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Clinostomum sinensis in Red-crowned Cranes (*Grus japonensis*) and in White-naped Cranes (*Grus vipio*) - Morphology and Molecular Characterization

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ABSTRACT

***Background*:** *Clinostomum* spp. are digenetic trematode parasites of freshwater snails and fishes, fish-eating birds, and humans. There are widely distributed around the world. The metacercariae of the *Clinostomum* can cause fish trematodes, and adults parasites parasitized in the throat and esophagus of fish-eating birds. The red-crowned crane (*Grus japonensis*) and white-naped cranes (*Grus vipio*) are internationally recognized as an endangered species. They are large wading bird species and listed as Class I protected animals in China. To date, there have been no reports of *Clinostomum* infection in red-crowned cranes (*Grus japonensis*) and white-naped cranes (*Grus vipio*) in China.

***Materials, Methods & Results*:** In this study, we isolated specimens of *Clinostomum sinensis* adults from the oral cavity of red-crowned cranes (*Grus japonensis*) and white-naped cranes (*Grus vipio*) and encysted "yellow grub" metacercariae from the forage fish *Pseudorasbora parva* and used morphological analysis and molecular characterization based on 18S ribosomal RNA and the mitochondrial cytochrome oxidase (COI) loci. The digestive system of the metacercariae was clear, whereas the genital system was not, with the exception of the vitellaria. Morphological features of adults included a small oral sucker, an unclear or absent pharynx, a medium-sized ventral sucker, an ovoid cirrus pouch near the anterior testis, and a uterine sac that extended to the ventral sucker with no folds, especially the entire anterior testis offset to the left by the uterine sac and cirrus sac, and genital pore opening on the right side of the anterior testis and papillae around, compared with the features of other species of *Clinostomum*. At the 18S loci, the species was in the same clade with *C. complanatum* from Italy and Israel. It speculated that although the specimens sequenced in this study grouped with the sequences of *C. complanatum* from Asia, there are so few species that have been sequenced (and verified) that identification of the sequences in this study is still problematic. At the COI loci, *Clinostomum* spp. were clearcutted into different clades depending on geographical regions. The species isolated in this study was in the same clade as isolates from Asia. While, based on 18S or COI loci sequences analysis, the isolated specimens were identified as *C. sinensis*. Morphological observation also identified the precise species was *C. sinensis*. Above of all, we isolated *C. sinensis* from cranes and *P. parva* in eastern China firstly, and firstly descripted the morphology of adult of *C. sinensis* in eastern China.

***Discussion*:** Precise species identification is crucial for controlling and treating parasite infections. Traditionally, morphological characteristics have been used to identify trematode species, although larval stages pose challenges due to fewer features. Molecular methods have proven effective, especially for morphologically similar species. This study identified *C. sinensis* in red-crowned (*Grus japonensis*) and white-naped cranes (*Grus vipio*) and their intermediate host *P. parva* in eastern China, using both morphology and molecular methods. Morphological analysis showed similarities with previous studies, with clear identification of the oral and ventral suckers in metacercariae, and specific features in adults such as the small oral sucker and ovoid cirrus pouch. Molecular analysis using 18S rRNA and COI loci revealed that the isolates clustered with *C. complanatum* and *C. sinensis* from Asia, with minimal sequence differences, confirming the specimens as *C. sinensis*.

Keywords: trematodes, *Clinostomum sinensis*, *Clinostomum* spp., crane, morphology, 18S rRNA, COI.

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INTRODUCTION

Clinostomum Leidy, 1856 is a genus of digenetic trematodes and one of the most common genus of Clinostomidae around the world [18,22]. Intermediate hosts include many freshwater snails and fishes, with metacercariae causing muscle damage and severe "yellow grub" disease in fish, such as *C. complanatum* [4,8,14,17,23]. The final hosts are fish-eating birds, where adults invade the oral cavity, pharynx, or esophagus [4]. Human infections, caused by consuming raw or undercooked parasitized fresh, have been reported in Thailand [25], Korea [16], and Japan [10,12], indicating some *Clinostomum* spp. are zoonotic and pose public health risks.

