

RESEARCH NOTE

First molecular identification of *Ascocotyle (Phagicola) longa* in its first intermediate host the mud snail *Heleobia australis*

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Abstract

This is the first study that used species-specific DNA primers to confirm the presence of the heterophyid *Ascocotyle (Phagicola) longa* Ransom, 1920 in its first intermediate host. The larval stages (rediae and cercariae) of this parasite were morphologically and genetically identified in the gonad of the intertidal mud snail *Heleobia australis* (d'Orbigny, 1835) (Cochliopidae) in the Bahía Blanca estuary, Argentina. In addition, we asked whether the prevalence in *H. australis* varied between seasons. Mulletts – the second intermediate host of this heterophyid – migrate in estuaries during the warmer seasons and it is expected that piscivorous birds and mammals – the definitive hosts – prey more intensively on this species at those times. Thus, the number of parasite eggs released into the tidal flat within their feces should be higher, thereby increasing the ingestion of the parasite by *H. australis*. We therefore expected a higher prevalence of *A. (P.) longa* in *H. australis* in the Bahía Blanca estuary during spring and summer than autumn and winter. We found that 16 out of 2,744 specimens of *H. australis* had been infected with *A. (P.) longa* (total prevalence of 0.58%). Nonetheless, the prevalence showed no significant variation between seasons. Hence, we discuss an alternative scenario where the lack of seasonal changes might be mostly related to the permanent residence of definitive hosts in the estuary and not to the seasonal recruitment of mulletts. Finally, we highlight the need for more experimental and comparative approaches in order to understand the diagnosis and geographical distribution of this worldwide heterophyid.

Keywords

Trematode, heterophyiasis, parasite distribution, Cochliopidae, Bahía Blanca estuary, Argentina

Introduction

The trematode *Ascocotyle (Phagicola) longa* Ransom, 1920 is a common parasite of a wide spectrum of fish-eating birds and mammals, with a few reports of its occurrence in humans (Hung *et al.* 2013) who can get infected by the consumption of raw mullet (Martorelli *et al.* 2012). This parasite is widespread in Asia, Africa, Europe, and America (Hung *et al.* 2013). The distribution of this heterophyid in South America includes reports from Rio de Janeiro, Brazil, to Bahía Blanca estuary, Argentina (Fig. 1) with mollusks, mulletts, birds, and

marine mammals as hosts (Chieffi *et al.* 1990; Etchegoin 1997; Antunes Barros *et al.* 2002; Carnevia *et al.* 2004; Morgades *et al.* 2006; Simões *et al.* 2010; Martorelli *et al.* 2012; Brandão *et al.* 2013; Pereira *et al.* 2013; Portes Santos *et al.* 2013; Alda and Martorelli 2014). The complete life cycle of *A. (P.) longa* has been described in the Rodrigo de Freitas Lagoon in Rio de Janeiro where the cochliopid mud snail *Heleobia australis* (d'Orbigny, 1835) serves as the first and the mullet *Mugil liza* Valenciennes, 1836 as the second intermediate hosts (Simões *et al.* 2010).

The available information on the first intermediate host deals with only the cercariae and rediae of *A. (P.) longa* also

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found in *H. australis* from Argentina but they were identified based on morphological characteristics only (Etchegoin 1997; Carnevia *et al.* 2004; Simões *et al.* 2010; Alda and Martorelli 2014). Since more species of heterophyid trematodes may occur in the same snail, in the present study we applied molecular markers to confirm the occurrence of larval stages of *A. (P.) longa* in *H. australis* from the Bahía Blanca estuary, Argentina. In addition, we compared the seasonal patterns in prevalence in the first intermediate host. In view of the known increase in the abundance of the mullet *M. liza* in estuaries during the warmer seasons (González-Castro *et al.* 2011), piscivorous birds and mammals would prey more intensively on this species at those times. Hence, the number of parasite eggs released into the tidal flat within bird and mammal feces should be higher, thereby increasing the ingestion of the parasite by *H. australis*. In this context, we expected that the prevalence of *A. (P.) longa* in *H. australis* would be higher in the spring-summer period than during the autumn-winter months.

