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Genetic characterisation of cercarial stages of *Choanocotyle* Jue Sue and Platt, 1998 (Digenea: Choanocotylidae) in a native Australian freshwater snail, *Isidorella hainesii* (Tryon)

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ABSTRACT

Isidorella hainesii (Tryon, 1866) is a native freshwater snail, belonging to the family Planorbidae, commonly found on aquatic vegetation in south eastern parts of Australia. In the present study, we report natural infection with a species of *Choanocotyle* Jue Sue and Platt, 1998 (Digenea: Choanocotylidae) parasite in inland Australia for the first time, followed by characterisation of the parasite using both morphological and molecular approaches. Snails (n = 150) were collected from recently drained, natural ponds at a local fish farm located in the Riverina region, New South Wales, Australia. Parasites were subjected to preliminary morphological examination followed by DNA extraction to obtain their ITS-2, 18S and 28S sequences. Based on their sequence data and phylogenetic analyses they were identified as *Choanocotyle hobbsi* Platt and Tkach, 2003, which has only previously been described from *Chelodina oblonga* Gray, 1841 (snake-necked turtle) in Western Australia. Previous researchers suggested that in Australia, C. *oblonga* and its parasite fauna are separated from their eastern counterparts due to formation of impenetrable waterless desert in the country during the late Cretaceous. Our study extends the distribution of *Choanocotyle hobbsi* from Western Australia to the Murray Darling Basin in New South Wales, however, the definitive host remains unknown in New South Wales.

1. Introduction

Parasites belonging to the genus *Choanocotyle* Jue Sue and Platt, 1998 (Digenea: Choanocotylidae) are endemic species in Australia infecting freshwater turtles. Jue Sue and Platt (1998) established the family Choanocotylidae to accommodate two species belonging to the Order Plagiorchiida: *Choanocotyle elegans* Jue Sue and Platt (1998) from the small intestine of the freshwater turtles, *Chelodina expansa* Gray, 1857 and *Emydura macquarii* (Gray, 1830), and *Choanocotyle nematoides* Jue Sue and Platt (1998) from the large intestine of *E. macquarii*. The genus *Choanocotyle* currently comprises 5 species (Table 1).

Like many other aquatic Trematoda, *Choanocotyle* spp. have a three-host life cycle; however, our knowledge on the details of their natural life cycle is limited. Jue Sue and Platt (1998) established the life cycle experimentally and showed that *C. elegans* eggs are fully embryonated, containing a motile miracidium, which were hatched only after being ingested by suitable snail host, *Glyptophysa gibbosa* (Gould, 1846), where they developed to cercariae. In their study, *Glyptophysa gibbosa* was also successfully infected with the eggs of *C. nematoides*, which passed cercariae utilising snails and tadpoles as second intermediate hosts in the laboratory, without infecting fish. They found the metacercariae to naturally infect a glossiphonid leech from Grafton, northern New South Wales. Species of *Glossiphonia* Johnson, 1816 feed on snails and the leech probably became infected by ingesting cercariae from an infected snail. Although large numbers of metacercariae of various ages (recovered from experimentally infected snails and tadpoles) were fed to three laboratory-reared *Chelodina longicollis* (Shaw, 1794), infection did not occur. Jue Sue and Platt (1998) reported that heavy infection with sporocysts has led to death of snails in their experiments.

Isidorella hainesii (Tryon, 1866) is a native freshwater snail, belonging to the family Planorbidae, which is commonly found on aquatic vegetation in ponds, billabongs, swamps and sluggish streams

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and rivers in south eastern part of Australia. In the present study, we report natural infection with a species of *Choanocotyle* parasite in *Isidorella hainesii* in inland Australia for the first time followed by its genetic characterisation using sequences of internal transcribed spacers (ITS), and small and large subunits ribosomal DNA (18S and 28S) regions.

2. Methodology

2.1. Sample collection

Snails (n = 150) were collected from a recently drained, natural pond at a local fish farm located in the Riverina region, New South Wales Australia. Combination of bore water and river water (Murrumbidgee River) was used for the pond which were mainly used to grow golden perch (*Macquaria ambigua,* (Richardson, 1845)), a native Australian fish species. The ponds were with soil bottom and were frequented by cormorants, duck, egrets, and pelicans, with turtles, yabbies, shrimp, small bivalves, water scorpions, and dipteran insects found at the bottom. Collection of snails took place late February–April 2019. The snails were collected in large specimen jars, approximately half-full of water, and were transported to the Parasitology Laboratory of Charles Sturt University. Snails were left in these jars with the lid loosely on (for air-flow), and with a lamp on over them for 12–48hrs. After this time, snails were examined for presence of parasites. Any parasite specimens found were preserved in 70 % ethanol. The best specimens were put on slides, some in lacto-phenol (25 % lactic acid, phenol, water and glycerine, each) and some in glycerine for morphology. The rest of the specimens were kept in 70 % ethanol for later molecular work.