Morphological differences, particularly in the genital complex (cirrus pouch shape and position, uterus shape, and genital pore position), are used to identify *Clinostomum* species. However, identification is challenging due to variations in developmental stage morphology and differing techniques among authors. Molecular data linked to morphological characteristics have successfully identified different *Clinostomum* species [17,23]. Researchers used 18S rRNA to identify *C. complanatum* from Israel (AY245760) and *C. marginatum* from the United States (AY245701) based on 22 nucleotide sequences [3,6]. Cytochrome oxidase subunit I (COI) loci have also been used to successfully identify these species [3]. In this study, we found specimens of *Clinostomum* adults in the oral cavity of red-crowned cranes (*Grus japonensis*) and white-naped cranes (*Grus vipio*) and progenetic metacercariae in the tegument of the forage fish *Pseudorasbora parva*. Using morphological analysis and molecular characterization based on 18S rRNA and COI loci, we identified the specimens as *C. sinensis*. This is the 1st report of *C. sinensis* in cranes in eastern China and 1st description of adult of *C. sinensis* in China, providing an important reference for the diagnosis, treatment, and controlling of this parasitic disease.

MATERIALS AND METHODS

Specimen collection

Six cranes (7-45 days old), including 4 white-naped cranes (*Grus vipio*) and 2 red-crowned cranes (*G. japonensis*), were reared in Wuxi Zoo (31.49117 S, 120.31191 E) in eastern China in 2017. On 7 June 2017, they all exhibited dyspnea one after another, and 5 died. Only 1 white-naped crane (45-day-old) survived with changing feed, and emergency treatment with anti-fluke drugs for 3 days. Necropsies of the deceased cranes showed large number of trematodes found in the tongue, mouth cavity, and larynx. The larynx was edematous and blocked with parasites. There were no significant lesions in other organs.

The worms were collected and washed with physiological saline (0.9% sodium chloride solution). Some of the specimens were fixed with 13% formalin for morphological description [2], and the rest were stored in 70% ethanol at -20°C for DNA extraction [7,15].

Considering the intermediate hosts of trematodes, the forage fish *Pseudorasbora parva* in the pool were also collected to find encysted progenetic metacercariae by using the isolation methods of other metacercariae decribed in previous study [20,24].

All animal procedures were carried out in accordance with the guidelines and regulations of the Animal Experiment Ethics Committee of Yangzhou University (Protocol No. 202103210), and all experimental protocols were approved by the Animal Experiment Ethics Committee of Yangzhou University. The study and all methods were reported in accordance with ARRIVE guidelines.

Morphological observations

Genomic DNA was extracted from adults and metacercariae using a TaKaRa Mini-BEST Universal Genomic DNA Extraction Kit[1] according to the manufacturer's instructions. A negative control containing no parasites was included.

Primers for *Clinostomum* 18S rRNA were designed using Primer 5[2] based on sequences from the following species: *C. marginatum* (AY245760), *C. cutaneum* (GQ339114), *C. complanatum* (FJ609420), *C. phalacrocoracis* (FJ609423), *C. tataxumui* (MF398349), *Clinostomum* sp. USA-PO-2003 (AY222095), and *Clinostomum* sp. Australia-PO-2003 (AY222094). PCR was performed with the forward primer Cli F1 5' ATGCCACTATGCCCTGAC 3' and the reverse primer Cli R1 5' ACGCTGACCCACGACCAC 3'. PCR reactions with a volume of 25 µL contained the following: 1 × PCR buffer, 10 µM of each primer, 1mM dNTPs, 1 U Taq DNA polymerase[1] and 50 ng sample DNA. PCR cycling conditions included a premelt at 95°C for 3 min, 30 cycles of 98°C for 10 s, 60°C for 5 s, and 72°C for 1 min, and a final elongation step at 72°C for 5 min. PCR for the partial COI loci was performed as described for 18S rRNA using the forward primer Cli F2 5' GGCGGGTTCTTAGGTTTA 3' and the reverse primer Cli R2 5' GCAGCCCTAAATTTACGATC 3'. PCR products were purified using a TaKaRa MiniBEST

DNA Fragment Purification Kit[1] and subcloned into the pGEM®-T easy vector[3] for sequencing in both directions by Invitrogen Corporation[4].

