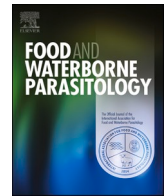




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Occurrence of digenean parasites in freshwater snails in the Murrumbidgee catchment area, Australia

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ABSTRACT

Freshwater snails are important hosts in the life cycles of many medically important parasites, particularly for digenetic trematodes such as liver flukes and schistosomes. The current study was conducted to determine the infection of freshwater snails with parasites that can potentially be transmitted to humans within the Murrumbidgee catchment area which is an area of widespread intensive aquaculture in Australia. A total of 116 freshwater snails, belonging to three species (*Isidorella hainesii*, *Glyptophysa novaehollandica* and *Bullastra lessoni*), were examined for the presence of parasites in both man-made and natural environments. The analysis of sequence data, including the internal transcribed spacers (ITS) of nuclear ribosomal DNA, small subunit (18S) ribosomal DNA, and large subunit (28S) ribosomal DNA, indicated that the collected parasites belonged to two distinct genera, namely *Clinostomum* and *Echinostoma*. It is noteworthy that species of both of these digenean parasites have the potential to be zoonotic. Cercariae of both *Clinostomum* and *Echinostoma* were observed in snails collected from aquaculture settings. It is important to highlight that infectious stages of *Clinostomum* has been previously detected in edible fish within Australia. This information raises concerns regarding the potential transmission of these parasites to humans through the consumption of contaminated fish. These findings emphasize the importance of monitoring and controlling the presence of *Clinostomum* and *Echinostoma* in aquaculture environments to minimise the risk of zoonotic infections and ensure food safety. Further research and surveillance are needed to better understand the prevalence, transmission dynamics, and potential public health implications associated with these parasites in the context of aquaculture in Australia.

1. Introduction

There are multiple notable zoonotic diseases of public health concern, which are caused by parasites that use snails as their intermediate hosts. Freshwater snails can serve as the transmission vectors and intermediate hosts for parasites that lead to severe diseases, such as clonorchiasis, fascioliasis, and opisthorchiasis (Lu et al., 2018). In this process, freshwater snails often are the first intermediate hosts in which a parasite develops into sporocysts and finally forms cercariae. These cercariae then infect freshwater fish which act as the second intermediate host (Zheng et al., 2017). Therefore, infection of freshwater snails with parasites also poses a risk

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to aquaculture and food safety. Since snail populations can become abundant in aquaculture ponds the likelihood of parasitic infection among stocked fish may increase.

In Australia, there have been several reports of snail borne parasites such as *Fasciola hepatica*, *Clonorchis sinensis* and *Paragonimus westermani* in humans (Attwood and Chou, 1978; Mukerjee et al., 1992; Hughes et al., 2003; Shamsi and Sheorey, 2018). However, limited research has been conducted on snails acting as intermediate hosts for these parasites. While freshwater snails play a significant

