prevalence of FZTs in freshwater fish in Jilin Province was 29.74% (1226/4122) (Table 1). The infection rates of *C. sinensis*, *M. orientalis* and *E. japonicus* were 22.59% (931/4122), 9.92% (409/4122) and 18.10% (746/4122), respectively (Table 1). The total coinfection rate of FZTs was 13.39% (552/4122). The coinfection rate of *C. sinensis* and *M. orientalis* was 4.95% (204/4122), *C. sinensis* and *E. japonicus* was 7.23% (298/4122), *M. orientalis* and *E. japonicus* was 3.81% (157/4122), and the coinfection rate of the three species FZTs was 2.60% (107/4122) (Table 1).

3.1.2. Difference in FZT prevalence in different wild freshwater fishes

FZT infection was observed in 9 out of 12 fish species, including *P. parva*, *H. leuciclus*, *P. glenii*, *R. sinensis*, *C. giurinus*, *C. auratus*, *S. dabryi*, *M. anguillicaudatus*, and *C. carpio*, while *S. asotus*, *O. argus Cantor* and *H. prognathus Regan* were not infected with FZTs. *C. sinensis* metacercariae were found in *P. parva*, *H. leuciclus*, *P. glenii*, *R. sinensis*, *C. giurinus*, *C. auratus*, *C. carpio* and *S. dabryi*, and the prevalence of *C. sinensis* among infected fishes ranged from 3.10% (*C. auratus*, 24/773) to 60.76% (*P. parva*, 607/999). The calculated intensity/100 g fish of *C. sinensis* varied from 0.06 to 6528.54, with the highest prevalence and the maximum metacercariae abundance observed in *P. parva* (Table 2). *P. parva*, *H. leuciclus*, *P. glenii*, *R. sinensis* and *S. dabryi* were infected with *M. orientalis* metacercariae. The prevalence of *M. orientalis* among infected fishes ranged from 2.00% (*P. glenii,* 5/250) to 28.73% (*P. parva,* 287/999), and the calculated intensity/100 g fish ranged from 0.30 to 366,78, with the highest prevalence and the maximum metacercariae abundance observed in *P. parva* (Table 2). *E. japonicus* metacercariae were detected in *P. parva*, *H. leuciclus*, *R. sinensis*, *C. giurinus*, *M. anguillicaudatus* and *S. dabryi*, and the prevalence of *E. japonicus* among infected fishes ranged from 0.81% (*C. giurinus*, 5/619) to 40.20% (*R. sinensis*, 318/791). The calculated intensity/100 g fish ranged from 6.76 to 700.89 (Table 2). *R. sinensis* was the freshwater fish with the highest prevalence and maximum metacercariae abundance of *E. japonicus* (Table 2).