2.2. Parasite study

Specimens were examined for distinguishing features of certain families of trematodes to estimate parasite identification. Where possible, total length (TotL), body length (BL), body width (BW), tail length (TL), tail width (TW), tail width with fins (TWF), oral sucker diameter (OS), ventral sucker diameter (VS), and stylet length (SL) were measured. Illustrations were made using a microscope equipped with a drawing tube. All measurements are given in micrometres, unless otherwise stated. Mean measurements were specified, followed by the range in parenthesis. Photos were taken using a 9 MP Microscope Digital Camera (AmScope Model MU900). To prepare for DNA extraction, specimens were placed in individual Eppendorf tubes and stored at −20 °C until DNA extraction. The samples did not need to be cut, as they were extremely small (<1 mm), and there were a multitude of available samples. DNA extraction was completed using the QIAGEN DNeasy Blood and Tissue Kit, following modified version of the manufacturer's instruction (Shamsi et al., 2017). Three nuclear gene regions namely ITS, 18S rRNA and 28S rRNA were amplified using the primer pairs listed in Table 2 with the following condition: Initial denaturation in 95 °C for 2 min; 40 cycles of denaturation (95 °C for 30 s), annealing for 30 s (please see Table 2 for annealing temperature) and extension (72 °C for 45 s), followed by a final extension in 72 °C for 10 min. PCR products were Sanger sequenced using the same primer at Australian Genome Research Facility (Brisbane). Sequences were aligned using BioEdit (Hall, 1999). Primer sequences were removed from analysis. Sequences of closely related taxa reported from definitive hosts, including fish, amphibians and reptiles (Iwaki et al., 2020; Kasl et al., 2014, 2018; Olson et al., 2003; Platt and Tkach, 2003; Pulis et al., 2011; Svinin et al., 2019; Tkach and Mills, 2011; Tkach et al., 2000, 2001; Tkach and Snyder, 2007) were obtained from GenBank for phylogenetic analyses (Supplementary Table 1). Alignment gaps were excluded for analyses. Pairwise genetic distances were calculated using MEGA X (Kumar et al., 2018). The GTR + G, GTR + I and HKY + I models were selected as best fit evolutionary models for ITS, 28S rRNA and 18S rRNA sequences, respectively, as inferred by jModelTest 2 (Darriba et al., 2012). *Brachycladium goliath* (KR703279, which includes all three gene sequences) was used as outgroup in the phylogenetic analyses. The phylogeny of selected sequences was calculated using MrBayes 3.2 for 2,000,000 generations until the average standard deviation is lower than 0.005. The tree was visualized using Figtree v 1.4.3 (Rambaut, 2014).

3. Results

Of the 150 *Isidorella hainesii* snails examined, 11 were infected by cercaria with the distinguishing characteristics of a stylet protruding from the oral sucker, a slightly larger oral sucker than ventral sucker, a short tail relative to body length and small tegumental spines covering the body of the cercaria (Fig. 1). Measurements are based on 11 samples mounted in glycerine and are presented in Table 3 in comparison to cercaria reported by Jue Sue and Platt (1998) obtained from experimental infections. *Description.* Tegument thick, armed with small spines extending to posterior end. Tail simple, slender, shorter than body. Oral sucker large, incised ventrally. Mouth ventral. Stylet antero-dorsal in oral sucker, with thickened cuff in distal third; dorsally curved in lateral view. Cercaria not examined alive, thus patterns of the excretory system and penetration glands could not be ascertained. Dark "patchy" area anterior to ventral sucker present, corresponding in location to penetration glands as described in Jue Sue and Platt (1998). Possible female genital rudiment immediately posterior to ventral sucker.

Sequences of the ITS, 18S and 28S regions for both cercariae and metacercaria were obtained and deposited in the GenBank (Supplementary Table 1 accession numbers: MW684083-9, MW686389-93 and MW682817-22). Alignment of our sequences with closely related species in the GenBank resulted in an alignment of 1318, 1215 and 1770bp for ITS, 28S rRNA and 18S rRNA regions, respectively. All three genes regions were identical for all our specimens. For the 18S region, our specimens had identical sequences with adult *C. hobbsi* reported from turtles, whereas for ITS and 28S regions, there was only 1 bp difference with adult *C. hobbsi*. Phylogenetic tree, using Bayesian inference, clustered our specimens into a single highly supported clade with *C. hobbsi* in all three gene regions, separate from other *Choanocotyle* species

Table 1
Previous reports of the taxa belonging to the family Choanocotylidae in Australia.