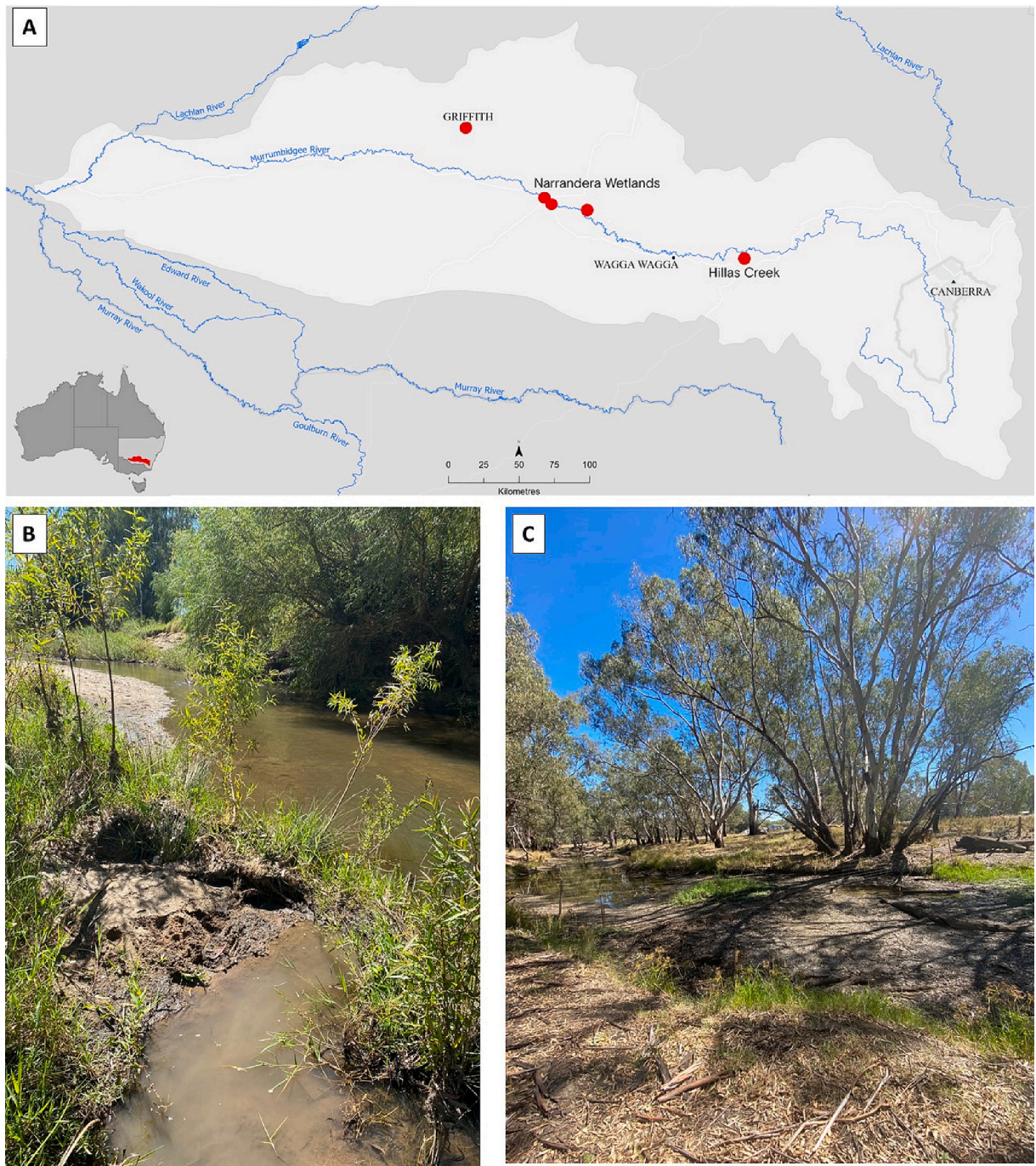


Fig. 1. A) A map of the Murrumbidgee catchment area with collection locations marked with red circles, sourced from Office of Research Services and Graduate Studies, Spatial Data Analysis Network (SPAN), Charles Sturt University. B and C) Two natural locations with moderate vegetation and areas of stagnant and flowing water where snails were collected, Hillas Creek (B) and Narrandera Wetlands (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

role in the life cycles of many parasites, only a few studies have attempted to identify larval stages of parasites in Australian freshwater snails. For instance, Johnston and Angel (1941a,b) conducted a comprehensive work on the Digenea of Australian animals in the South Australian part of the Murray-Darling river system, into which the Murrumbidgee catchment flows. Koch (2003) conducted a study on parasites of *Gabbia vertiginosa*, a freshwater snail in the New England Tablelands, in which he reported an unknown *Echinostoma* sp. in an unknown snail species belonging to the genus *Glyptophysa*. Recently, other snail borne parasites such as *Clinostomum*, were found in Australian freshwater fish (Shamsi et al., 2021b) which prompted the present study which aims to determine whether the larval stages of food-borne parasites are present in the freshwater snails species in the Murrumbidgee catchment area. The Murrumbidgee catchment area is one of the most important agricultural regions in Australia where several aquaculture industries operate. These aquaculture industries supply products to both domestic and international markets.

