Zoonotic Trematode Prevalence In *Galba Pervia* (Lymnaeidae) And Experimental Infection Of Three Isolated *Trematodes* In The Intestine Of Duck

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Research Article

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Abstract

Background: Food-borne diseases cause serious harm to public health and food safety. The snail species *Galba pervia* is an intermediate host for an array of parasitic trematodes. In this study, we performed a prevalence investigation on zoonotic trematode in *G. pervia* in Guangxi, China, and assessed the zoonotic potential of Trematode in the region.

Methods: *G. pervia* was collected from 61 sites in 9 cities throughout Guangxi Provinces between 2012 to 2014. The larvae species were determined by combing morphological and molecular characteristics. Phylogenetic trees were constructed using neighbor-joining method with ITS2 sequences. The developmental cycles of the isolated trematodes were examined by experimental infection in ducks. The developmental characteristics of *Echinostoma revolutum was* recorded by dissecting infected ducklings from 1 dpi to 10 dpi.

Results: Species identification showed *E. revolutum*, *Australapatemon* sp., *Hypoderaeum conoideum*, *Pharyngostomum cordatum* and *Echinostoma* sp. parasitized in *G. pervia. H*owever, no *Fasciola* larvae had been detected. A Neighbor-Joining tree analysis of ITS2 sequences resulted in monophyletic clades comprised of all sequences from isolated larvae with high bootstrap support. The overall prevalence of Trematode larvae in *G. pervia* was 22.0% (1818/8258), while *E. revolutum* presented with the highest infection rate of 12.9% from 11 sampling sites. Ducklings exposed to *Echinostoma* sp., *E. revolutum*, and *H. conoideum* larvae were successfully infected. *E. revolutum* larvae matured at 10 dpi in the intestine of the duck, and the developmental characteristics of *E. revolutum* were characterized by the maturation of the reproductive and digestive organs around 6~8 dpi.

Conclusions: The present investigation revealed the high prevalence of trematodes in the *G. pervia* in Guangxi, China. With existing trematode infection human cases together with wide geographical distribution of *G. pervia*, more insight into the risks of human health and its link to human infections are needed.

Background

Food-borne trematode is a parasitic disease that seriously harms humans and animals [1, 2, 3]. Common food-borne fluke disease are *fasciolosis*, *paragonimosis*, *schistosomatosis*, g*astrodiscosis*, etc. Corresponding pathogens include *fasciolidae*, *echinostomatidae*, *opisthorchiidae*, *heterophyidae,* etc[3, 4, 5]. Food-borne trematodes can infect a wide range of mammals, including livestock and humans, causing severe veterinary and public health problems worldwide [6, 7]. Although the infection rates are low, several outbreaks have been reported recently [8, 9].

Galba pervia belongs to Mollusca, Gastropoda, Pulmonata, Basommatophora, Lymnaeidae, *Galba* [10]. It is an intermediate host for a variety of trematodes, some of which are zoonotic, such as *F. gigantica*, *F. hepatica*, *Echinostoma* revolutum, *Echinochasmus perfoliatus* and plays a vital role in the transmission and prevalence of these diseases [11]. The shell of *G. pervia* is thin and translucent with an ear-shaped

aperture; the ratio apex/body is 10/8mm. Its natural habitats ranged from lakes, canals, ponds, and rice fields. Oviparous hermaphroditic *G. pervia* lives in large aggregation in suitable environments such as sewage sludge bottom or broken bricks and feeds on algae, hummus, and aquatic plants [12]. *G. pervia* is widely distributed in China and is the dominant host snail for transmitting *Fasciola* spp [13].

Food-borne trematode is often infected by eating raw vegetables such as fish mint (*Houttuynia cordata*), lettuce (*Lactuca sativa*), parsley (*Petroselinum crispum*), and watercress (*Nasturtium officinale*) [14]. From 2011 to 2012, there was an outbreak of *F. gigantica* infection in Binchuan County, Dali Prefecture, Yunnan Province in China, and then the authors think that fish mint was most likely the source of diseases [9, 15]. Guangxi Zhuang Autonomous Region is contiguous to Yunnan and shares a similar climate, as well as lifestyles and dietary habits of the local people. Given that the *Galba pervia* is the important intermediate host of *Fasciola* in China [11, 16], it also has a wide distribution in Guangxi, representing a potential risk of parasitic zoonosis. Therefore, the main objective of this study was to investigate and identify presence of various trematode larvae in *G. pervia* in Guangxi, and assess the zoonotic potential of trematode for both animal and human in this area.