Parasite	Host	Host type	Stage	Locality	Reference
Auriculotrema lechneri	*Elseya latisternum*	Turtles	Adult	Queensland, Australia	Platt (2003)
	Emydura krefftii	Turtles	Adult	Queensland, Australia	Platt (2003)
C. elegans	*Emydura macquarii*	Turtles	Adult	Queensland, Australia	Jue Sue and Platt (1998)
	Bufo marinus	Toad	Metacercaria	Queensland, Australia	Jue Sue and Platt (1998)
	Chelodina expansa	Turtles	Adult	Queensland, Australia	Jue Sue and Platt (1998)
	Cherax sp.	Crayfish	Metacercaria	Queensland, Australia	Jue Sue and Platt (1998)
	Glyptophysa gibbosa	Snail	Larval	Queensland, Australia	Jue Sue and Platt (1998)
C. hobbsi	*Chelodina rugosa* (syn. *oblanga*)	Turtles	Adult	Western Australia	Platt and Tkach, 2003a
C. juesuei	*Chelodina rugosa* (syn. *oblanga*)	Turtles	Adult	Western Australia	Platt and Tkach (2003)
C. nematoides	*Emydura macquarii*	Turtles	Adult	Queensland, Australia	Jue Sue and Platt (1998)
C. platti	*Chelodina rugosa*	Turtles	Adult	Northern Territory, Australia	Tkach and Snyder (2007a)

Table 2
- Details of primers used in this study for PCR amplification and sequencing.

Targeted gene	Direction	Forward primer name	Forward primer sequence (5′-3′)	Annealing temperature	References
ITS	F	D1 (F)	AGGAATTCCTGGTAAGTGCAAG	58	Hillis and Dixon (1991)
ITS	R	D2 (R)	CGTTACTGAGGGAATCCTGG	58	Hillis and Dixon (1991)
ITS	F	18SDigenea-F1	GTCGTAACAAGGTTTCCGTAGG	58	Present study
ITS	R	28SDigenea-R1	GTGATATGCTTAAGTTCAGCGG	58	Present study
18S rRNA	F	WormA	GCGAATGGCTCATTAAATCAG	55	Littlewood and Olson (2001)
18S rRNA	R	WormB	CTTGTTACGACTTTTACTTCC	55	Littlewood and Olson (2001)
28S rRNA	F	LSU-5m	TAGGTCGACCCGCTGAAYTTAAGCA	50	Olson et al. (2003)
28S rRNA	R	1500Rm	GCTATCCTGAGGGAAACTTCG	50	Olson et al. (2003)

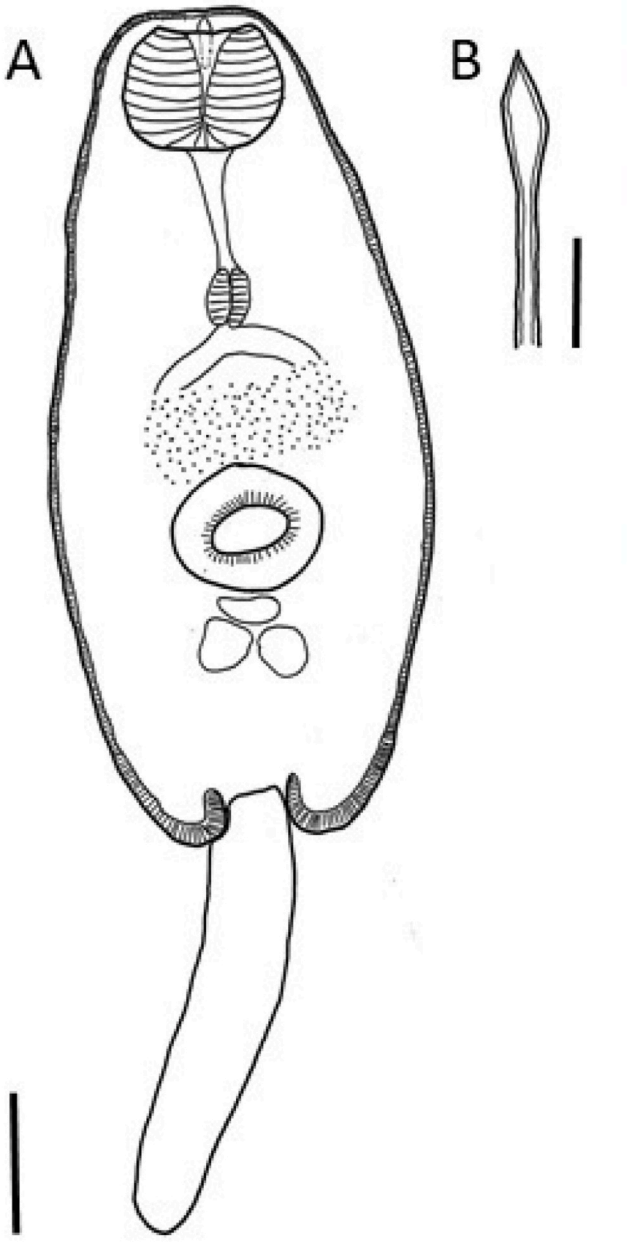

Fig. 1. Cercaria of *Choanocotyle hobbsii*. A. Ventral view of whole mount. Scale bar 100 μm. B. Stylet. Scale bar 10 μm.

