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First case report of *Metorchis orientalis* from Black Swan

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ABSTRACT

Metorchis orientalis belongs to the genus *Metorchis* of Opisthorchiidae, which mainly parasitizes in liver and bile ducts of waterfowl, causing liver dysfunction of the host. It has been reported that *M. orientalis* also infects humans. As a natural species in Australia and a popular ornamental animal, Black Swan (*Cygnus atratus*) has been imported into many countries. At present there has been no report of *M. orientalis* infection in Black Swan. In the present study *M. orientalis* infection in Black Swan was identified by a combination of different techniques, including morphological observation and molecular analysis. *M. orientalis* adults were found in the gallbladder and bile duct of a three-year-old female Black Swan, which was further confirmed by internal transcribed spacer (ITS) sequence analysis. In addition, the intermediate and definitive hosts of *M. orientalis* from the 'Qing' lake (a man-made lake in Changchun, China) that Black Swan lived were investigated and the infection route was preliminarily determined. *Parafossarulus striatulus* functioned as the first intermediate host which contained *M. orientalis* DNA, and fishes such as *Pseudorasbora parva* and *Rhodeinae* served as the second intermediate hosts with *M. orientalis* metacercariae in the fish flesh. *M. orientalis* eggs were found in the feces of three other Swans and six ducks that lived in the 'Qing' lake. This was the first reported case about *M. orientalis* infection of Black Swan. Our study described the course of the infection and provided new information about potential carriers and disseminators of *M. orientalis*.

1. Introduction

Metorchis orientalis belongs to *Metorchis* of Opisthorchiidae, Digenea, Trematoda, Platyhelminthes (Chen et al., 2013, 2017). This species infects a wide variety of vertebrate definitive hosts. It was reported that poultry (ducks, chicken, geese), wild birds (*Pavonini*, *Grus japonensis*, *Alcedo*), and mammals (*Felinae*, *Canis lupus familiaris, Mus musculus, Caviaporcellus*) including human could serve as the definitive hosts of *M. orientalis* (Gao et al., 2017; Cheng et al., 2005; Lin et al., 2001a, b; Chen et al., 2018). Adults of *M. orientalis* live in bile ducts and gallbladder of the definitive hosts, consequently cause thickened cystic wall, enlarged gallbladder, biliary obstruction, bile cholestasis and even other serious illnesses (Zhan et al., 2017; Na et al., 2016; Qiu et al., 2017). *Parafossarulus striatulus* is the first intermediate host (Chen et al., 2013) and freshwater fishes such as *Pseudorasbora parva, Misgurnus anguillicaudatus, Abbottina rivularis,* etc, are the second intermediate hosts of *M. orientalis* (Lin et al., 2001b; Yang et al., 2019; Chen et al., 2013; Sohn, 2009; Sohn et al., 2019). Black Swan is a natural species in Australia. As a popular ornamental animal, Black Swan is imported into many countries. So far, there has been no report of *M. orientalis* infection in Black Swan. In previous studies, infection of *M. orientalis* was identified by the examination of external morphology and internal anatomic features (Lin et al., 2001a, b). To investigate the transmission route, the larvae in its intermediate hosts could be examined (Qiu et al., 2017). PCR analysis for *M. orientalis* has been established for the detection of metacercariae and adult worm DNA (Ai et al., 2010).

Here, a dead Black Swan is dissected and analyzed. External morphology, internal anatomic features and PCR sequence analysis are carried out to determine the parasite in bile duct of the Black Swan. This study will provide valuable information on the infection and

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transmission of *M. orientalis*.

2. Materials and methods

2.1. Morphological observation of adults and eggs isolated from a Black Swan

A three-year-old female Black Swan died in August 2018 in Changchun, China (43°82′N, 125°27′E), and an autopsy was performed on the dead Black Swan to determine the causes of death. Approximately 2.00 g (g) of liver tissue was removed under sterile condition and fixed at 10% formalin. In order to observe the pathological change of liver, tissue sections were prepared and stained by hematoxylin-eosin (HE) staining. The remaining liver tissue and gallbladder were soaked in sterile phosphate buffer solution (PBS), then the adult worms in the tissues were removed with ophthalmic forceps. The contents of the bile duct and gallbladder were repeatedly washed by centrifugation with sterile PBS to collect the eggs. The collected adult worms and eggs were immediately observed and measured under a microscope (OLYMPUS CX43, Japan). The remaining samples were stored in the refrigerator at −80 °C.