Methods

Study areas and snail collection

To investigate the potential vector capacity of *G. pervia* in Guangxi Province, snails were collected from 54 sites in 9 cities, namely, Beihai, Fangchenggang, Guigang, Guilin, Liuzhou, Laibin, Nanning, Qinzhou, Wuzhou, and Yulin, from 2012 August to 2014 August (the number of snail samples per site was about 200). Two types of areas were included: Type 1 areas were rice cultivation areas (contains 51 sites, marked by circular shapes in Fig. 1G); Type 2 areas were the vegetation areas of crops which often used as the raw food (10 sites, marked by triangular shape in Fig. 1G). Details of each locality sampled are given in Table S1. In each sampling site, the snails were collected manually by the plastic scoop, transported to the laboratory, cleaned and rinsed five times in sterilized water, and then placed in plastic trays for subsequent experiments.

Fig. 1

Identification of snails and isolation of trematodes larvae

The snails were identified morphologically as *G. pervia* depending on systematic keys of the shell [12]. Then collected *G. pervia* were dissected under a stereomicroscope and carefully checked for trematodes larvae (rediae, cercariae, or metacercariae), and the larvae were separated from the tissue. We used MoticBA400 microscope to observe and record the body length and body width of each isolated trematode. The body length and body width of rediae, and the body length, body width, tail long and tail width of cercariae at each site were measured. The diameter and wall thickness of metacercaria were also measured.

Molecular examinations of the trematodes

Next, a single larva with the identical morphology at each sampling site was selected and rinsed with sterilized distilled water three times before being used to extract parasite genomic DNA by a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA samples were stored at −20°C until PCR amplification. The PCR assay targeting the sequence of the internal transcribed spacer 2 (ITS2) gene was used to amplify trematode larvae. The universal primer pairs were designed as described by McManus et al. [17]. All the PCR products were directly sequenced after being purified. The obtained sequences were edited using DNASTAR software (www.dnastar.com/software/lasergene/) and aligned using ClustalX (http://www.clustal.org/clustal2/). The identity of individual specimens was ascertained by comparison with the sequences available in 'non-redundant' database in GenBank by BLAST (http://www.ncbi.nlm.nih.gov/blast/). The nucleotide sequences obtained in the present study have been deposited in the GenBank database under the accession numbers.

Phylogenetic tree construction with ITS2

Phylogenetic trees were constructed using the neighbor joining (NJ) method in MEGAX (26). The *F. gigantica* (MK321643), isolated from a cattle, was used as an out-group for the construction of the phylogenetic trees of *Echinostoma* sp. (KJ848453, KJ848454, and KJ848455), *E. revolutum* (AY168930, KM980474, KM980476 and KM980477), *H. conoideum* (AJ564385, KJ944311, KJ944312, and KJ944313), *E. robustum* (LC224084), *E. friedi* (AJ564383), *E. miyagawai* (MW199188), *E. paraensei* (AF336232), *E. caproni* (AJ564382), *E. trivolvis* (GQ463127), *E. malayanum* (JF412727), and *Echinoparyphium recurvatum* (AY168931 and KJ435270). For the construction of phylogenetic tree of *Australapatemon* sp. (KM980467, KM980468, KM980469, KM980470, and KM980471), *Pharyngostomum cordatum* (OL870492 and KJ137231), *A. burti* (KU950451), *Austrodiplostomum ostrowskiae* (KT72878), *Alaria americana* (MH521246), *Diplostomum paracaudum* (KJ889013) and *Cyathocotyle prussica* (MH521249), the *Brachylaima* sp. (JX010634) and *Schistosoma japonicum* (S72866) were used as out-groups. The phylogeny was tested with 1,000 bootstrap replicates, using the Kimura two-parameter model as a nucleotide substitution model and gamma distribution as rates among sites.

Experimental infections of isolated trematodes in the intestine of duck

Five-day-old ducklings were fed with snails parasitized by isolated trematodes in the field. Each duckling was fed 20 *G. pervia*, and one duckling was dissected every day from the 1st to 10th day after ingestion. The trematodes were collected from the duck intestines using a complete helminthological dissection method [5], and high-resolution pictures of the collected trematodes were taken with the Motic BA400 microscope and additional accessories. The carmine staining of the press-and-fixed specimen was made according to the method provided by Kong Fanyao [6], and collar, spines, oral sucker, acetabulum, prepharynx, esophagus, testis, ovary was measured from digital images during daily observations. In addition, a single trematode was selected, and a small amount of tissue from the tail of the parasite was

cut out aseptically. After repeated rinsing with sterilized distilled water 2-3 times, DNA extraction was carried out according to the above method. ITS2 gene was amplified and sequenced using the same method, and the trematode species was verified.

Results

Overall information on the sampling and survey data

G. pervia samples were collected from 54 sites (as shown in Fig. 1G) with about 38-214 snails in each site. Trematodes were found in 17 sites investigated following dissection, including Tianbao Reservoir and Hede village in Nanning city, Liushan Town, Liutang Village, Guangrong village, and Cha Village in Liuzhou City, and Maling Town in Guilin City. Various stages in the life history of this trematode (*redia*, *cercaria* and *metacercariae*) were found during dissection procedures.