The 18S and COI loci of *Clinostomum* spp. and additional isolates from GenBank were used to build phylogenetic trees with the MEGA 7 program[5] [29]. The phylogenetic trees were constructed using maximum likelihood (ML) and assessed by bootstrap resampling on 1,000 replicates [17]. The best nucleotide substitution model was tested referencing previous research [1,13,19]. As results, the K2 (Kimura 2-parameter) model was chosen for 18S analysis and HKY (Hasegawa-Kishino-Yano) + G (Gama Distributed) model for COI analysis.

RESULTS

Morphological observations

Encysted "yellow grub" metacercariae [21] were found in the forage fish *Pseudorasbora parva*. The metacercariae were surrounded by yellow spherical or elliptical cysts in the fish tegument, located at the base of the anus, and measured 6.0 mm in diameter (Figure 1A & B). The cysts were broken to release the metacercariae, and each cyst contained only 1 parasite. The metacercariae were alive and moving (Figure 2A & B). These were smaller than adults, and the widest part of the body was at the level of the ventral sucker. The oral collar was well developed and visible, and the oral sucker was smaller than the ventral sucker. The intestinal ceca were yellow within the body of the parasite, lateral to the ventral sucker, and extended to the end of the body. The shape of the testis and ovary were not clear but located in the posterior end of the middle 3rd of the body, and were smaller than in adults. The vitellaria was lateral to the intestinal ceca from the ventral sucker to the end of the body.

According to necropsy results of the dead crane, there were a number of larvae and adult parasites in the oral cavity of the cranes (Figure 3). Morphological observation of adults showed that body linguiform, stout, and anterior body was bend to 1 side, the posterior body region was flatter than the anterior region, widest at the gonadal region, and distinctly constricted at the ventral sucker. The oral collar was well developed. The pharynx was unclear or absent, and the esophageal bulb was not clear. The ventral sucker located in the end of the anterior 3rd of the body and was larger than the oral sucker. The intestinal ceca ran laterally to the posterior end of the body, and the testis triangular and branched to the lump. The anterior testis was in the posterior end of the middle 3rd of the body and positioned slightly to the left. The posterior testis was in the anterior end of the posterior 3rd of the body and on midline. The cirrus pouch was ovoid and located near the anterior testis, between the right side of the anterior testis and the intestinal ceca and overlapping with the right side of the ovary, and had a clear cirrus sac and a coiled seminal vesicle that opened to a genital pore. The genital pore was located on top of the cirrus pouch, pointing anteriorly. The ovary was small and located between the anterior and posterior testes. The vitellarium was extensive, from the shorter distance of the posterior of the ventral sucker to the end of the intestinal ceca. The utero duct opened into a uterine sac that extended to a ventral sucker with no folds. There were number of mature eggs in the uterine sac (Figures 4 & 5). Measurements of 12 adults are shown in Table 1.

Remark: Morphological observations of adults showed that the cirrus pouch was ovoid, and the uterine sac extended to the ventral sucker with no folds. Especially the entire anterior testis offset to left by uterine sac and cirrus sac, and genital pore opened in the right side of the anterior testis, these features were different to *C. marginatum* and similar to *C. complanatum* preferring previous research [3]. Genital pore has some small papillae at periphery, which was similar to *C. sinensis* and different to *C. complanatum* [13]. It is regrettable that we did not get live adults to obtain eggs to measure size, which could double certain about the species [11]. While, morphological analysis identified the precise species was *C. sinensis*.

Table 1. Measurements of *Clinostomum sinensis* adults.