2.2. Investigation of the intermediate hosts and definitive hosts of *M. orientalis* in the living environment of the Black Swan

The freshwater snails: *P. striatulus*is (35 samples), *Radix auricularia* (12 samples), *Cipangopaludina chinensis* (44 samples), and fishes: *M. anguillicaudatus* (28.56 g fish flesh obtained from 4 fishes), *Carassius auratus* (81.00 g fish flesh obtained from 18 fishes), *P. parva* (23.32 g fish flesh obtained from 22 fishes), *Rhodeinae* (58.00 g fish flesh obtained from 51 fishes) and *A. rivularis* (17.00 g fish flesh obtained from 20 fishes), were collected from the 'Qing' lake where the Black Swan lived. The collected snails were shucked and their axe-feet were removed, and the lung, liver and digestive system were separated. About 0.10 g of the fish flesh in the back and tail was compressed into thin slices and the larvae of *M. orientalis* were observed directly under a microscope (OLYMPUS CX43, Japan). Metacercariae was purified by digestion method as described previously (Nguyen et al., 2015; Sohn, 2009; Sohn et al., 2019). The prevalence and the mean intensity was calculated using a previously published method (Bush et al., 1997). The number of metacercariae per gram fish flesh was calculated.

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

$$\text{Mean intensity} = \frac{\textit{The total number of metacercariae}}{\textit{The number of metacercariae} - \textit{infected fish}}$$

$$\text{The average number of metacercariae (fish flesh /g)} = \frac{\textit{The total number of metacercariae}}{\textit{The weight of fish flesh}}$$

The fecal samples from other Swans and ducks (including one Black Swan, two White Swans and six ducks) were collected immediately after defecation (Phan et al., 2010). Stool samples were recorded in detail and stored in sample collection bags respectively. All samples were put into the sample collection boxes and sent to the laboratory immediately. *M. orientalis* eggs were observed immediately and measured under a microscope. The remaining samples were stored in the refrigerator at −80 °C.

2.3. ITS sequences amplification of *M. orientalis* and analysis

DNA was isolated from the samples (adults, egg, metacercariae and snail tissues) using the TIANGEN Genomic DNA Kit (TIANGEN BIOTECH (BEIJING) CO., LTD, Beijing, China) following the instructions of the manufacturer. All DNA precipitates were dissolved in 50 μl of nuclease-free water and the DNA concentration was determined using the NanoDrop 2000 instrument (Thermo Scientific, Waltham, MA, USA). The primers F (5-ACAATGACGGTTTCAGCGAGTTT-3) and R (5-CACAAACAACCCGACTCCAAGG-3) (Su et al., 2018) were used to amplify the sequences of *M. orientalis* ITS. The PCR products were run on a 2% agarose gel and purified using a PCR purification kit (Sangon Biotech (Shanghai) CO., LTD, Shanghai, China) which were then cloned into the pMD®18-T vector (Takara Bio, Dalian, China). The constructed plasmid was sequenced using the M13 primer.

The 12 ITS sequences of *M. orientalis* from Black Swan and other hosts were obtained and compared. They had the same similarity and were deposited to National Center for Biotechnology Information (NCBI, GenBank accession no. **MT231323**). An ITS gene phylogenetic tree was drawn using the MEGA X software (Kumar et al., 2018; Qiu et al., 2020). For multiple repeats, ITS sequences were automatically aligned using Muscle and unaligned flanking sequences were deleted. Gblocks was used to extract conservative sites from multiple sequence alignments to facilitate further evolutionary analysis. The MT231323 sequence was analyzed via Maximum parsimony (MP), Neighbor-Joining (NJ) and Maximum likelihood (ML) with other Opisthorchiidae trematodes including *Metorchis, Clonorchis*, *Opisthorchis*, and *Erschoviorchis*. *Metagonimus pusillus* belonging to Heterophyidae, Digenea was used as the outgroup. Initial trees were obtained automatically by selecting the topology with superior log likelihood value, and the phylogenetic analyses were conducted in MEGA X.