Morphological characters and molecular identification of trematodes larvae

The rediae of echinostomes were cylindrical, blunt at both ends, slightly pointed at the head and more pointed at the tail. The body was curved to the ventral surface with muscular feet, and the movement was slow. The tail of the cercariae is not forked. The head of *H. conoideum* cercariae shows prominent spines, as well as well-developed ventral suckers, pharynx, and intestines (Fig. 2D-F). The metacercaria were round and have two transparent walls (the outer wall was thicker than the inner wall). Abdominal suckers and refractive granules of larvae could be seen inside the cyst. Due to the movement of the larvae inside the sac, the small spines around its head were not easily observed. The rediae of *Australapatemon* sp. forms a distinct bulge at the head. The cercaria larvae had a forked tail, which was obviously longer than the body length. The cercaria of *P. cordatum* also had visible forked-tail, oral sucker and pharynx (Fig. 2J-L).

Figure 2

Based on morphologically available keys, the species of isolated trematodes were identified by amplification of ITS2 region and verified through BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi) with the highest identity after sequencing. Finally, we identified five different species of trematodes including *Australapatemon* sp., *Echinostoma* sp., *E. revolutum*, *H. conoideum*, and *P. cordatum*. The lengths of ITS2 were 292 bp, 429bp, 430bp, 432 bp and 294 bp, respectively. The nucleotide sequences obtained in the present study have been deposited in GenBank database under the accession numbers KM980466-KM980471 (*Australapatemon* sp.), KJ848453-KJ848455 (*Echinostoma* sp.), KM980474 and KM980476-KM980477 (*E. revolutum*), KJ944311-KJ944313 (*H. conoideum*) and OL870492 (*P. cordatum*).

The prevalence of trematodes in G. pervia

The overall trematodes infection rate was 22.0% (1818/8258). *Echinostoma revolutum* were detected in the snails from 11 sampling sites, with an infection rate of 12.9% (1069/8258); *Hypoderaum conoideum* infection was detected in the snails from two sampling sites, with an average infection rate of 3.8%

(315/8258). Infection of *Australapatemon* sp. was detected in the snails form 2 sampling sites, with an infection rate of 2.5% (206/8258); Infection of *Pharyngostomum cordatum* and *Echinostoma* sp. were detected at 1 sampling site with an infection rate of 0.4% (34/112) and 2.3% (194/8258), respectively.

Phylogenetic analyses

In total, 15 representative high-quality ITS2 sequence data was obtained. Figure 2 shows an NJ tree based on the submitted sequences and relevant GenBank sequences. The ITS2 sequences of *Echinostoma* sp. constituted a monophyletic clade (Fig. 3A shaded pink area), distinct from the clade formed by *E. robustum*, *E. friedi* and *E. miyagawai*. The sequences of *E. revolutum* and *H. conoideum* constituted a monophyletic group together with *E. revolutum* (AY168930) and *H. conoideum* (AJ564385) references (Fig. 3A shaded blue area). The ITS2 sequences of *Australapatemon* sp. formed a group with *A. burti* (KU950451) at 99% bootstrap value but formed a unique clade at 75% bootstrap value. Figure 3B showed that the ITS2 sequences of *P. cordatum* were identical to the reference sequences of *P. cordatum* (KJ137231).

Figure 3

Laboratory infection experiment with Echinostoma sp., E. revolutum, and H. conoideum

Because there was no suitable second intermediate host for *P. cordatum* and definitive host for *Australapatemon* sp., we conducted an infection experiment for isolated three kinds of trematode to evaluate rates of parasite establishment in ducklings. Ducklings were individually exposed to *Echinostoma* sp., *E. revolutum*, and *H. conoideum* larvae and all were successfully infected. Subsequent observation on Ducklings (17 dpi) fed with *Echinostoma* sp. infected *G. pervia*, we detected eggs (195.8×143.8 μm) in the feces, and the morphological characteristics of adult *Echinostoma* sp. were presented as measures: body length 9.8 mm, width 1.2 mm, oral suker 638.9×399.2 μm, acetabulum 1591.2×1338.2 μm, pharynx 492.6×331.7 μm, anterior testis 1120.5×707.4 μm, posterior testis 1274.9×880.4 μm, ovary 818.9×527.9 μm. In contrast, we found *H. conoideum* eggs in ducklings fed with infected *G. pervia* from three sites from a median of 12 dpi (range: 9 dpi to 14 dpi). The morphological characteristics of adult *H. conoideum* were: body length 1.05 mm, width 1.5 mm, oral suker 424.2×293 μm, acetabulum 1610.2×1594.6 μm, pharynx 379.8×253.6 μm, anterior testis 1902.6×875.3 μm, posterior testis 2045.2×898.2 μm, ovary 751.7×553.9 μm, and also characterized by the possession of 50 spines. *E. revolutum* eggs (104.1×63.1 μm) were found on 10 dpi. The morphology of adult *E. revolutum* was characterized by: body length 8.5 mm, width 2.2 mm, oral suker 260×180.1 μm, acetabulum 741.6×598.3 μm, pharynx 193.1×150.6 μm, anterior testis 628×459.4 μm, posterior testis 725.5×557.9 μm, ovary 411.8×311.2 μm, and presence of a head collar with 37 spines.