2. Materials and methods

2.1. Study area and snail collection

Freshwater snails ($n = 116$) were collected between January and April 2021 at five locations throughout the Murrumbidgee catchment area (Fig. 1). The locations were a combination of natural creek and wetland sites (Fig. 1B&C) and man-made ponds in finfish aquaculture facilities. Fish Farm 1, located in Narrandera, breeds a variety of native fish species: Murray cod (*Maccullochella peelii*), trout cod (*Maccullochella macquariensis*), silver perch (*Bidyanus bidyanus*) and golden perch (*Macquaria ambigua*). Fish Farm 2 and Fish Farm 3 both grow and supply Murray cod for commercial sale for human consumption. Snails were collected either by scraping a small bucket along the bottom of the water body and physically sorting through the sediment for snails, or by sighting and collecting them where the water was clear.

Following collection, the snails were transported to the Parasitology Laboratory at Charles Sturt University in Wagga Wagga, and identified to species using the Illustrative Key for Freshwater and Estuarine Molluscs in New South Wales (Ponder et al., 2020).

2.2. Parasite collection and identification

Snails were examined for parasites in accordance with previous studies (Caron et al., 2008; Shamsi et al., 2021c; Barton et al., 2022). Parasites were preserved in 70% ethanol. Collected parasites were examined and separated preliminarily into morphotypes. Representative samples of each morphotype, locality and host were selected for morphological and molecular analyses to determine parasite species.

DNA was extracted using DNeasy Blood and Tissue Kits (Qiagen, Australia), with alterations to the manufacturer's instructions (Shamsi et al., 2018). Various regions of ribosomal DNA, including Internal Transcribed Spacers (ITS), 18S, and 28S were amplified using primers sets D1 & D2 (Hillis and Dixon, 1991), WormA & WormB (Littlewood and Olson, 2001) and LSU-5 m & 1500Rm (Olson et al., 2003), respectively. PCR conditions were as follow: 95 °C for 2 min (initial denaturing) then 40 cycles of 95 °C for 30 s (denaturing), 56 °C for 30 s (annealing), 72 °C for 45 s (extension), 72 °C for 10 min (final extension). PCR amplicons were sequenced at the Australian Genome Research Facility (AGRF) using the same primers as used in PCR. Sequences were quality checked using SequenceScanner (Applied Biosystems/Thermo Fisher), and then were aligned with MUSCLE program (MEGA-X). BLAST search was used (Altschul et al., 1990) to determine if they matched any known species. DNA pairwise distances were calculated with the MEGA-X software using the Tamura-Nei substitution model (Tamura et al., 2013). Maximum likelihood (ML) analyses were performed. Bootstrap values based on 1000 resampled datasets were generated. The ML tree was visualised using the tree explorer of MEGA-X (Tamura and Nei, 1993; Kumar et al., 2016).

3. Results

Three species of freshwater snails were collected. The details of the collected snails and the parasites found in them are provided in Table 1. Preliminary examination showed cercariae could be divided into two digenean morphotypes. Morphotype 1 was found in snails belonging to species *Isidorella hainesii*, only from aquaculture facilities. The distinguishing characteristics of this morphotype included a small body relative to tail length, a slightly larger ventral sucker than oral sucker and the presence of collar spines. Morphotype 2 was found only in *Bullastra lessoni* and had a long tail, which was forked at the tip of the tail. They were significantly

Table 1

Snail species, corresponding locations, parasites found and prevalence of infection (P) in the present study.