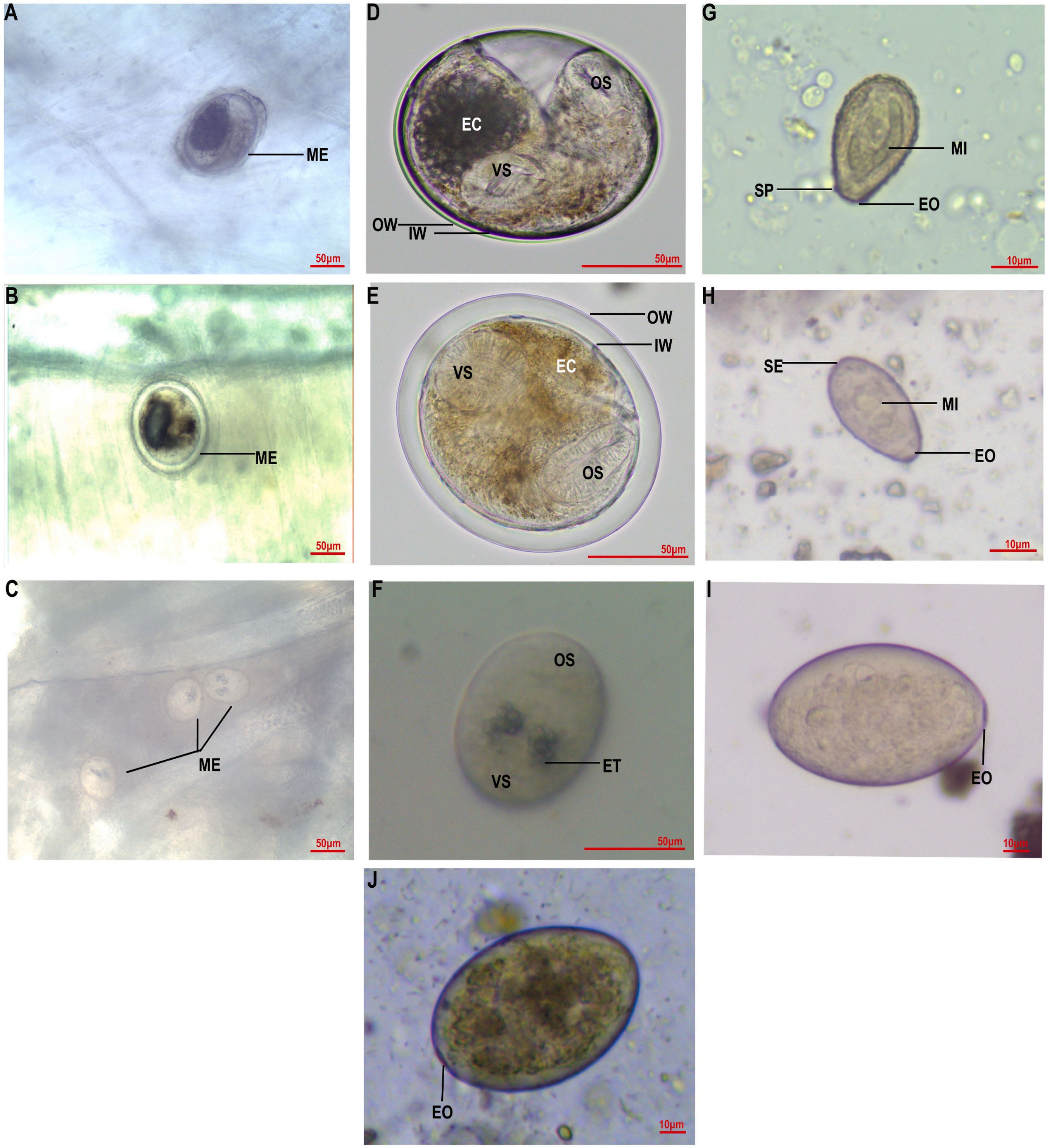

Fig. 2. The metacercariae and eggs of *C. sinensis, M. orientalis* and *E. japonicus.C. sinensis* metacercariae encysted on the flesh of *P. parva*, scale bar = 50 μm (A). *M. orientalis* metacercariae encysted on the flesh of *P. parva*, scale bar = 50 μm (B). *E. japonicus* metacercariae encysted on the gills of *R. sinensis*, scale bar = 50 μm (C). *C. sinensis* metacercariae isolated from *P. parva*, scale bar = 50 μm (D). *M. orientalis* metacercariae isolated from *P. parva*, scale bar = 50 μm (E); *E. japonicus* metacercariae isolated from *R. sinensis*, scale bar = 50 μm (F). *C. sinensis* egg in feces of canine, scale bar = 10 μm (G). *M. orientalis* egg in feces of duck, scale bar = 10 μm (H). *E. japonicus* eggs in feces of canine and duck, scale bar = 10 μm (I, J). EC, excretion cyst; ET, excretion tube; EO, egg operculum; IW, inner cyst wall; OW, outer cyst wall; ME, metacercariae; MI, miracidium; SP, shoulder peaks; SE, small spine at the rear end; OS, oral sucker; VS, ventral sucker.