The developmental characteristics of E. revolutum in duckling host from juvenile to adult

As there were not sufficient metacercariae of other trematodes, experiments were only designed to gain insight into how *E. revolutum* developed in duckling hosts. The developmental characteristics of *E.*

revolutum was recorded by dissecting infected ducklings from 1 dpi to 10 dpi (eggs in the feces were first detected). *E. revolutum* could be obtained in the small intestine from 1 to 7 dpi and then migrate and reside in the cecum and colon around 8-10 dpi. The body length developed from 490 μm to 8500.5 μm (a dramatic 17-fold increase). At 1 dpi, juveniles presented a circumoral collar bearing 37 spines in a double circle and characterized by clearly visible oral suckers, acetabulum, pharynx, esophagus, and cecum. At 1 dpi, the tiny structure of the testis appeared. By 4 dpi, the ovaries were beginning to organize and develop, and the seminal receptacle began to form. The tubular-shaped uterus loomed at 4 dpi, and maturation of the reproductive and digestive organs occurred around 6~8 dpi. The vitelline glands were the last to appear, and several eggs deposited in the uterus could be observed at 9 dpi. *E. revolutum* larvae matured at 10 dpi and excreted eggs (Fig. 4). The daily measurement of *E. revolutum* development was recorded in detail, as shown in Table S2.

Figure 4

Discussion

Numerous species of food-borne trematodes are endemic in developing nations and significantly impact public health [18, 19, 20]. *Austropeplea*, *Galba*, *Lymnaea*, *Radix* and *Stagnicola* etc. from the families Lymnaeidae act as intermediate hosts of trematodes with substantial implications for human health [10, 13]. The primary research focused on the capability of transmitting *Fasciola* sp., and at least 20 species of Lymnaeidae have been described as potential vectors of fascioliasis [21]. The results reported in the present paper demonstrate the presence of five trematode species in *G. pervia*. Morphological characteristics identified the larvae to species level by combining unequivocal molecular markers, which identified as *E. revolutum*; *H. conoideum*; *Australapatemon* sp. *P. cordatum* and *Echinostoma* sp., respectively. Different collection sites differed concerning the larvae species and intensity of snails present, which would link with meteorological parameters and habitat types.

However, other trematode fauna, such as Fasciola, has not been detected, although Guangxi is one of the important regions of ruminant fascioliasis prevalence in the previous reports [22]. Our investigation indicated that *E. revolutum* was the most prevalent trematode species in Guangxi Province, with an infection rate of 12.9% among collected snails. In consideration of previous studies that Echinostomatidae have low intermediate host specificity [23]. In addition, *Radix plicatula*, *R. swihoei*, *Gyraulus conrexiusculus* etc. can also act as intermediate hosts [24], and all of above-mentioned snail species also have a wide distribution in Guangxi Province, so it implicates that the actual infection rate of Echinostomatidae trematodes may be much higher than the results found in this study.

There are many species of echinostomes, which are tiny parasites that mainly parasitize the intestines of birds, mammals, and humans [21, 25, 26]. However, due to the high diversity of species and similar morphology, some species have not been fully morphologically described by the most used morphological traits, with a precise classification elusive. In addition, it is time-consuming to identify the adults by reintroducing the larvae to complete their life cycles, and the improper selection of the definitive

experimental host will also lead to the failure of entering the next stage of the life cycle. Given these facts, Jonsson et al. proposed to apply gene markers or restriction fragment length polymorphism (RFLP) for molecular identification [17, 21, 27, 28]. ITS2 species-specific markers have been proven as suitable genetic markers for identifying and differentiating trematode species. Because the external morphology of trematode metacercariae from this study was quite similar, the ITS2 gene sequences of metacercariae were amplified to identify the metacercariae. The species identification results are consistent with morphological analyses, and the evolutionary relationships of trematode species were successfully elucidated and compared with reference sequences deposited in the public databases.

