An Acad Bras Cienc (2021) 93(3): e20191017 DOI 10.1590/0001-3765202120191017
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ECOSYSTEMS

The first record of *Centrocestus formosanus* (Trematoda: Heterophyidae) in southern Brazil supported by molecular data

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Abstract: *Centrocestus formosanus* is a digenean parasite first described from Asia, which parasitizes *Melanoides tuberculata* (Gastropoda: Thiaridae) at the first intermediate stage, and different fish species as second intermediate host. *C. formosanus* was previously recorded in Brazilian states, but never before in the southern region of this country. Recording and identification of digenean species through morphological identification is a taxonomic challenge. In light of this, we use an integrative taxonomic approach to report the occurrence of cercariae and metacercariae of *C. formosanus* in molluscs and fish, respectively, in an urban park located in southern Brazil. Specimens of *M. tuberculata* and the fishes *Poecilia reticulata* and *Xiphophorus* sp. (Poeciliidae) were collected for screening for parasites and molecular analyses using partial fragments of the 28S rDNA gene. The identification of *C. formosanus* obtained from molluscs and fish specimens permitted us to partially solve the life cycle of this parasite for the first time in the studied environment, demonstrating the necessity of monitoring and controlling molluscs populations. Nevertheless, our results will support future studies aiming to elucidate the life-cycle of *C. formosanus* in this region, since all sampled hosts' species are invasive in this environment.

Key words: Digenea, zoonotic potential, integrative taxonomy, 28S rDNA, Thiaridae.

INTRODUCTION

Melanoides tuberculata (Müller, 1774) (Gastropoda: Thiaridae) is a freshwater gastropod occurring in lentic and lotic environments in Asia and East Africa (Cheng & Malek 1974, Paula-Andrade et al. 2012). In Brazil, this species was introduced in the 1960s, and was first recorded in Santos city, São Paulo State (Vaz et al. 1986), and since then in other regions of this country (Fernandez et al. 2003, Coelho et al. 2018).

Melanoides tuberculata is recognized as an intermediate host of at least 37 species of trematodes around the world, 11 of which have zoonotic potential (Ben-Ami & Heller 2005, Pinto & Melo 2011). In the Americas, four species of trematodes were identified in *M. tuberculata*: *Centrocestus formosanus* (Nishigori, 1924) (Heterophyidae), *Haplorchis pumilio* (Looss, 1896) (Heterophyidae), *Philophthalmus gralli* Mathis & Leger, 1910 (Philophthalmidae) and *Renicola* sp. Cohn 1904 (Renicolidae) (Pinto & Melo 2011, 2013). Among these, *C. formosanus* has a wide distribution, recorded from many countries, including India, Japan, China, Mexico, Italy, Colombia, Croatia, Thailand, Costa Rica, United States, Vietnam, Lao People's Democratic Republic, Turkey, Philippines and Egypt (Komiya & Suzuki 1966, Rekharani & Madhavi 1985, Chen et al. 1991, Tampieri et al. 1999, Salgado-Maldonado et al. 2005, Mitchell et al. 2005, Velásquez et al. 2006, Gjurčević et al. 2007, Han et al. 2008, Chuboon & Wongsawad 2009, Cortés et al. 2009, De & Le 2011, Chai et al. 2013, McDermott et al. 2015, Yousif et al. 2016).

In Brazil, *C. formosanus* was recorded in molluscs in Minas Gerais, Rio Grande do Norte, Brasília and Rio de Janeiro (Pinto & Melo 2010, 2012b, Paula-Andrade et al. 2012, Ximenes et al. 2016). Studies carried out in Goiás (Pinto et al. 2013) and Rio de Janeiro States (Thiengo et al. 2001, Boaventura et al. 2002, Bogéa et al. 2005) reported only the occurrence of the larval stage identified as pleurolophocercous cercariae, and it was not possible to identify these to species level. Pinto & Melo (2010), Paula-Andrade et al. (2012), and Ximenes et al. (2016) carried out experimental infections for the study of the life cycle. Additionally, other studies contributed to understanding the damages caused by *C. formasanus* to its definitive hosts, mainly related to the immune system and susceptibility to worms (Chen 1942, Mati et al. 2013).

These parasites have molluscs as their first intermediate host, and fish or amphibians as second intermediate host (Han et al. 2008). The definitive host varies from piscivorous birds to mammalian species, as well as humans, presenting zoonotic potential (Chen 1942, Pinto & Melo 2010). In different hosts, *C. formosanus* is characterized morphologically by a double crown of acicular spines composed of 26-36 spines in the oral suction cup (Pinto & Melo 2010, Yousif et al. 2016), and the identification of species belonging to *Centrocestus* depends on the number of spines on the oral sucker (Yousif et al. 2016).