3. Results and discussion

3.1. *M. orientalis* infection in Black Swan was identified by pathohistological analysis and morphological observation of adults and eggs

An autopsy on a three-year-old female Black Swan was performed to determine the death causes. The results showed that the liver and gallbladder of the Black Swan were highly swollen, with cholestasis in the gallbladder, and a large number of trematodes in the gallbladder and bile duct (Fig. 1 A and B). In order to observe the pathological changes, we performed pathohistological analysis on the Black Swan liver and found hyperplasia of the bile ducts, obstruction of the biliary tract by parasites, and focal necrosis of liver cells (Fig. 1 C). A total of 167 parasites were collected from the bile duct and gallbladder of the Black Swan. The parasites were yellow-brown colored and the tegument was covered with spines. The oral and ventral suckers were about equal in size. The pharynx was spherical and adjacent to the oral suckers. The vitellaria was granular and bunchy, lying on both sides of the body. The tubular uterus containing eggs twisted through the ovary towards the gonopore, which was located at the anterior ventral sucker. The ovary was oval shaped and located in front of the testis and the seminal receptacle arose at the back of the ovary and was slightly curved. Two testes presented in tandem in petaloid fashion at the posterior 1/4 of the body (Fig. 2 A, G). The eggs were yellow-brown colored, sized at 26.53 ± 1.55μm long and 13.94 ± 0.81μm wide containing a well-developed

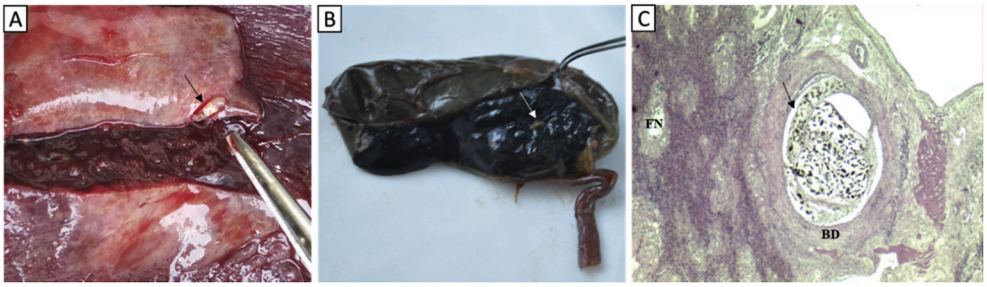
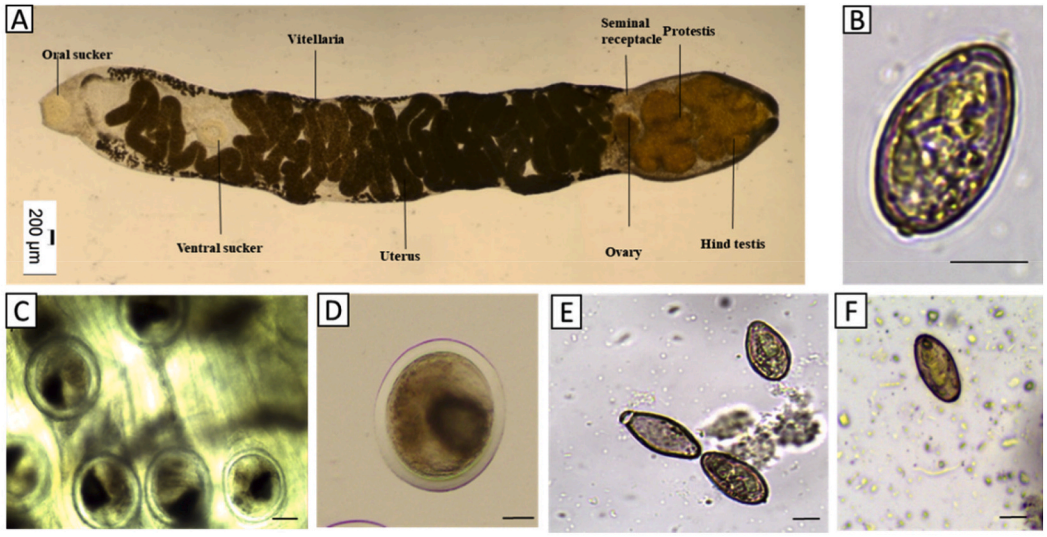