As early as 1968, Lie [29] et al. proposed that the development of trematodes may be restricted by others due to the competition inside the snail when they take the same species of snail as the intermediate host. Subsequent studies revealed a similar competition relationship in the intermediate host of echinostomes [30] and schistosomes [31]. In our study, different trematode species have not been detected in one snail simultaneously. Meanwhile, although *G. pervia* snail can also serve as the intermediate host for Fasciola in Guangxi Province, we have not observed Fasciola infected *G. pervia.* This phenomenon may be caused by the cross-species competitive antagonisms of echinostomes with other trematodes, which led to a generally low or non-infection of Fasciola. Trematode is a parasite that can cause severe zoonotic diseases. Lie proposed that the transmission of the disease could be contaminated through competition among trematode larvae in intermediate hosts in 1973 [32]. Although theoretically, echinostomes could be used to reduce the economic losses caused by Fasciola, however, given its great harm to the livestock and poultry, echinostomes are not sound biological control agents for the control of Fasciola in practice. To unveil the trematode infection rate in larger areas in Guangxi Province, it is necessary to expand the sampling sites and select more species of snails for investigation. Further research is needed to determine the coexistence and coevolution of competitive species, especially two or more trematodes that reside in one snail host in natural communities.

Echinostomes are a common intestinal parasitic trematode in poultry, which mainly affects the growth and development of the young while is less harmful to the adult. The developmental cycle of echinostomes in its terminal host is short and uncomplicated. Therefore, the animal developmental model of the echinostomes in its terminal host is suitable for studying the immune response between trematode and its host. The research results can also be used as a reference for other small intestinal flukes which induce the terminal host immune response, and related research has also been reported in recent years [3–4]. This study is mainly aimed at the observation of the growth and development of echinostomes from decapsulation of the cyst to the sexually mature adult stage in the intestinal tract of the terminal host. For the selection of experimental animals, mammals are not susceptible to echinostomes infection, so ducklings were used as the definitive host in our study. To provide a basis for subsequent related research, it needs to explore more animal models for echinostomes infection in the future.

Conclusions

The present investigation revealed the prevalence of five trematodes species in the *G. pervia* in Guangxi Province, China. The results from our study not only provide a baseline information but also offered laboratory experimental models for assessing the potential zoonotic echinostomiasis from *G. pervia*. Further research is needed toward the understanding risk of human infection in combination with risk evaluation to ameliorate unwanted adverse effect during casual contact or exposure to infected *G. pervia*.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All participants consented to have their data published.

Availability of data and materials

The sequences data has already submitted to GenBank, and will be released to the public database until Dec 1, 2016. The GenBank accession numbers are KX781395 for the ITS2 of *Australapatemon* sp., KM980463~KM980465, KM980478~KM980479 for the *Hypoderaeum conoideum*, KM980474 and KM980476~KM980477 for the *Echinostoma revolutum*.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributors

LJ and RYJ conceived and designed the study. LJ, RYJ, LY, GJN and CHY, LJN, THQ, ZQA and HWY collected and identified the snails, cercariae and metacercariae. RYJ and LJ analyzed the data and drafted the manuscript. LJ, FXY and HW helped in study design, study implementation and manuscript revision. LJ, FXY and HW critically revised the manuscript. All authors read and approved the final manuscript.