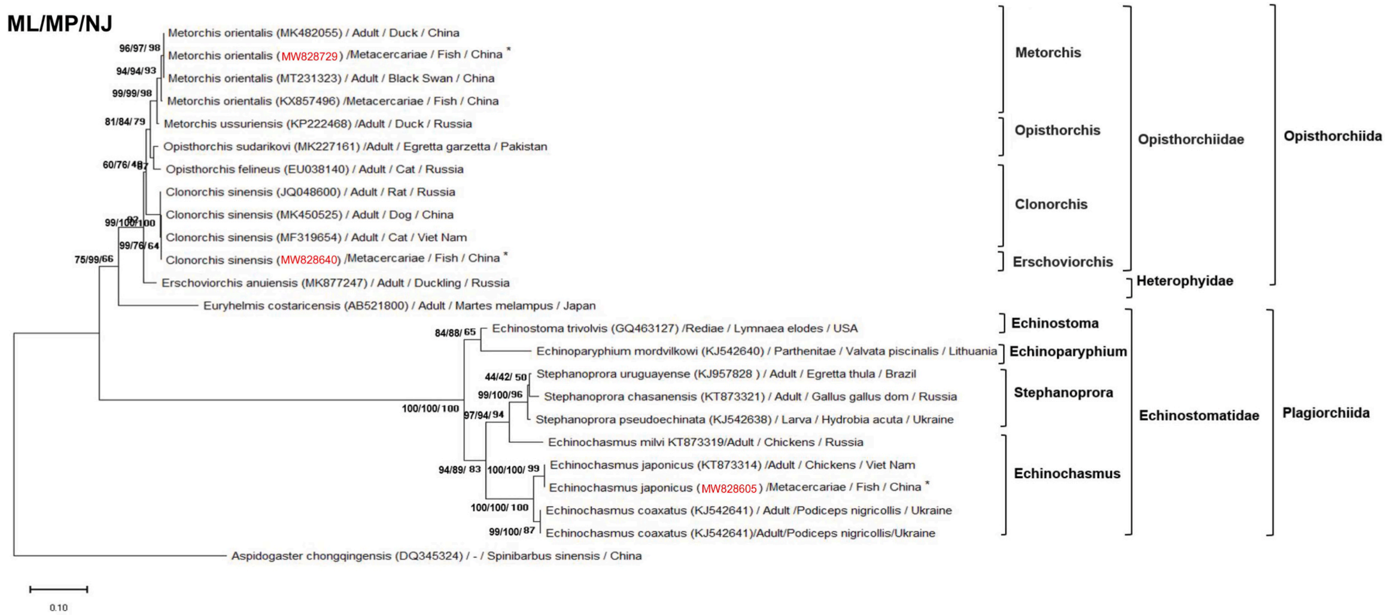

Fig. 3. The phylogenetic tree of FZTs obtained in this study with other trematodes based on ITS sequences.
ITS sequences of *C. sinensis, M. orientalis* and *E. japonicus* from fish were obtained and compared. They had the same similarity and were deposited into NCBI (No. **MW828640**, **MW828729** and **MW828605**). The phylogenetic relationship between the FZTs obtained in this study and other trematodes based on ITS sequences was analyzed via MP, NJ and ML using *A. chongqingens* as the outgroup. The scale bar indicates an evolutionary distance of 0.10 substitutions per site in the sequence. The ITS sequences of *C. sinensis*, *M. orientalis* and *E. japonicus* obtained in this study (marked with *) was 100% consistent with the sequences of *C. sinensis* (**MF319654**), *M. orientalis* (**MK482055**) and *E. japonicus* (**KT873314**) deposited in NCBI GenBank.

Table 1
The prevalence of FZTs in freshwater fish in Jilin Province of China.