Materials and Methods

We collected snails in 2012 during late summer (March, $n = 691$), autumn (July, $n = 378$), winter (September, $n = 1,088$), and spring (December, $n = 587$) from a tidal flat located close to the mouth of the Bahía Blanca estuary, Argentina ($38^{\circ}51'S$; $62^{\circ}07'W$; Fig. 1). On each sampling date, we took nine replicates from puddles using core samples (10 cm diameter and 2 cm deep) during low tide. The snails obtained were transported to the laboratory, kept alive in aquaria with water from the estuary, and dissected within the following 20 days.

To morphologically identify larval trematodes, we followed the histological, staining, and drawing procedure fully described by Alda and Martorelli (2014). Snail tissue infected with *A. (P.) longa* was preserved in 96% (v/v) ethanol at $-20^{\circ}C$ for molecular-genetic identification. In addition, we considered four infected snails for molecular analysis. From each snail, we collected four mature rediae ($n = 4$) that were pipetted into a 1.5 ml microtube. Rediae from each individual host were considered a clone derived from a single miracidium. The rediae were placed in a Speedvac evaporator centrifuge for drying until total ethanol evaporation. DNA extraction, PCR, purification, and sequencing were done as previously described by Martorelli *et al.* (2012). We used species-specific primers provided by Dzikowski *et al.* (2004) to amplify a fragment of the ribosomal 18S-DNA gene of *A. (P.) longa*: Pha1463F (5' ACT CGT GCG GGT GGC GGT ATT CT 3') and Het1824R (5' AAT CGG TAG TAG CGA CGG GCG GT 3'). Sequences obtained were built and analyzed with BIOEDIT. We then compared the sequences obtained with those already available in GenBank (Dzikowski *et al.* 2004, Martorelli *et al.* 2012) using BLAST. We built a phylogenetic tree between the sequences from *A. (P.) longa* and five other heterophyid species (Dzikowski *et al.* 2004; Martorelli *et al.* 2012) by the Neighbor Joining method using MEGA 4 (Tamura *et al.* 2007). Finally, to analyze seasonal variations in prevalence, we fitted a generalized linear model with binomial errors, a logit link, and an overdispersion parameter, considering infected snails and the total number of snails examined as the response variables and the seasons as the explanatory variable. The statistical analysis was performed by means of the JMP statistical software (v11.2 SAS Institute).

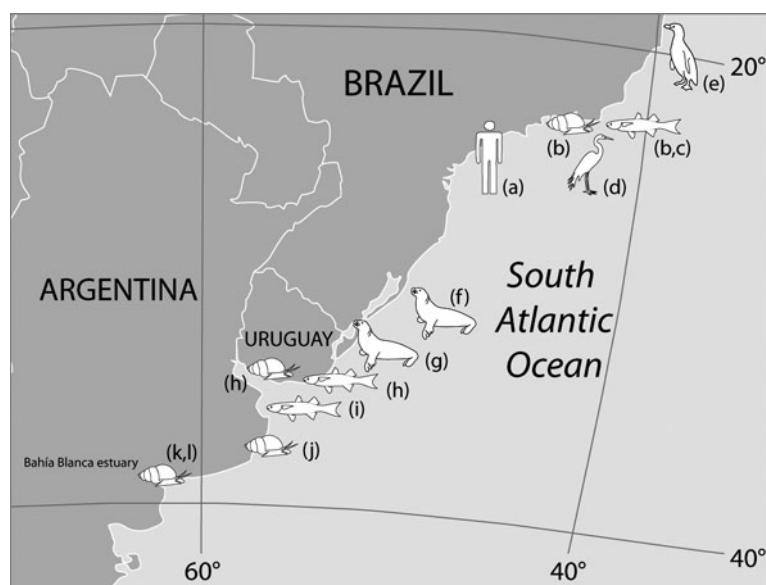


Fig. 1. Reports of *Ascocotyle (Phagicola) longa* in Brazil: a Chieffi *et al.* (1990), b. Simões *et al.* (2010), c. Portes Santos *et al.* (2013), d. Antunes Barros *et al.* (2002), e. Brandão *et al.*, 2013, f. Pereira *et al.* (2013); in Uruguay: g. Morgades *et al.* (2006), h. Carnevia *et al.* (2004); and in Argentina: i. Martorelli *et al.* (2012), j. Etchegoin (1997), k. Alda and Martorelli (2014), l. the present study