Fig. 1. Examination of the flukes in the Black Swan's liver and gallbladder.
A: The fluke in the Black Swan's bile duct. B: The fluke in the Black Swan's swollen gallbladder. C: Tissue sections showed that the Black Swan's bile duct was blocked by a trematode. The bile duct (BD) was thickened and the liver tissue had focal necrosis (FN). The magnification was 100×. The flukes were pointed by the arrow.



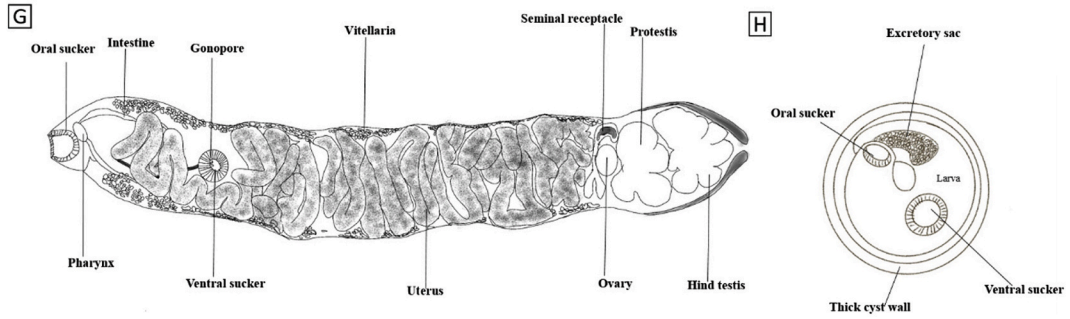


Fig. 2. The morphology and internal structure observation of *M. orientalis* in the present study.
A: The morphology and internal structure of *M. orientalis* adults isolated from the Black Swan. The worms were brown colored, measuring 7040.00 ± 2060.30 μm in length and 975.12 ± 365.00 μm in width. Oral sucker, ventral sucker, vitellaria, uterus, ovary, seminal receptacle, protestis and hind testis of this adult were marked respectively. Scale bar = 200 μ m. B: The morphology and internal structure of *M. oriental* eggs from the Black Swan. The egg was yellow-brown colored, containing a well-developed miracidium, the egg operculum was large and a small spine was at the rear end. Scale bar = 10 μ m. C: The metacercariae in fish flesh of *P. parva*. They were oval shaped with thick cyst wall containing a movable larva with a brownish yellow excretory sac. Scale bar = 50 μ m. D: An isolated metacercariae. Scale bar = 20 μ m. E: Eggs of *M. orientalis* in feces of other Swans where the Black Swan lived. Scale bar = 10 μ m. F: Egg of *M. orientalis* in feces of ducks where the Black Swan lived. Scale bar = 10 μ m. G: Drawings of specimens of *M. orientalis* adult. Oral sucker, pharynx, intestine, ventral sucker, gonopore, vitellaria, uterus, ovary, seminal receptacle, protestis and hind testis were marked respectively. H: Drawings of specimens of *M. orientalis* metacercariae. The thick cyst wall, larva, oral sucker, ventral sucker and excretory sac were marked respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
The sizes of adult, adult's internal structures and egg of *M. orientalis* isolated from the Black Swan.

Items	Sizes (μm)
Adults	7040.00 ± 2060.30 × 975.12 ± 365.00
Oral sucker	377.33 ± 54.08 × 336.04 ± 27.18
Ventral sucker	348.35 ± 51.65 × 328.66 ± 58.06
Protestis	999.65 ± 166.02 × 700.24 ± 186.76
Hind testis	1048.02 ± 171.02 × 841.65 ± 235.87
Ovary	441.49 ± 90.59 × 298.26 ± 54.74
Seminal receptacle	513.02 ± 184.02 × 229.2 ± 53.75
Egg	26.53 ± 1.55 ×13.94 ± 0.81

miracidium. The operculum was large with a small spine at the rear end (Fig. 2 B). Data for the trematode and egg isolated from Black Swan were described in detail in Table 1.