FZT species	No. of fish examined	No. of fish infected	Prevalence (%)	95% CI
C. sinensis	4122	931	22.59	21.30–23.86
M. orientalis	4122	409	9.92	9.01–10.83
E. japonicus	4122	746	18.10	16.92–19.28
Total	4122	1226	29.74	28.34–31.14
C. sinensis* and *E. japonicus	4122	298	7.23	6.44–8.02
C. sinensis* and *M. orientalis	4122	204	4.95	4.29–5.61
M. orientalis* and *E. japonicus	4122	157	3.81	3.23–4.39
Total	4122	552	13.39	5.83–20.95
C. sinensis, M. orientalis* and *E. japonicus	4122	107	2.60	2.11–3.09

3.1.3. Association of the size of fish with FZT prevalence

To understand the relationship between fish size and FZT metacercariae infection, we counted FZT metacercariae in fish of different weights. The results showed that small-sized fish (0<fish≤5 g) were more vulnerable to three FZTs than middle-sized fish (5<fish≤50 g), and no FZT metacercariae infection was observed in large-sized fish (>50 g) (Fig. 4).

3.1.4. The FZT prevalence in different reaches and water sources

Wild freshwater fish from upstream, midstream and downstream were examined. The results showed that *C. sinensis, M. orientalis* and *E. japonicus* were present in all three reaches of the Yitong River basin. The prevalence rates of *C. sinensis*, *M. orientalis* and *E. japonicus* were 19.38% (304/1569), 17.46% (274/1569) and 15.55% (244/1569) upstream; 20.35% (292/1435), 7.11% (102/1435) and 17.70% (254/1435) midstream; and 29.96% (335/1118), 2.95% (33/1118) and 22.18% (248/1118) downstream. The highest prevalence of *M. orientalis* was upstream (17.46%, 274/1569). The highest prevalence of *C. sinensis* and *E. japonicus* was downstream, with prevalence rates of 29.96% (335/1118) and 22.18% (248/1118), respectively (Table 3).

The sampling sites were classified according to different water sources (reservoirs, ponds, river mainstems, river tributaries, streams and wetlands). The prevalence among different water sources ranged from 8.24% (22/267 in streams) to 34.44% (186/540 in ponds) for *C. sinensis*, from 4.12% (11/267 in streams) to 12.56% (57/454, in reservoirs) for *M. orientalis* and from 10.49% (28/267 in streams) to 25.19% (237/941 in wetlands) for *E. japonicus* (Table 4).

3.1.5 The effect of different months on FZT prevalence in wild freshwater fish.

We continuously examined FZT prevalence in freshwater fish from July to November. The prevalence of *C. sinensis*, *M. orientalis* and *E. japonicus* in wild freshwater fish exhibited similar trends, increasing from July to August or September. The highest prevalence of *C. sinensis* (31.96%) and *E. japonicus* (30.25%) was observed in August, while that of *M. orientalis* (19.03%) was observed in September (Fig. 5).

3.2. Infection rate of FZTs in wild freshwater snails

We determined FZT infection rates in freshwater snails using direct microscopic observation combined with ITS sequence analysis. No FZT larvae were detected by direct observation in snails. ITS sequence analysis showed that *C. sinensis* ITS sequences were amplified in two *P. striatulus* samples (4.35%, 2/46), the *M. orientalis* ITS sequence was amplified in one *P. striatulus* sample (2.17%, 1/46), and no *E. japonicus* ITS sequences were amplified in snail samples. The overall infection rate of FZTs in snails was 2.27% (3/132), and no coinfection was observed.

3.3. FZT infection in the definitive hosts

Morphological identification and ITS sequence analysis were combined to assess FZT infection in fecal samples from canines, swine and ducks. The infection rates of *C. sinensis* and *E. japonicus* in canines were 46.43% (13/28) and 64.29% (18/28), respectively, and the infection rates of *M. orientalis* and *E. japonicus* in ducks were 19.23% (15/78) and

Table 2
The prevalence of FZTs in different freshwater fishes in Jilin Province of China.