Clinostomum sinensis (n=12)	Average (μM)
Oral collar length	533.0
Oral collar width	311.6
Body length	6,081.5
Body width	1,167.4
Oral sucker length	271.4
Oral sucker width	127.9
Ventral sucker length	713.4
Ventral sucker width	623.1
Distances between suckers	1,419.8
Anterior testis length	606.2
Anterior testis width	443.7
Posterior testis length	557.9
Posterior testis width	357.4
Distances between testes	570.5
Ovary length	249.2
Ovary width	214.2
Cirrus pouch length	661.8
Cirrus pouch length	216.6

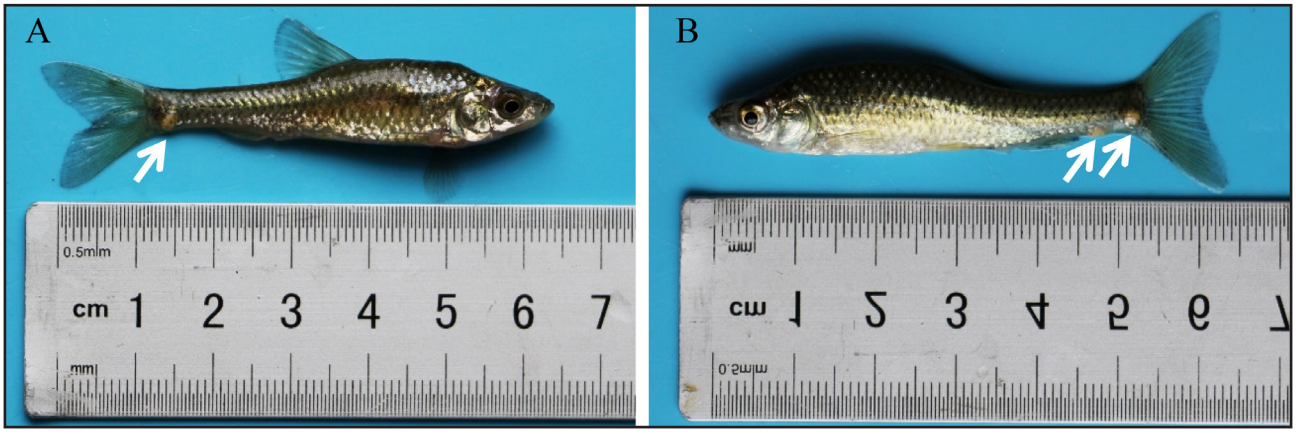

Figure 1. Photographs of one forage fish (*Pseudorasbora parva*) infected with encysted *Clinostomum sinensis* metacercariae (yellow grubs). The metacercariae were surrounded by yellow spherical or elliptical cysts located at the base of the anal fin. [A- Right side of fish. B- Left side of fish].

Figure 2. Encysted progenetic *Clinostomum sinensis* metacercariae obtained from forage fish (*Pseudorasbora parva*). The excysted metacercariae were alive and moving, as shown in pictures A and B, and the oral sucker and ventral sucker were observed very clearly.

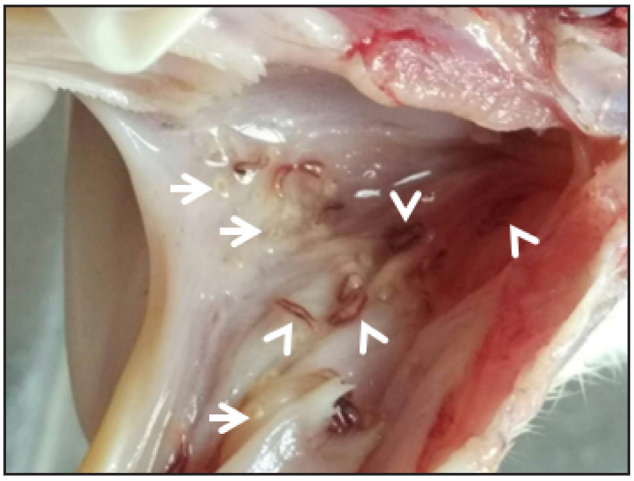

Figure 3. The oral cavity of *Grus japonensis* showing *Clinostomum sinensis* larvae (arrows) and adults (arrow heads).