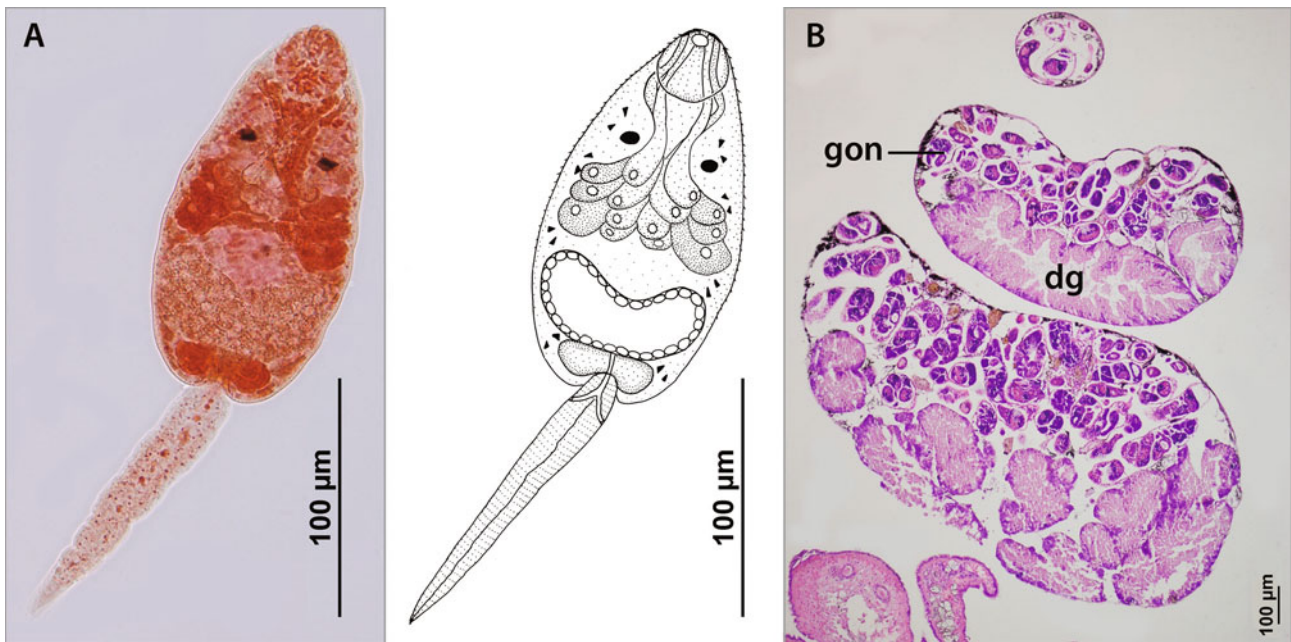


Fig. 2. *Ascocotyle (Phagicola) longa*. A) cercaria, B) rediae parasitizing the gonad of *Heleobia australis*