FZT species	Fish species	No. of fish examined	No. of fish infected	Prevalence (%)	95% CI	Metacercariae abundance	Calculate intensity/100 g fish
C. sinensis	*P. parva*	999	607	60.76	57.73–63.79	0–1044	6528.54
	H. leuciclus	365	103	28.22	23.60–32.84	0–65	119.96
	P. glenii	250	34	13.60	9.35–17.85	0–37	4.10
	R. sinensis	791	86	10.87	8.70–13.04	0–18	55.91
	C. giurinus	619	31	5.00	3.28–6.72	0–11	10.13
	C. auratus	773	24	3.10	1.88–4.32	0–9	0.31
	S. dabryi	153	44	28.76	21.59–35.93	0–68	317.57
	M. anguillicaudatus	60	0	0.00	0.00–0.00	0	0
	C. carpio	44	2	4.55	1.61–10.71	0–13	0.06
	S. asotus	18	0	0.00	0.00–0.00	0	0
	O. argus Cantor	2	0	0.00	0.00–0.00	0	0
	H. prognathus Regan	48	0	0.00	0.00–0.00	0	0
M. orientalis	*P. parva*	999	287	28.73	25.91–31.53	0–127	366.78
	H. leuciclus	365	44	12.05	8.71–15.39	0–36	6.58
	P. glenii	250	5	2.00	0.26–3.73	0–8	0.30
	R. sinensis	791	53	6.70	4.96–8.44	0–16	18.94
	C. giurinus	619	0	0.00	0.00–0.00	0	0
	C. auratus	773	0	0.00	0.00–0.00	0	0
	S. dabryi	153	20	13.07	7.73–18.41	0–54	144.72
	M. anguillicaudatus	60	0	0.00	0.00–0.00	0	0
	C. carpio	44	0	0.00	0.00–0.00	0	0
	S. asotus	18	0	0.00	0.00–0.00	0	0
	O. argus Cantor	2	0	0.00	0.00–0.00	0	0
	H. prognathus Regan	48	0	0.00	0.00–0.00	0	0
E. japonicus	*P. parva*	999	327	32.73	29.82–35.64	0–46	396.38
	H. leuciclus	365	75	20.55	16.40–26.70	0–25	30.68
	P. glenii	250	0	0.00	0.00–0.00	0	0
	R. sinensis	791	318	40.20	36.78–43.62	0–76	700.89
	C. giurinus	619	5	0.81	0.10–1.52	0–8	6.76
	C. auratus	773	0	0.00	0.00–0.00	0	0
	S. dabryi	153	15	9.80	5.09–14.51	0–14	60.22
	M. anguillicaudatus	60	6	10.00	2.40–17.59	0–28	11.63
	C. carpio	44	0	0.00	0.00–0.00	0	0
	S. asotus	18	0	0.00	0.00–0.00	0	0
	O. argus Cantor	2	0	0.00	0.00–0.00	0	0
	H. prognathus Regan	48	0	0.00	0.00–0.00	0	0

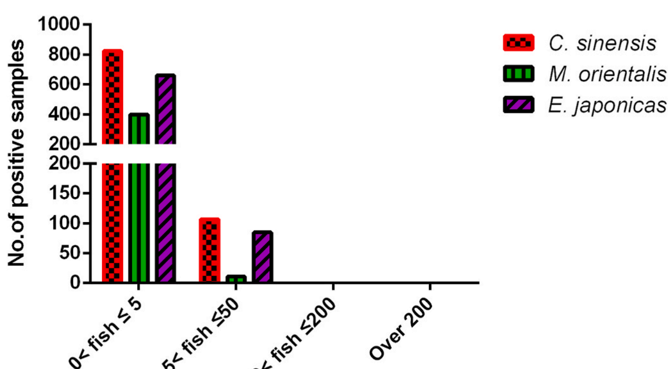

Fig. 4. The number of FZTs infecting wild freshwater fish of different sizes.

25.64% (20/78), respectively. The coinfection rate of *C. sinensis* and *E. japonicus* in canines was 35.71% (10/28), and the coinfection rate of *M. orientalis* and *E. japonicus* in ducks was 7.69% (6/78). No FZT infection was detected in swine.