Since the morphology of adults and eggs was consistent with that of *M. orientalis* (Zhan et al., 2017; Lin et al., 2001b; Chen et al., 2013, 2017; Zhang et al., 2013), we identified the trematode as *M. orientalis*. Previous studies have shown that the sizes of the adults isolated from different hosts varied (Lin et al., 2001b; Chen et al., 2013). It was reported that adult *M. orientalis* obtained from artificially infected ducks was measured at 2.83–6.81 mm in length and 0.61–1.73mm in width (Chen et al., 2017; Zhan et al., 2017). We found that the adults were leaf-shaped and brown colored, measuring at 7.04 ± 2.06 × 0.98 ± 0.37 mm in size. The adult size we observed from Black Swan was longer than that of *M. orientalis* in mammals (human 2.21–2.87 mm and cat 1.96–4.00 mm) (Chen et al., 2017; Lin et al., 2001a, b) and poultry (chicken 3.20–5.20 mm and duck 2.83–6.81 mm) (Zhan et al., 2017; Chen et al., 2013, 2017; Zhang et al., 2013).

3.2. *M. orientalis infection in Black Swan was further confirmed by ITS sequence analysis*

In order to determine the species of the trematode, we performed PCR amplification and sequence analysis for ITS sequence of *M. orientalis*. A DNA fragment of 1725 base pairs was amplified from adults, eggs, metacercariae and snail tissues. The 12 nucleotide sequences obtained had the same similarity and which were deposited to Genbank with the accession no. **MT231323**. Phylogenetic tree showed that ITS sequence of *M. orientalis* from Black Swan was classified to the group of *Metorchis*. And it belonged to a branch (99% similarity) with reference sequences of *M. orientalis* (GenBank accession no. **KX832894** and **MK482055**) (Fig. 3). ITS sequence analysis confirmed that the trematode parasitized in the liver and gallbladder of Black Swan was *M. orientalis*.

3.3. *The intermediate hosts and definitive hosts of M. orientalis were found in the lake where Black Swan lived*

To understand the infection route of the Black Swan, we investigated the intermediate hosts and other definitive hosts of *M. orientalis* in the 'Qing' lake where the Black Swan lived (Fig. 4 A–J). Metacercariae were found in fish flesh of *P. parva* and *Rhodeinae* by microscopic observation. The metacercariae in fish flesh were oval or rounded, 156.77 ± 4.01 μm × 133.89 ± 12.47 μm in size with thick cyst wall (15.42 ± 0.25 μm) and contained a movable larva with a brownish yellow excretory sac (Fig. 2 C, D, H). It was consistent with the morphology of metacercariae as previously described (Sohn, 2009). We found that *P. parva* had a higher mean intensity of 740.90, whereas *Rhodeinae* was 0.20 (Table 2). The metacercariae per gram fish flesh in *P. parva* was 698.97, and 0.17 in *Rhodeinae* (Table 2). *P. parva* has been reported as the most susceptible fish with a maximum prevalence at 97% (Zhan et al., 2017; Sohn, 2009; Sohn et al., 2019; Yang et al., 2019; Chen et al., 2018), whereas the prevalence detected in our study was 100% which was substantially higher (Table 2). No suspected larvae of *M. orientalis* was observed in the snail by microscope. PCR analysis found that three *P. striatulusis* samples contained *M. orientalis* DNA among the thirty-five snail tissue samples. The sequence of *M. orientalis* from snail and fish was consistent with that of Black Swan. We examined the feces of three other Swans and six ducks that lived in the same lake (Fig. 4 I, J). The eggs of *M. orientalis* were found in the feces of all Swans and ducks (Fig. 2 E, F), which were further confirmed by ITS sequence analysis.

In summary, the present results suggested that a complete life cycle of *M. orientalis* could be present in the 'Qing' lake in Changchun of China, with *P. striatulusis* as the first intermediate host, *P. parva* and *Rhodeinae* as the second intermediate hosts, and Swans and ducks as the definitive hosts.