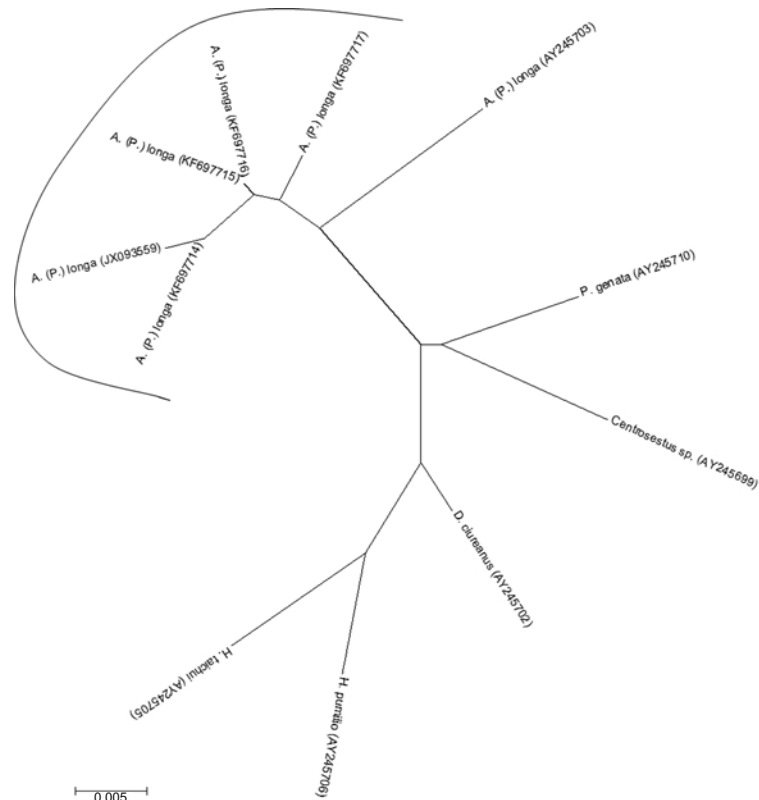


Fig. 3. Phylogenetic tree of 18S-rDNA sequences available in GenBank from *Ascocotyle (Phagicola) longa* and five other heterophyid species: (i) *Centrocestus* sp. (Looss, 1989), (ii) *Haplorchis pumilio* (Looss, 1896), (iii) *Haplorchis taichui* (Nishigori, 1924), (iv) *Dexiogenimus ciureanus* Witenberg, 1929, and (v) *Pygidiopsis genata* Looss, 1907. References in the figure indicate species' name and the accession number in GenBank in parentheses. Those sequences from *A. (P.) longa* from Argentina clustered together and are enclosed in a semicircle

Results

We found *A. (P.) longa* parasitizing the gonad of 16 of 2,744 specimens of *H. australis* (total prevalence of 0.58%) from the Bahía Blanca estuary. The morphologic features of the redia and cercaria (Fig. 2) were consistent with the characteristics of *A. (P.) longa* described by Simões *et al.* (2010) and Alda and Martorelli (2014). The PCR yielded four fragments of the 18S-rDNA gene of 360 base pairs, whose four sequences were uploaded to GenBank (accession numbers: KF697714, KF697715, KF697716, and KF697717). These sequences had a 98% identity with the individual of *A. (P.) longa* collected from *Phalacrocorax carbo* (Linnaeus, 1758) in Israel (accession number: AY245703) and 98–99% identity with the individual of *A. (P.) longa* collected from *M. liza* at Samborombón bay, Argentina (accession number: JX093559). Based on the phylogenetic tree, all the sequences from *A. (P.) longa* clustered together showing a minimum value of distance (Fig. 3)

The mean prevalence (\pm standard error, number of replicates = 9) for summer was $0.67 \pm 0.35\%$, for autumn $0.29 \pm 0.19\%$, for winter $0.49 \pm 0.27\%$, and for spring $0.27 \pm 0.27\%$. The low number of infected snails in each replicate accounted for the large standard errors estimated. The general-linear-model (GLM) test indicated no significant differences in the prevalence of *A. (P.) longa* between the different seasons (GLM: binomial errors, $X^2_{(3)} = 1.386$, $P = 0.7089$).

Discussion

The work reported here has confirmed the presence of the fish-borne trematode *A. (P.) longa* in the Bahía Blanca estuary – the southernmost record of the species – in the first intermediate host *H. australis* by means of both morphological and molecular-genetic data. Our results demonstrate that four rediae contain enough DNA for amplification and sequencing in order to identify the intramolluscal stage of *A. (P.) longa* genetically. The sequences amplified were similar to those obtained from adults present in birds in Israel (Dzikowski *et al.* 2004) and from metacercariae in mullets in Argentina (Martorelli *et al.* 2012). They also clustered together in the phylogenetic tree (Fig. 3). In general, the ribosomal 18S-DNA gene is an appropriate molecular marker for phylogenetic studies of digeneans families (Olson *et al.* 2003) and has been applied to interspecies analyses of some heterophyids – as has been done here. Nonetheless, we think that a multigene analysis – that is, one based on different genetic markers for the individuals under study, such as *A. (P.) longa*, from different sites worldwide – would enable a better understanding of the genetic polymorphism of this species, or maybe species complex, and the potential risk of zoonosis by these trematodes around the world.