The eggs of *C. sinensis* were bulb-shaped and yellow colored, approximately 24 ± 2.58 μm × 15.6 ± 2.33 μm in size, with egg operculum and shoulder peaks, containing a well-developed miracidium and a small spine at the bottom (Fig. 2 G). *M. orientalis* eggs were oval and yellow–brown colored, 25.87 ± 3.21 μm × 16.32 ± 1.65 μm in size, with egg operculum and without shoulder peaks, containing a well-developed miracidium and a small spine at the bottom (Fig. 2 H). The eggs of *E. japonicus* were oval and large, golden yellow colored, 84.25 ± 3.17 μm × 62.5 ± 2.55 μm in size, with a small egg operculum and containing yellow follicles (Fig. 2 I and J).

Table 3
The prevalence of FZTs in wild freshwater fish from different reaches of Yitong River basin.

FZT species	Drainage reaches	No. of fish examined	No. of fish infected	Prevalence (%)	95% CI
C. sinensis	Upstream	1569	304	19.38	11.64–20.48
	Midstream	1435	292	20.35	18.37–27.77
	Downstream	1118	335	29.96	24.61–34.46
M. orientalis	Upstream	1569	274	17.46	12.41–20.41
	Midstream	1435	102	7.11	4.47–10.07
	Downstream	1118	31	2.95	0.94–4.43
E. japonicus	Upstream	1569	244	15.55	9.98–18.22
	Midstream	1435	254	17.70	14.79–23.27
	Downstream	1118	248	22.18	17.72–26.68

Table 4
The prevalence of FZTs in wild freshwater fish from different water sources in Jilin Province of China.

FZT species	Water sources	No. of fish examined	No. of fish infected	Prevalence (%)	95% CI
C. sinensis	Reservoirs	454	63	13.88	10.70–17.06
	Ponds	540	186	34.44	30.43–38.45
	River mainstems	600	202	33.67	29.89–37.45
	River tributaries	1320	199	15.08	13.15–17.01
	Streams	267	22	8.24	4.94–11.54
	Wetlands	941	259	27.52	24.67–30.37
M. orientalis	Reservoirs	454	57	12.56	9.51–15.61
	Ponds	540	26	4.81	3.01–6.61
	River mainstems	600	62	10.33	7.89–12.77
	River tributaries	1320	157	11.89	10.14–13.66
	Streams	267	11	4.12	1.74–6.50
	Wetlands	941	96	10.20	8.27–12.13
E. japonicus	Reservoirs	454	54	11.89	8.91–14.86
	Ponds	540	135	25.00	21.53–28.85
	River mainstems	600	133	22.17	18.85–25.49
	River tributaries	1320	159	12.05	10.29–13.81
	Streams	267	28	10.49	6.81–14.17
	Wetlands	941	237	25.19	22.31–27.85

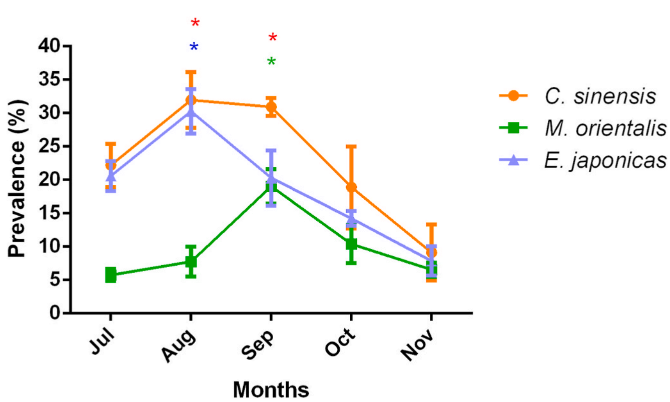

Fig. 5. The prevalence of FZTs in different months in wild freshwater fish in Jilin Province, China.
The prevalence of FZTs in freshwater fish gradually increased and then decreased, with the highest prevalence of *C. sinensis* and *E. japonicus* in August and the highest prevalence of *M. orientalis* in September. *$p < 0.05$ was considered to be a significant difference, and the prevalence in November was used as a control. The significances of *C. sinensis, M. orientalis* and *E. japonicus* in different months are marked in red *, green * and blue *, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

In the present study, infection by FZTs in the first intermediate hosts (snails), the second intermediate hosts (freshwater fish) and the definitive hosts (canines, swine and ducks) in Jilin Province of China was investigated. Three trematodes (*C. sinensis*, *M. orientalis* and *E. japonicus*) were detected to be prevalent in this region. Nine species of fish could serve as the second intermediate hosts of FZTs, with *P. striatulus* serving as the first intermediate host and canines and ducks serving as the definitive hosts.