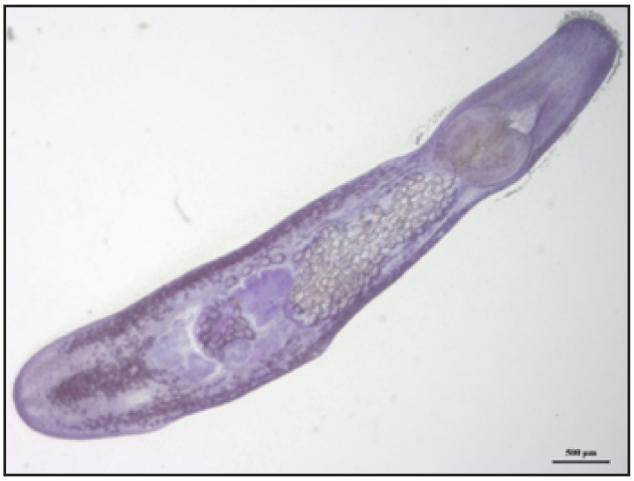

Figure 4. *Clinostomum sinensis* adult worm. [Delafield's hematoxylin stain, scale bar=500 µm].

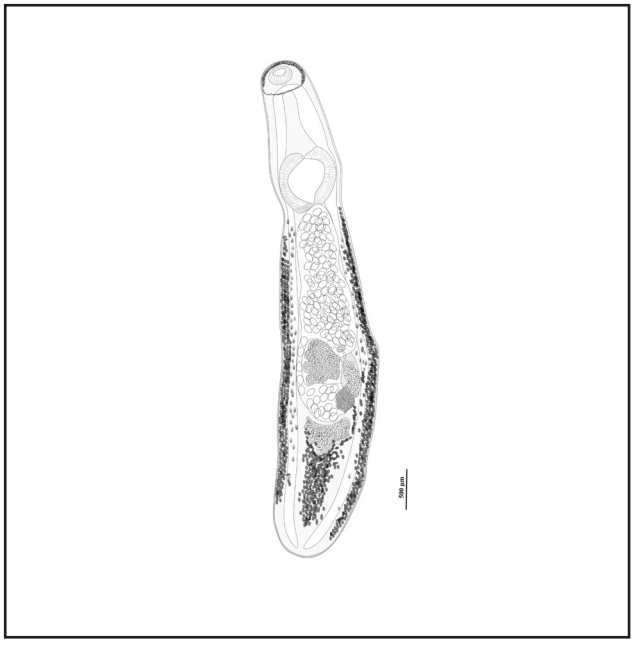

Figure 5. Line drawing of *Clinostomum sinensis* adult worm. [Scale bar = 500 μm].

Phylogenetic analysis of Clinostomum sinensis *at the 18S rRNA loci*

The same 1372-bp 18S rRNA sequences were obtained from metacercariae and adults and given the GenBank accession number ON507988 and MK490986. They aligned with 27 other *Clinostomum* spp. sequences from GenBank, and *Euclinostomum* sp. was used as the outgroup (Figure 6). According to ML tree analysis, sequences were divided into different clades depending on geographical regions in some degree. *Clinostomum* sp. isolated in this study (labeled with a black triangle in Figure 6) were in the same clade with *C. complanatum* from Italy and Israel. *Clinostomum* sp. isolated from Australia were in one clade. *Clinostomum* spp. from North America, containing USA and Mexico, were in one clade. *C. cutaneum* and *C. phalacrocoracis* from Africa were in one clade. *C. brieni* from Africa were in one clade, and from Asia were in another sister clade. The 18S rRNA sequence obtained in this study had the support of 98.0% bootstrap in the same sister clade as 3 other geographical isolates, *C. complanatum* (FJ609420.1) [9] and *C. complanatum* (MK811210.1) isolated in Italy and *C. complanatum* (AY245701.1) isolated in Israel [5], and had 99.7% identity to these isolates. It had different degrees of identity, from 93.6% to 98.6%, to other *Clinostomum* spp. in the phylogenetic tree.