The prevalence of *A. (P.) longa* in its first intermediate host *H. australis* exhibited no significant differences between

seasons. This result suggest that this lack of seasonal variation in prevalence might be associated to other aspects of the trematode's life cycle and not strictly related to mullet's recruitment in estuaries and coastal lagoons as we stated initially. The piscivorous birds *Ardea cocoi* Linnaeus, 1766 and *Spheniscus magellanicus* (Forster, 1781) and marine mammals *Otaria flavescens* Shaw, 1800 play the role of definitive host of *A. (P.) longa* in Brazil and Uruguay (Antunes Barros *et al.* 2002; Morgades *et al.* 2006; Brandão *et al.* 2013; Pereira *et al.* 2013; *cf.* Fig. 1). Unfortunately, no such studies regarding the definitive host in Argentina have been reported. Nevertheless, we suggest that the definitive host of *A. (P.) longa* in the Bahía Blanca estuary would most likely be the birds – herons, egrets, skimmers, terns, and cormorants (Pablo Petracci, personal communication) – and/or that species of the sea lion (*O. flavescens*) that eat mullets. All of the above-mentioned species are permanent residents of the estuary (Petracci and Delhey 2005; Petracci *et al.* 2010). These possible definitive-host species might generate a continuous year-round input of eggs of *A. (P.) longa* into the environment masking to some extent the expected seasonal differences in the prevalence in *H. australis*. We believe that this alternative scenario might prevail over the one that focuses on seasonal migration of mullets. Nevertheless, the feasibility of both scenarios needs to be more closely examined in future studies to gain a significant insight into the host range of this parasite.

In summary, our results definitively confirm the existence of the fish-borne trematode *A. (P.) longa* in the Bahía Blanca estuary and highlight that we are only just beginning to understand the diagnosis and geographical distribution of this worldwide heterophyid trematode.

Acknowledgements. We thank anonymous reviewers for their helpful comments that significantly improved this work. We also thank Drs. Andrea López Cazorla and Mariano González Castro for contributing information on the ecology and biology of mullets and Pablo Petracci for providing information regarding the feeding biology of sea birds in the Bahía Blanca estuary. We also thank Tim Janicke and Julio Lorda for statistical support. This work was partially funded by a research grant from the Agencia Nacional de Promoción Científica y Técnica (PICT 34412/05) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PID 0257) to SRM. AP, CJP, and SRM are staff researchers in CONICET; NB is a postdoctoral fellow in CONICET; and NJC is a staff researcher in the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Argentina. Dr. Donald F. Haggerty, a retired career investigator and native English speaker, edited the final version of the manuscript.

References

- Alda P., Martorelli S. 2014. Larval trematodes infecting the South American intertidal mud snail *Heleobia australis* (Rissooidea: Cochliopidae). *Acta Parasitologica*, 59, 50–67
- Antunes Barros L., Santos de Arruda V., Corrêa Gomes D., Magalhães Pinto R. 2002. First natural infection by *Ascocotyle (Phagicola) longa* Ransom (Digenea, Heterophyidae) in an avian host, *Ardea cocoi* Linnaeus (Aves, Ciconiiformes, Ardeidae) in Brazil. *Revista Brasileira de Zoologia*, 19, 151–155