The different prevalences in different regions might be attributed to geographical conditions and local ecological status (climate, temperature, water quality, environment, etc.). The prevalence of *C. sinensis* was 37.09% (2160/5824) in Guangdong (Chen et al., 2010), 16.97% (52/307) in Guangxi (Sohn et al., 2009,b) and 19.96% (643/3221) in Heilongjiang (Zhang et al., 2014). The prevalence of *M. orientalis* was 10.54% (718/6815) in Heilongjiang (Qiu et al., 2017) and 6.33% (19/300) in Anhui (Li et al., 2017). Although *E. japonicus* metacercariae was found in freshwater fish (Sohn, 2009), the prevalence data for this trematode in freshwater fish are still lacking. In this study, the prevalence rates of *C. sinensis*, *M. orientalis*, and *E. japonicus* in freshwater fish in Jilin Province were 22.59%, 9.92% and 18.10%, respectively. Among 4122 samples, 13.39% of fish were infected with two species of trematodes, and 2.60% of fish were coinfected with three species of trematodes. This is the first report of the coinfection rate of FZTs in freshwater fish in Jilin Province. The suggests that ingestion of small amounts of raw fish might also cause FZT coinfection in definitive hosts.

A total of nine species of fish were found to be infected with FZTs in our survey, of which eight species were infected with *C. sinensis*, five species with *M. orientalis* and six species with *E. japonicus*. Unlike the survey in Heilongjiang Province (Qiu et al., 2017; Zhang et al., 2014), we did not find *C. sinensis* and *M. orientalis* in *M. anguillicaudatu* and *C. auratus.* In Guangdong Province, *C. sinensis* was prevalent in *C. idellus, H. nobilis, C. molitorella, T. mossambica, C. auratus, H. molitrix, P. pekinensis* and *P. parva* (Chen et al., 2010). The prevalence of FZTs in different fishes in Jilin Province was not consistent with that in other regions, which might be influenced by factors such as sampling time, sampling location, sampling water sources and sample size. Of note, *P. parva* was the most vulnerable fish to *C. sinensis* and *M. orientalis* in Jilin Province, which is consistent with observations in other provinces (Chen et al., 2010; Qiu et al., 2017; Zhang et al., 2014). Locally, the most commonly eaten fish are *P. parva*, *H. leuciclus*, *R. sinensis*, *C. auratus*, *S. dabryi*, and *C. carpio*. *P. parva*, *H. leuciclus*, *R. sinensis* and *S. dabryi,* which are susceptible to FZTs, are often eaten by deep-frying or stewing. However, insufficient cooking can result in incomplete extinction of the metacercariae in the muscle of fish and cause infection. Additional advice should be given to strengthen local disease publicity to avoid raw and accidental ingestion of susceptible fish. This is the first study to report the prevalence of *E. japonicus* in *R. sinensis, H. leuciclus, C. giurinus,* and *S. dabryi*, which enriches data on the second intermediate host of *E. japonicus*.