4. Conclusion

The present case was the first report of *M. orientalis* isolated and identified from Black Swan in Changchun of China. It described the course of the infection and brought new information about potential carriers and disseminators of *M. orientalis*.

Ethics statement

No specific permits were required for the described field research in this study. Before the study started, owner of Swans and ducks had been informed about the study and oral consent had been obtained.

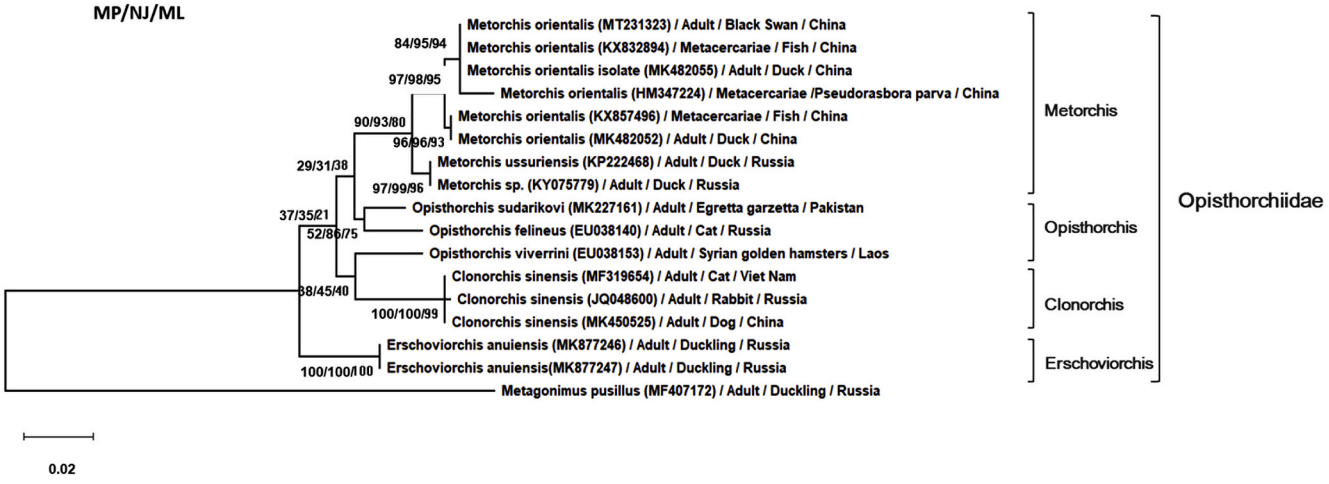


Fig. 3. Phylogenetic relationships of *M. orientalis* from Black Swan with other Opisthorchiidae trematodes based on ITS sequences.