- Brandão M., Luque J.L., Scholz T., Kostadinova A. 2013. New records and descriptions of digeneans from the Magellanic penguin *Spheniscus magellanicus* (Forster) (Aves: Sphenisciformes) on the coast of Brazil. *Systematic Parasitology*, 85, 79–98
- Carnevia D., Perreta A., Venzal J.M., Castro O. 2004. *Heleobia australis* (Mollusca, Hydrobiidae) y *Mugil platamus* (Pisces, Mugilidae), primer y segundo hospedador intermediario de *Ascocotyle (Phagicola) longa* (Digenea, Heterophyidae) en Uruguay. *Revista Brasileira de Parasitologia Veterinária*, 13, 283
- Chieffi P.P., Leite O.H., Dias R.M.D., Torres D.M.A. V., Mangini A. C.S. 1990. Human parasitism by *Phagicola* sp. (Trematoda, Heterophyidae) in Cananésia, São Paulo State, Brazil. *Revista do Instituto Medicina Tropical de São Paulo*, 32, 285–288. DOI: 10.1590/S0036-46651990000400008
- Dzikowski R., Levy M.G., Poore M.F., Flowers J.R., Paperna I. 2004. Use of rDNA polymorphism for identification of Heterophyidae infecting freshwater fishes. *Diseases of Aquatic Organisms*, 59, 35–41
- Etchegoin J.A. 1997. Sistemas parasitarios presentes en la albufera Mar Chiquita. PhD Thesis, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Argentina
- González-Castro M., Macchi G.J., Cousseau M.B. 2011. Studies on reproduction of the mullet *Mugil platamus* Günther, 1880 (Actinopterygii, Mugilidae) from the Mar Chiquita coastal lagoon, Argentina: Similarities and differences with related species. *Italian Journal of Zoology*, 78, 343–353
- Hung N.M., Madsen H., Fried B. 2013. Global status of fish-borne zoonotic trematodiasis in humans. *Acta Parasitologica*, 58, 231–258
- Martorelli S.R., Lino A., Marcotegui P., Montes M., Alda P., Panei C. J. 2012. Morphological and molecular identification of the fish-borne metacercaria of *Ascocotyle (Phagicola) longa* Ransom, 1920 in *Mugil liza* from Argentina. *Veterinary Parasitology*, 190, 599–603
- Morgades D., Katz H., Castro O., Capellino D., Casas L., Benítez G., Venzal J. M., Morana A. 2006. Fauna parasitaria del lobo fino *Arctocaphalus australis* y del león marino *Otaria flavescens* (Mammalia, Otariidae) en la costa uruguaya. In: (Eds. R. Menafrá, L. Rodríguez-Gallego, F. Scarabino and D. Conde) Bases para la conservación y el manejo de la costa uruguaya. Vida Silvestre, Uruguay, 89–96
- Olson P.D., Cribb T.H., Tkach V.V., Bray R.A., Littlewood D.T.J. 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *International Journal of Parasitology*, 33, 733–755
- Pereira E.M., Müller G., Secchi E., Pereira Jr.J., Valente A.L.S. 2013. Digenetic trematodes in South American Sea Lions from Southern Brazilian Waters. *Journal of Parasitology*, 99, 910–913
- Petracci P.F., Delhey K. 2005. Guía de Aves Marinas y Costeras de la ría de Bahía Blanca. Asociación Industrial Química, Bahía Blanca, 96 pp
- Petracci P.F., Sotelo M., Massola V., Carrizo M., Scorolli A., Zalba S., Delhey V. 2010. Actualización sobre el estado del apostadero de lobo marino de un pelo sudamericano (*Otaria flavescens*) en la Isla Trinidad, estuario de Bahía Blanca, Argentina. *Mastozoología Neotropical*, 17, 175–182
- Portes Santos C., Corrêa Lopes K., da Silva Costa V., Nunes dos Santos E. G. 2013. Fish-borne trematodosis: Potential risk of infection by *Ascocotyle (Phagicola) longa* (Heterophyidae). *Veterinary Parasitology*, 193, 302–306
- Simões S.B.E., Barbosa H.S., Portes Santos C. 2010. The life cycle of *Ascocotyle (Phagicola) longa* (Digenea: Heterophyidae), a causative agent of fish-borne trematodosis. *Acta Tropica*, 113, 226–233
- Tamura K., Dudley J., Nei M., Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24, 1596–1599

Received: February 9, 2015

Revised: March 20, 2015

Accepted for publication: April 29, 2015