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Figures

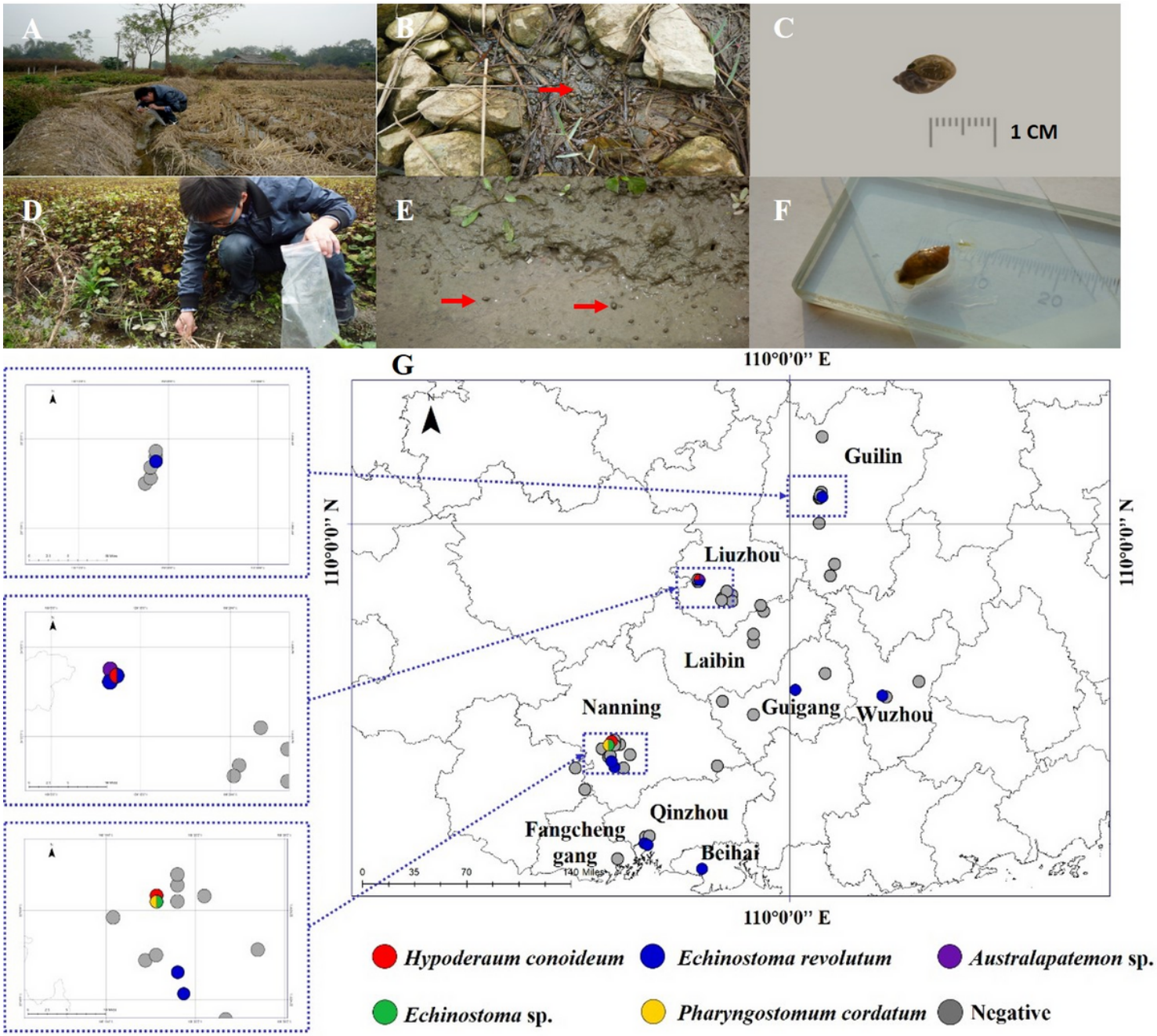

Figure 1

A, B: Type 1 areas were rice cultivation areas; C: *G. pervia* image in anterior view; D, E: Type 2 areas were the vegetation areas of agricultural crops which often used as the raw food; F: *G. pervia* image posterior view; G: 54 *G. pervia* snail collection sites in 9 cities in Guangxi Province.