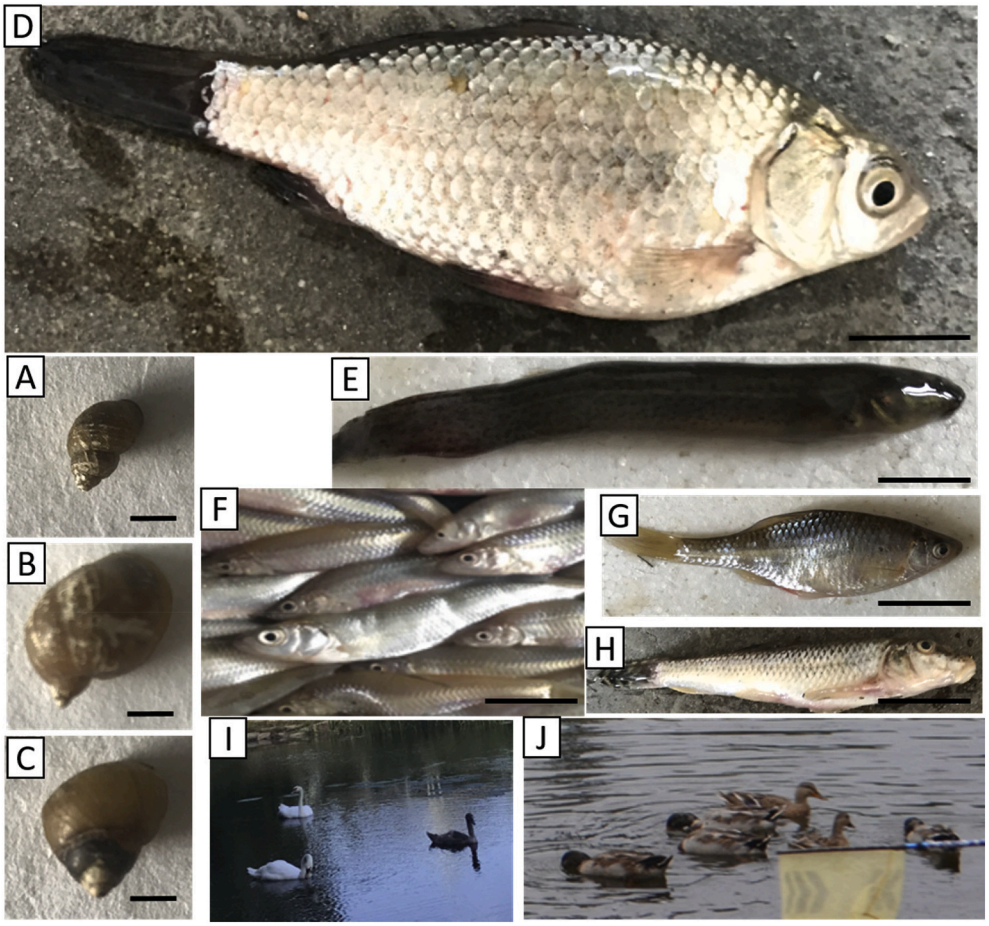


Fig. 4. The investigation of the intermediate and definitive hosts of *M. orientalis* in the lake where the Black Swan lived.
A: *P. striatulusis.* Scale bar = 0.5 cm. B: *R. auricularia.* Scale bar = 0.5 cm. C: *C. chinensis.* Scale bar = 0.5 cm. D: *C. auratus.* Scale bar = 1 cm. E: *M. anguillicaudatus.* Scale bar = 1 cm. F: *P. parva.* Scale bar = 1 cm. G: *Rhodeinae.* Scale bar = 1 cm. H: *A. rivularis.* Scale bar = 1 cm. I and J: Waterfowl.

Table 2
The infection of *M. orientalis* metacercariae in different freshwater fishes in the lake where the Black Swan lived.

Fish species	The number of fish	The weight of fish flesh (g)	The total number of metacercariae	The average number of metacercariae (fish flesh/g)	Mean intensity	Prevalence (%)
P.parva	22	23.32	16300	698.97	740.90	100.00
A. rivularis	20	17.00	0	0.00	0.00	0.00
C. auratus	18	81.00	0	0.00	0.00	0.00
Rhodeinae	51	58.00	10	0.17	0.19	5.80
M. anguillicaudatus	4	28.56	0	0.00	0.00	0.00

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the conclusions of this article are provided within the article. The newly generated sequences were submitted to the GenBank database under the accession numbers **MT231323**.The adults and eggs specimens are deposited in the Key Laboratory of Zoonosis Research, Ministry of Education; College of Veterinary Medicine, Jilin University.

Authors' contributions

Yuru Wang and Xin Li performed all the laboratory work and manuscript writing; Pengtao Gong and Qingsong Sun carried out the data analysis. Nan Zhang collected freshwater snailes, fishes and feces samples; Xichen Zhang and Xiaocen Wang revised the manuscript, and Guojiang Li and Jianhua Li supervised the study, intellectual interpretation and critical. All authors read and approved the final version of the manuscript.

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Phylogenetic relationship of *M. orientalis* with other Opisthorchiidae trematodes inferred were analyzed via Maximum parsimony (MP), Neighbor-Joining (NJ) and Maximum likelihood (ML) using *M. pusillus* as the outgroup. Scale bar indicates an evolutionary distance of 0.02

substitutions per site in the sequence. ITS sequence of *M. orientalis* (GenBank accession no. MT231323) in the present report shared 99% homology with reference sequences of *M. orientalis* (GenBank accession no. KX832894 and MK482055).

Declaration of competing interest

The authors declare that they have no competing interests.

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