Phylogenetic analysis of Clinostomum sinensis *at the COI loci*

The same 528-bp COI loci sequences were obtained from metacercariae and adults and given the GenBank accession number ON506262.1 and MK501949. They were compared with 41 other *Clinostomum* spp. sequences from GenBank, and *Euclinostomum heterostomum* was used as the outgroup (Figure 7). According to ML tree analysis, 3 main clades were identified. Sequences from Asia and Europe as sister clade were in 1st clade. Sequences from Africa were divided into 2 clades and had the nearest phylogenetic distance. The COI loci obtained in this study (labeled with a black triangle in Figure 7) was in the 1st clade and they had the support of 62.0% bootstrap in the same sister clade with other geographical isolates from Asia. It showed 99.2% to 99.8% similarities with Asia isolates. It also showed different levels of identity, ranging from 97.2% to 98.1%, to sister clade isolated from Europe. According to sequences analysis, COI loci obtained in this study only had 1 to 4 bp sequence difference with Asia isolates and showed 10 to 15 bp sequence difference with Europe isolates in sister clade.

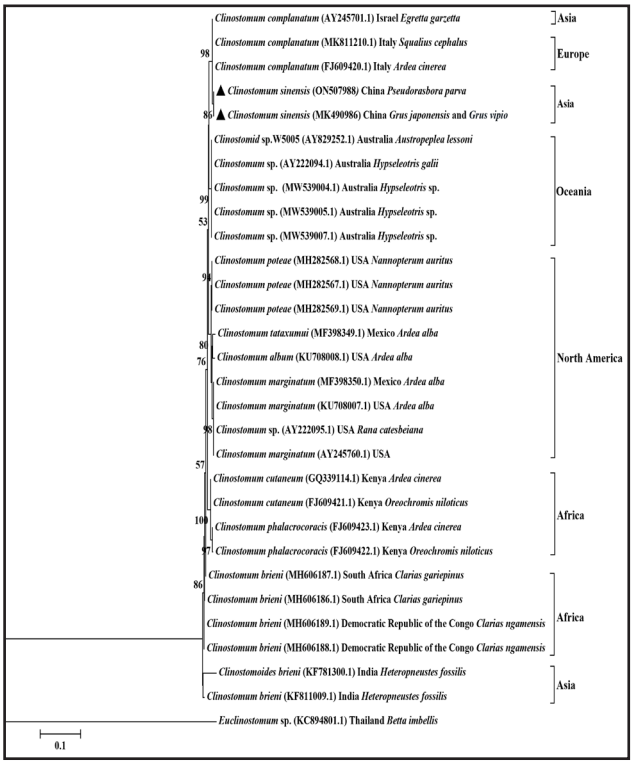

Figure 6. Phylogenetic relationships of *Clinostomum sinensis* inferred by distance analysis of 18S rRNA sequences. The 664-bp alignment was analyzed using K2 model in maximum likelihood analysis (ML). Percentage support (>50%) from 1,000 pseudo-replicates. Sequences are given as GenBank number, country, and host. [Sequences labeled with black triangles were obtained in this study].

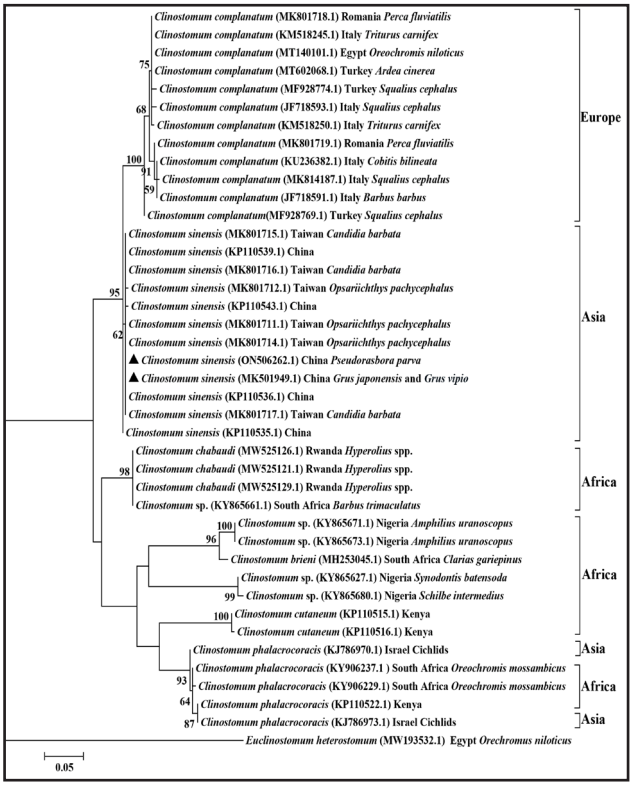

Figure 7. Phylogenetic relationships of *Clinostomum sinensis* inferred by distance analysis of COI sequences. The 282-bp alignment was analyzed using HKY+G model in maximum likelihood analysis (ML). Percentage support (>50%) from 1,000 pseudo-replicates. Sequences are given as GenBank number, country, and host. [Sequences labeled with black triangles were obtained in this study].