Snail family	Snail species	Locality	Snail count	Number infected (P)	Parasites found
Planorbidae	<i>Isidorella hainesii</i> (Tyron, 1866)	Fish farm 1	52	2 (3.8%)	<i>Echinostoma</i> sp. (morphotype 1)
Planorbidae	<i>Glyptophysa novaehollandica</i> (Bowdich, 1822)	Hillas creek, Narrandera Wetlands; Fish farm 1, 2 and 3	33	0 (0%)	–
Lymnaeidae	<i>Bullastra lessoni</i> (Deshayes, 1831)	Fish farm 1	31	2 (6.4%)	<i>Clinostomum</i> sp. (morphotype 2)
Total in the study			116	4 (3.4%)	

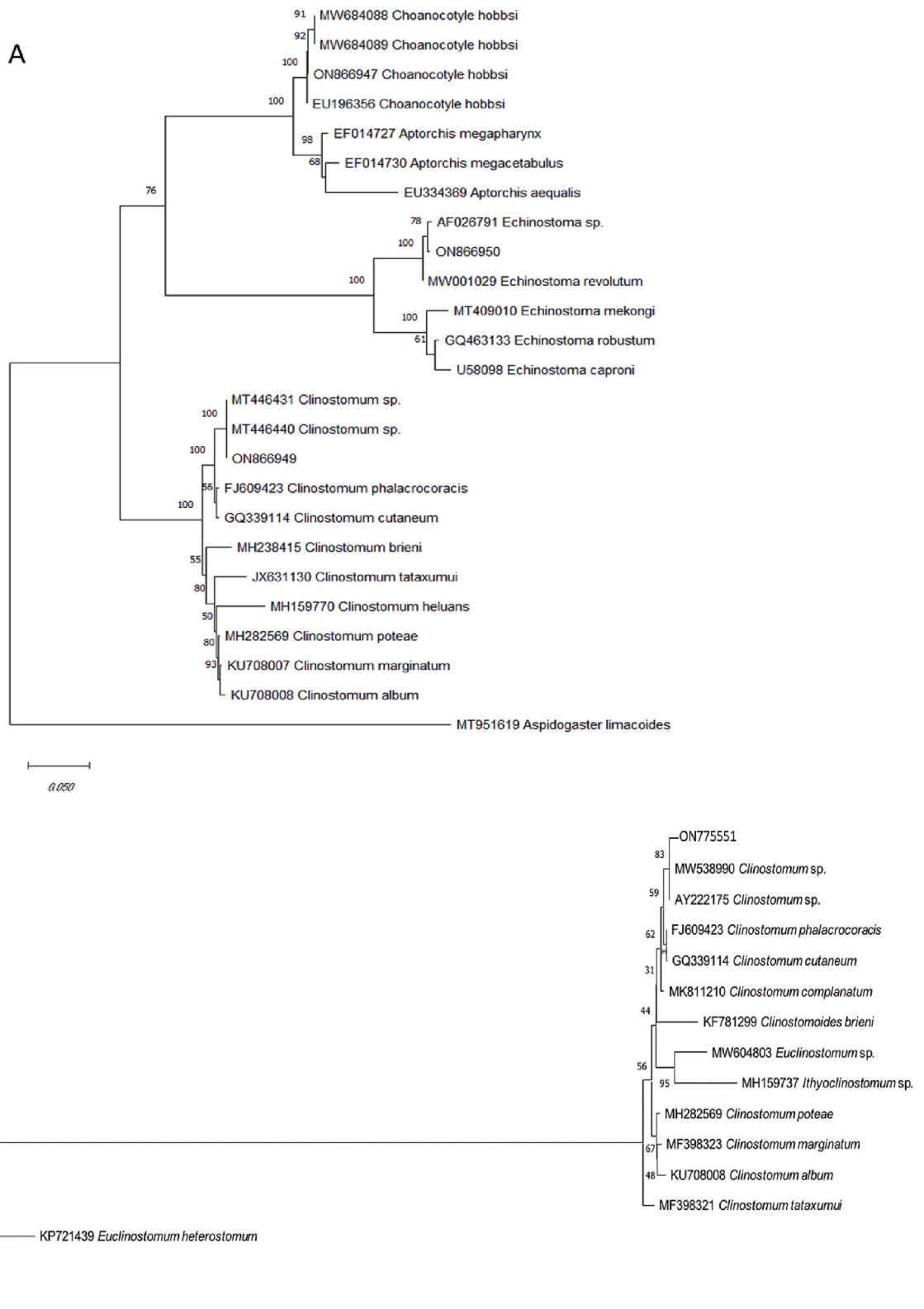