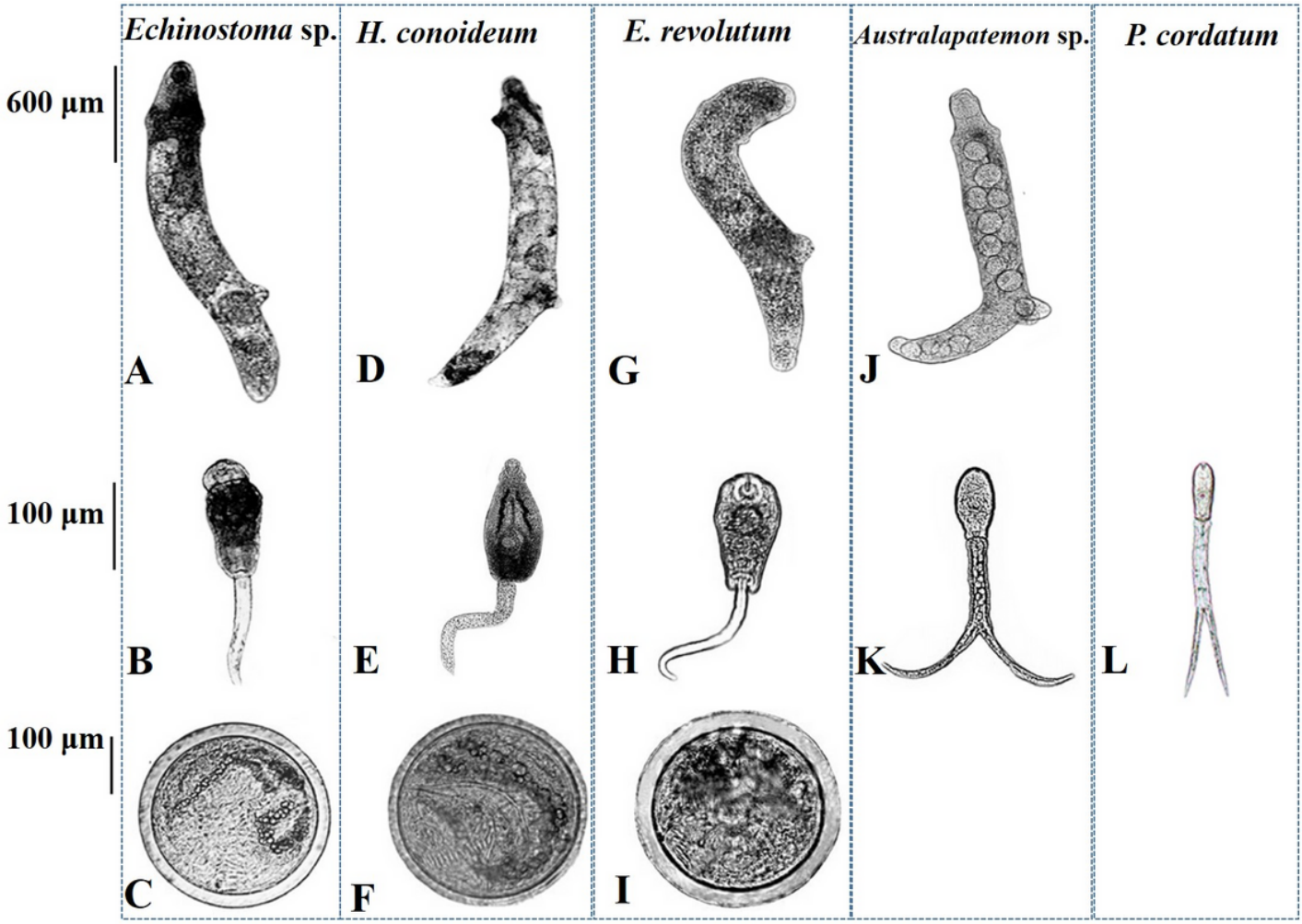

Figure 2

Morphology of rediae, cercariae and metacercariae collected in *G. pervia*. A-C: The rediae, cercariae and metacercariae of *Echinostoma* sp.; D-F: The rediae, cercariae and metacercariae of *Hypoderaeum conoideum*; G-I: The rediae, cercariae and metacercariae of *Echinostoma revolutum*; J, K: The rediae and cercariae of *Australapatemon* sp.; L: The cercariae of *Pharyngostomum cordatum*.