DISCUSSION

Precise species identification is very important for the control and treatment of parasite infections [14]. Morphological characteristics have successfully been used to identify different species of trematodes [17]. However, because larval stages have fewer morphological features, species identification depends mainly on adult morphology [23]. Molecular methods have been widely used to identify trematode species, especially morphologically similar species. In this study, we identified *Clinostomum* sinensis adults in the oral cavity of red-crowned cranes (*G. japonensis*) and white-naped cranes (*G. vipio*) and metacercariaes in the intermediate host *Pseudorasbora parva* in eastern China and used morphology combined with molecular methods to describe their characteristics.

According to morphological analyses, observations of metacercariae and adults were similar with those of previous studies [14,17,23,28]. The metacercariae was contained in a "yellow grub" on the fish tegument. In this study, the metacercariae was alive and continuously moving, so the oral sucker and ventral sucker were clearly identified. The digestive system was also clear, but the genital system was not clear, with the exception of the vitellaria. Further observation was obtained from adult parasites. The oral sucker of adults was small, the pharynx was absent or unclear, the ventral sucker was medium in size, the cirrus pouch was ovoid, and the uterine sac extended to the ventral sucker with no folds, compared with other species of *Clinostomum* [15]. And the position of cirrus sac was different with *C. marginatum* and similar with *C. complanatum*. Small papillae rounded genital pore made it was identified the precise species as *C. sinensis*.

Two loci, 18S rRNA and COI, were used for further molecular definition. The 18S rRNA phylogenetic tree showed that *Clinostomum* sp. isolated in this study were in the same clade with *C. complanatum* from Europe and Asia. It speculated that there are so few species that have been sequenced (and verified) that identification of the sequences in this study is still problematic.

Recently, the COI loci has been frequently used for species-based identification, and shows better resolution than ITS for exploring intraspecific genetic divergence [26,27]. The COI phylogenetic tree showed that *Clinostomum* sp. isolated in this study were in the same clade with Asia isolates, containing *C. complanatum* and *C. sinensis*. *Clinostomum* species were grouped according to geographical regions of collection [18], which was same with our study in Figure 7, and *C. complanatum* was a European species [13]. *Clinostomum* sp. in this study was in the same clade with *C. sinensis* isolated from Asia, and had similar morphometric feature with it, while, they only had 1 to 4 bp sequence difference. Based on the COI molecular sequences, the specimens collected in this study are *C. sinensis*.

CONCLUSION

This study provided the 1st report of the adult specimens of *Clinostomum sinensis* from the red-crowned cranes (*G. japonensis*) and white-naped cranes (*G. vipio*) and the progenetic metacercariae of *C. sinensis* from the forage fish *Pseudorasbora parva*. We also firstly descripted the morphology of adult of *C. sinensis* in Asia. Based on morphological and molecular characteristics in this study, the specimen

isolated in this study was identified as *C. sinensis*. The prevalence of and harm caused by metacercariae in forage fish of waterfowl and adults in fish-eating birds requires further study. This study provides helpful tools for the identification, treatment, and control of this zoonotic parasite.

MANUFACTURERS

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[4]Invitrogen. Shanghai, China.

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***Ethical approval*.** All animal procedures were carried out in accordance with the guidelines and regulations of the Animal Experiment Ethics Committee of Yangzhou University (SYXK(Su)2016-0020), and all experimental protocols were approved by the Animal Experiment Ethics Committee of Yangzhou University. The study and all methods were reported in accordance with ARRIVE guidelines.

***Declaration of interest*.** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors alone are responsible for the content and writing of the paper.

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