Table 3
Measurements of cercaria of *Choanoctyle* species.

Characteristic	*C. hobbsi*	*C. elegans*	*C. nematoides*
Reference	This study	Jue Sue and Platt (1998)	Jue Sue and Platt (1998)
Host	*Isidorella hainesii* (natural infection)	*Glyptophysa gibbosa* (experimental infection)	*Glyptophysa gibbosa* (experimental infection)
Body Length	338.6 (265–410)	423 (396–460)	251 (223–281)
Body Width	144.1 (85–200)	113 (104–117)	81 (71–92)
Tail Length	242 (220–260)	317 (288–347)	175 (161–184)
Tail Width	38.1 (32.5–52.5)	37 (34–39)	–
Oral Sucker Diameter	73.4 (65–95)	70 (66–77)	52 (49–53)
Stylet Length	25.4 (20–35)	37 (36–38)	26 (25–27)
Ventral Sucker Diameter	63.3 (57.5–72.5)	63 (58–66)	40 (33–42)

(Supplementary Fig. 1), suggesting our specimens belong to *C. hobbsi*.

4. Discussion

Choanocotyle hobbsi has only previously been described from *Chelodina oblonga* in Western Australia (Platt and Tkach, 2003). Our study extends the distribution of the parasite from Western Australia to the Murray Darling Basin in New South Wales. The adult fluke of *C. hobbsi* has been described in *Chelodina oblonga* (Gray, 1841), a freshwater turtle endemic to the south-west of Australia, in Melaleuca swamp near Perth (Platt and Tkach, 2003). The definitive host remains unknown in New South Wales, however is likely to be a freshwater turtle of similar anatomy and physiology to *C. oblonga* as was found in Western Australia. Three species of turtle are found throughout the Murray Darling Basin (including in the ponds where snails were collected): *Chelodina longicollis*, *C. expansa* and *Emydura macquarii macquarii* (Cann, 1998), the latter two of which have been reported as hosts for other *Choanoctyle* species in eastern Australia (Table 1).

Platt and Tkach (2003) suggested *C. oblonga* and its parasite fauna have been separated from their eastern counterparts for well over 100 million years, resulting in speciation and providing ample time for the accumulation of genetic and morphological differences in both host and parasite lineages. This was based on a review by Manning (1996) who suggested that southwestern Australia became isolated from the eastern portion of the continent by an inland sea during the early Cretaceous, which then was subsided during the late Cretaceous forming "waterless desert equally impenetrable''.

This is also the first study providing measurements, description, images and drawings for the cercarial stage of this species. Although cercaria have been described for *C. elegans* and *C. nematoides*, none have previously been described for either *C. hobbsii* or *C. juesuei*. Measurements of the cercaria collected in this study were intermediate between *C. elegans* and *C. nematoides* for overall body and tail dimensions. The stylet measurements for *C. hobbsii* in this study were closer to those reported for *C. nematoides*. Unfortunately, the cercaria collected in this

study were not examined alive so the patterns of the excretory system and penetration glands could not be ascertained. Additionally, cercaria were dissected from the snail, rather than after release, which may have influenced the range of measurements recorded. Future work should include study of live free-swimming cercaria to enable a more accurate description.

In experimental infections conducted by Jue Sue and Platt (1998), *C. elegans* infected *Glyptophysa gibbosa*, but not *Austropeplea lessoni* or *Glyptophysa* sp.; metacercaria were found in naturally infected *Glyptophysa* spp., including *G. gibbosa*. Similarly, *C. nematoides* infected *G. gibbosa* but not *A. lessoni* nor *Glyptophysa* sp. *Choanoctyle elegans* were found to apparently naturally infect *Isidorella newcombi* (mentioned in abstract by Jue Sue and Platt, 1998; but no details provided in the text of the paper).

In this study, *Choanaoctyle* cercariae were found in *Isidorella hainesii* (Planorbidae) in aquaculture ponds. Although Jue Sue and Platt (1998) stated that *I. newcombi* was infected with *C. elegans*, the distribution of this species is more inland, in arid to semi-arid areas; *I. hainessi*, on the other hand, is found along the coastal edge of southern Queensland and NSW (Ponder et al. 2016) and is more likely to be the snail host studied by Jue Sue and Platt (1998). If this is the case, then this study confirms the presence of *Choanaoctyle* cercaria in naturally infected *Isidorella hainessi* specimens.

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Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the conclusions of this article are included in the article and its additional files. Raw data are available upon request to the first author.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

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