Fig. 2. Maximum Likelihood trees of ITS rRNA sequences of Digenea found in the present study, *Clinostomum* and *Echinostoma* (A), 28S rRNA sequences of Digenea, *Clinostomum* (B), and 18S of Digenea *Clinostomum* (C). Bootstrap support probabilities are listed above branches. GenBank accession numbers produced in this study include ON866949, ON866950, ON775551 and ON775556.

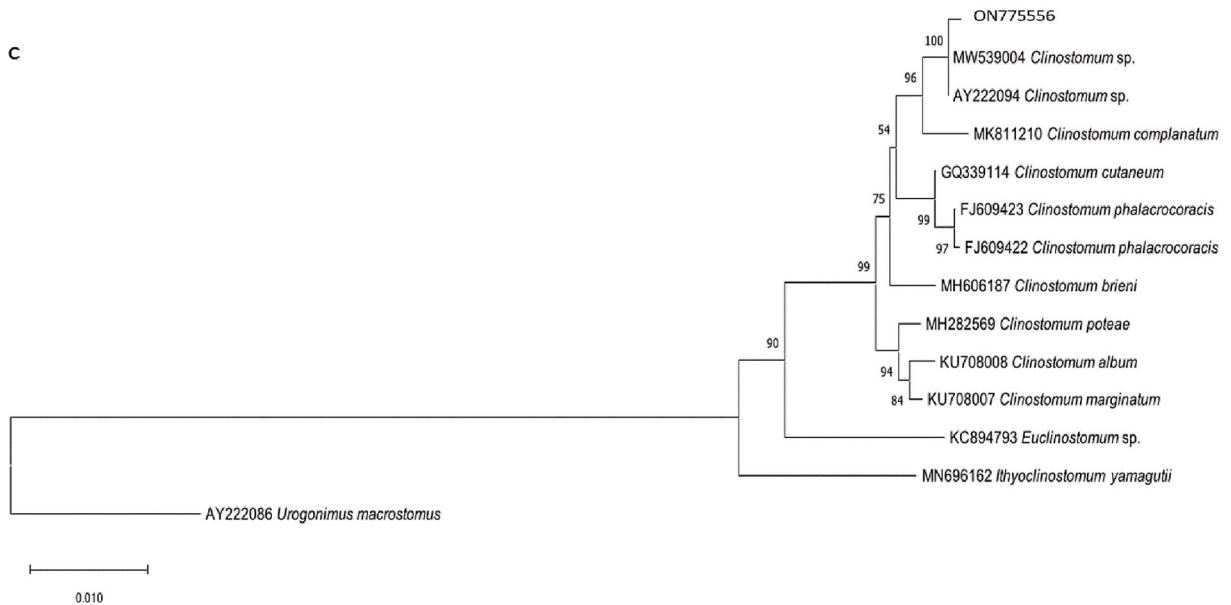


Fig. 2. (continued).

smaller (57–91.1 μ) than morphotype 1 (217.8–324.5 μ).