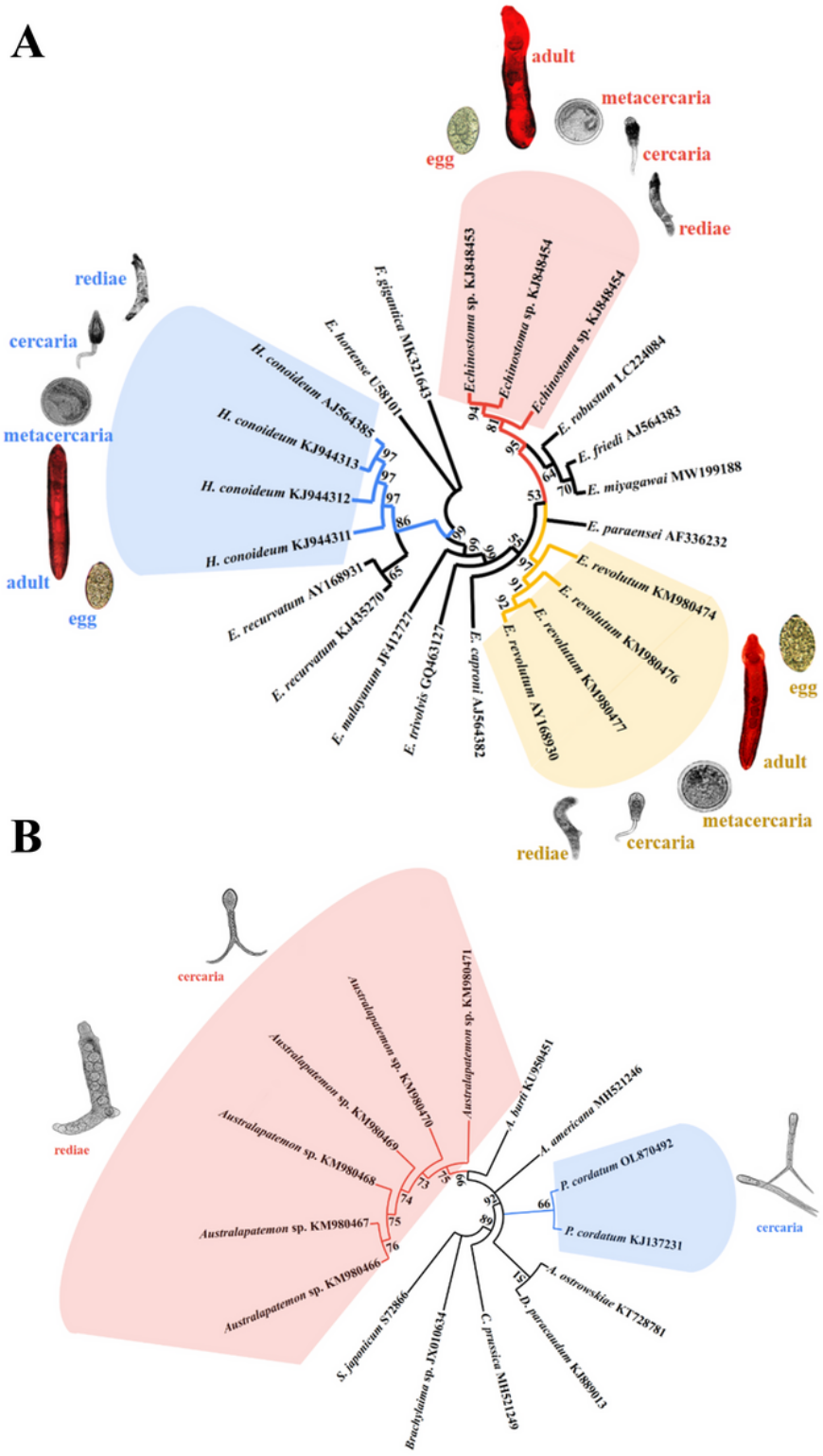

Figure 3

Phylogenetic analyses of isolated trematodes based on the ITS2 sequences and relevant GenBank sequences. A: Neighbor joining bootstrap consensus tree with 1000 bootstrap iterations for the rediae of echinostoms; B: Neighbor joining bootstrap consensus tree with 1000 bootstrap iterations for *Australapatemon* sp. and *Pharyngostomum cordatum*.

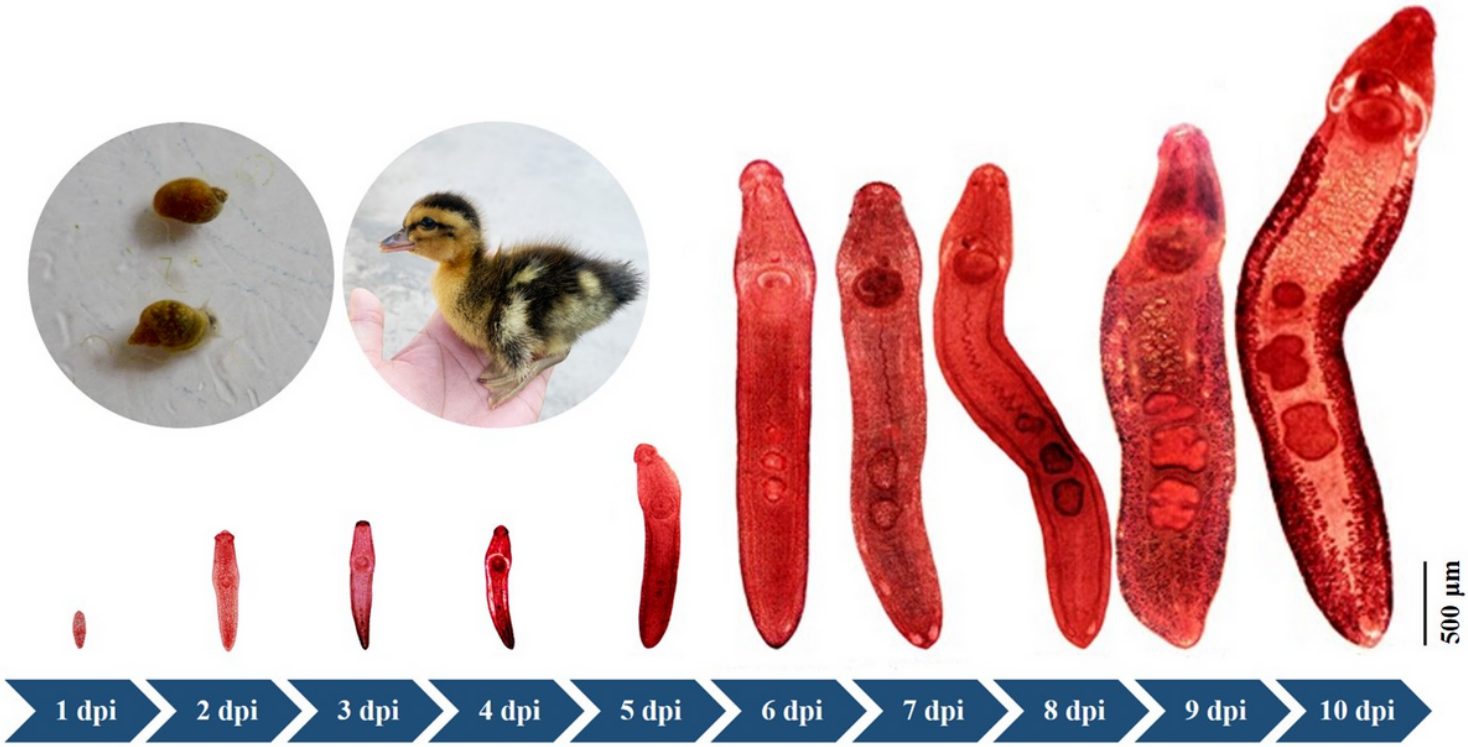

Figure 4

Development of *E. revolutum* in duckling host from 1 dpi to 10 dpi

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx
- TableS2.docx