Interestingly, our study found that the sampling water sources acted as constraints on the prevalence of FZTs, demonstrating that *C. sinensis*, *E. japonicus* and *M. orientalis* have selectivity for the environment, which has not been reported in previous investigations. A previous report demonstrated that the prevalence of *C. sinensis* varied in different water sources, with the prevalence of *C. sinensis* in rivers being much higher than that in ponds, reservoirs and streams (Zhang et al., 2020). In Heilongjiang Province, the prevalence of *C. sinensis* and *M. orientalis* was higher in the Songhua and Nenjiang Rivers than in lakes and ponds (Qiu et al., 2017; Zhang et al., 2014). In this study, the prevalence of *C. sinensis* was higher in ponds than in other water sources in Jilin Province. We found that the ponds had freshwater snails living in them with the necessary conditions for the transmission of *C. sinensis*. Additionally, the definitive hosts around the ponds may be responsible for the high prevalence of *C. sinensis* in the ponds. The prevalence of *M. orientalis* was higher in the reservoirs than in other water sources in our study due to the abundance of water, fish resources, and a large number of wild birds. *M. orientalis* is transmitted in a variety of wild birds (Wang et al., 2020), which may be responsible for the high prevalence of *M. orientalis* in these reservoirs in our study. The prevalence of *E. japonicus* was higher in the wetlands than in other water sources due to the rich biodiversity of the wetland park, which had all the hosts necessary for the prevalence of *E. japonicus*. Wetland waters are virtually immobile, making them more conducive to *E. japonicus* infection. We also found that *M. orientalis* was primarily prevalent upstream, while the prevalence of *C. sinensis* and *E. japonicus* was similar among the three reaches. It is recommended that surveys of FZTs should cover various water sources and basins as much as possible to avoid data bias or errors.

The differences in FZT prevalence in wild fish in Jilin Province among different months reflect the important influence of temperature and precipitation on FZT prevalence. Analyzing the prevalence of *C. sinensis* in freshwater fish in different seasons revealed that the prevalence of *C. sinensis* was higher in summer, but there were no significant differences among the four seasons (Zhang et al., 2020). However, that study did not mention sampling locations, and the influence of sampling time on the prevalence of FZTs may not be suitable for Jilin Province, China. It is known that precipitation and temperature variation affect the prevalence of *C. sinensis* metacercariae in fish (Zhang et al., 2020). The prevalence of FZTs in cultured freshwater fish in Jilin Province was highest in July (Yang et al., 2019), which was earlier than our finding (the prevalence of FZTs was highest in August and September), which may be caused by the lower temperature in 2020. The earlier increases in temperature and precipitation are more favorable for the development of FZTs in freshwater snails.

Transmission of FZTs is strictly restricted by the first and second intermediate hosts and the definitive hosts (Zhang et al., 2020). Our study identified FZT infection in the first intermediate hosts and in the definitive hosts in Jilin Province. These findings suggested that a complete life cycle of FZTs has formed in Jilin Province. The activities of definitive hosts may pose new challenges to local public safety to enact measures against FZTs, and the surveillance and treatment of FZTs in local livestock and poultry may be needed. The results of two FZT coinfections in canines and ducks further supported the inference that FZT coinfection in freshwater fish could ultimately result in FZT coinfection in definitive hosts.

This study identified three FZTs in intermediate and definitive hosts in Jilin Province. However, the low number of samples of the definitive hosts and the first intermediate hosts may affect the assessment of the prevalence of FZTs among the first intermediate hosts and definitive hosts in Jilin Province, and more work is required in future studies to confirm these findings.

5. Conclusion

This study is a detailed epidemiological investigation of FZTs in Jilin Province, including the first and second intermediate hosts, definitive hosts, species and prevalence of FZTs and coinfection rates.

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Data availability statement

The newly generated sequences were submitted to the NCBI GenBank database under the accession No. **MW828640**, **MW828729** and **MW828605**.

Authors' contributions

Jianhua Li, Yuru Wang and Xin Li supervised the study and designed the experiments. Yuru Wang, Yanhui Yu and Yanyan Ren conducted these experiments and wrote the manuscript. Xiaocen Wang, Xichen Zhang, Jianhua Li and Zhiteng Zhao revised the manuscript. Nan Zhang, Pengtao Gong and Yeting Ma provided technical assistance.

Ethics statement

The project was approved by the animal welfare and research ethics committee of Jilin University. Prior to the start of the study, written informed consent was obtained from the owners of dogs, ducks and swine.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2022.04.004.

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