The ITS sequence data revealed two genetically distinct taxa (Fig. 2), including a distinct sequence in specimens collected from *Isidorella hainesii*, whose closest related species was *Echinostoma revolutum*, with a genetic divergence of 0.003. However, this was not supported by bootstrap probability. Therefore, it will be referred to as *Echinostoma* sp. in this study. Sequences from specimens belonging to morphotype 2 grouped with sequences identified as *Clinostomum* sp. for each of the ITS, 28S and 18S analyses. ITS and 28S regions were a 100% match with previously reported *Clinostomum* sp. in the area.

4. Discussion

This study revealed that freshwater snails in the Murrumbidgee catchment area harbour a diverse range of parasites in their larval stage, indicating the need for further investigation. Despite examining a relatively small sample size, the study demonstrated a high abundance and diversity of parasites, highlighting the potential for more extensive future studies. It is suggested that future research should include a larger sample size collected over an extended period and from a wider range of locations within the study area.

Echinostoma sp. and *Clinostomum* sp., are known zoonotic parasites. Members of the family Echinostomatidae (Poche, 1926) are intestinal parasites of aquatic associated birds and mammals, with over 30 known genera and 200 species (Tkach et al., 2016), approximately 15 of which can infect humans (Chai et al., 2005). Human infection occurs by consuming an infected second intermediate host, such as snails, mussels, tadpoles, frogs, and fish (Bettioli et al., 2003). Echinostomiasis, the disease caused by *Echinostoma* spp., is considered a globally important public health concern. The disease is generally mild, but ulcerations and bleeding in the stomach or duodenum may occur, as in *E. hortense* infection (Chai and Lee, 1991).

In terms of the specific identity of the echinostome found in the present study, the ITS sequence of the cercaria formed a close relationship with species from the family Echinostomatidae, particularly the species *Echinostoma revolutum*, with only 4 base pair difference. In a study by Morgan and Blair (1995) sequences from *Echinostoma revolutum*, *E. friedi*, *E. caproni*, *E. paraensei*, and *E. trivolvis* were classified within this group and utilized to construct a phylogenetic tree of closely related species.

Members of the genus *Echinostoma* are differentiated by the number of collar spines on cercaria, with many studies focused on the 37-collar spined group. Due to high morphological uniformity among species of *Echinostoma*, it is necessary to have information about all stages in the life history for their proper identification and classification (Morgan and Blair, 1995). Unfortunately, due to the low number and poor quality of cercaria in the present study we could not perform a detailed morphological examination.

The cercaria identified as *Clinostomum* sp. in the present study is the first report in Australia. The metacercaria and immature adult of this taxon has been previously reported from cormorants and fish in the same geographical area (Shamsi et al., 2021a,b) and from firetail gudgeon (*Hypseleotris galii*) in the Brisbane area in Queensland, Australia. The life cycle of *Clinostomum* involves multiple hosts. The adult worms reside in the oral cavity or pharynx of various vertebrate hosts, such as birds, reptiles, and mammals, including humans (Miller et al., 2004; Shamsi et al., 2013; Kim et al., 2019; Kanev et al., 2002). Intermediate hosts include snails, and fish and amphibians (Aghmandi et al., 2018; Kanev et al., 2002). The parasites occur as large, often yellow metacercariae in the tissues or body cavity of the second intermediate host. Human infections by adult clinostomids have been reported from several countries, such as Japan, Korea, Thailand and India (Cameron, 1945; Tiewchaloern et al., 1999; Kitagowa et al., 2003; Park et al., 2009) and it is considered as an important parasitic zoonosis for public health (Kagei et al., 1988). In the literature *C. complanatum* is the only species

that has been reported as a causative agent of the human infections. This might be because medical professionals usually identify the parasite based on the assumptions rather than through correct taxonomic identification. In summary, the study highlighted the presence of zoonotic parasites, in freshwater snails in the Murrumbidgee catchment area. It emphasized the importance of conducting further research with larger sample sizes and from a wider range of locations to gain a comprehensive understanding of the parasites in the region.

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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