Records about the occurrence of a parasite in a region represent a taxonomic challenge while solving its life cycle and stages are important for public health policies (Chen 1942, Esch et al. 2002). Using only the traditional taxonomy can be controversial since many morphological characters could be subjective because of sampling methods, coloration techniques, and variation of morphological structures because of methods used during microscope slide preparation. The use of molecular data only can be challenging since the DNA extraction can be difficult, as well as tissue obtention, and some groups present a small amount of molecular data available in public databases. Thus, the use of morphological and molecular data allows a more assertive identification considering an integrative taxonomy approach (Perkins et al. 2011, Chen et al. 2011). In this sense, the molecular characterization can be helpful during the identification of different groups of organisms, including digeneans, that are difficult to characterize morphologically during intermediate life stages (Pavanelli et al. 2002, Olson & Tkach 2005).

This study aimed to record the occurrence of *Centrocestus formosanus* using morphological and molecular analyses, investigating possible intermediate hosts obtained in an urban park.

MATERIALS AND METHODS

Collection

Melanoides tuberculata (n = 57), *Poecilia reticulata* Peters, 1859 (n = 52) and *Xiphophorus* sp. Heckel, 1848 (n = 2) were collected between August and November 2018, totalizing three samplings. The collection sites were ponds “Gruta da Santa” and “Jardim Japonês” (23º25’29”S, 51º55’48”W), at Parque do Ingá, an urban park considered as a conservation area in Maringá city, Paraná, Brazil, also used for recreation by local and regional population. Fish and molluscs were collected using landing nets. *M. tuberculata* and *P. reticulata* were collected in all samplings, while *Xiphophorus* sp. was collected only in one of the three samplings. All specimens were transported to the Laboratório de Ictioparasitologia of the

Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupélia) of the Universidade Estadual de Maringá (UEM); specimens obtained were maintained in an aquarium for subsequent examination. These fishes species were analyzed because they were the main species collected during a short-term monitoring program carried out in this environment.

During screening for parasites, molluscs were eviscerated to remove the digestive gland, used as an infection site by cercariae; fish were screened in the branchial arch, used as an infection site by metacercariae. Cercariae and metacercariae found were morphologically identified previously according to Pinto & Melo (2010) and individually separated for subsequent DNA extraction.

All the procedures were approved by the Ethics Committee on Animal Use from the Universidade Estadual de Maringá (protocol CEUA 8244220618) and by the environmental council of Maringá, Paraná, Brazil (32520/2018).

Molecular analyses

The DNA extraction was carried out using the *ReliaPrep™ gDNA Tissue Miniprep System* (Promega™) kit, according to the manufacturer's instructions. Polymerase chain reactions (PCR) were carried out in an Applied Biosystems ProFlex PCR (Life Technologies) thermocycler, with the final volume of reactions of 20 µL, containing Tris-KCl [20mM Tris-HCl (pH 8.4), 50mM KCl], $MgCl_2$ (1.87 mM), primers (2.5 pmoles), dNTPs: dATP, dCTP, dGTP and dTTP (0.5 mM), Taq Polymerase Platinum – Invitrogen™ (1 U), DNA template (3 µL) and ultrapure water *q.s.* 20 µL.

Following primers were used in the PCR reactions of the 28S rDNA gene, responsible by the large subunit (LSU) of the ribosome, U178 (5'GCA CCC GCT GAA YTT AAG-3') and L1642 (5'-CCA GCG CCA TCC ATT TTC A-3') (Lockyer et al. 2003), considering following conditions: initial denaturation at 94 ºC for 5 min, followed by 30 cycles at 94 ºC for 30 s, 56 ºC for 1 min, and 72 ºC for 1 min; and a final cycle at 72 ºC for 5 min (modified from Graça et al. 2018). Amplicons were visualized in an electrophoresis gel (1%) and purified with polyethylene glycol 8000 (Rosenthal et al. 1993). Sequencing reactions were prepared using the BigDye™ Terminator v 3.1 kit and sent to an automated sequencer ABI 3500 Applied Biosystems™, at the Complexo de Centrais de Apoio à Pesquisa (COMCAP) at the Universidade Estadual de Maringá. Sequences were visually edited and aligned using BioEdit (Hall 1999) and MEGA 7 (Kumar et al. 2016) software, respectively, and compared to sequences available previously in GenBank using the BLASTn tool <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Sequences obtained in this study were deposited in GenBank (MK876840-MK876845).

RESULTS

Among all specimens of *M. tuberculata*, only one was parasitized by cercariae (1.8%) and were morphologically classified as pleurolophocercous (Figure 1a). These cercariae present spherical eyespots on the body, a ventral sucker and an oral sucker with spines surrounding it, pharynx, penetration glands, rostellar hooks, genital primordium and a tail, with finfolds throughout tail and body. Metacercariae were obtained in all individuals of *Xiphophorus* sp. (n = 2) and four individuals of *P. reticulata* (7.7%) (Figure 1b-d). The metacercariae has a visible oral sucker with two rows armed with spines.

The partial fragments of the 28S gene presented 732 bp and were obtained from six specimens of parasites (three cercariae and three metacercariae). All sequences were identical and confirmed the identification of *Centrocestus*

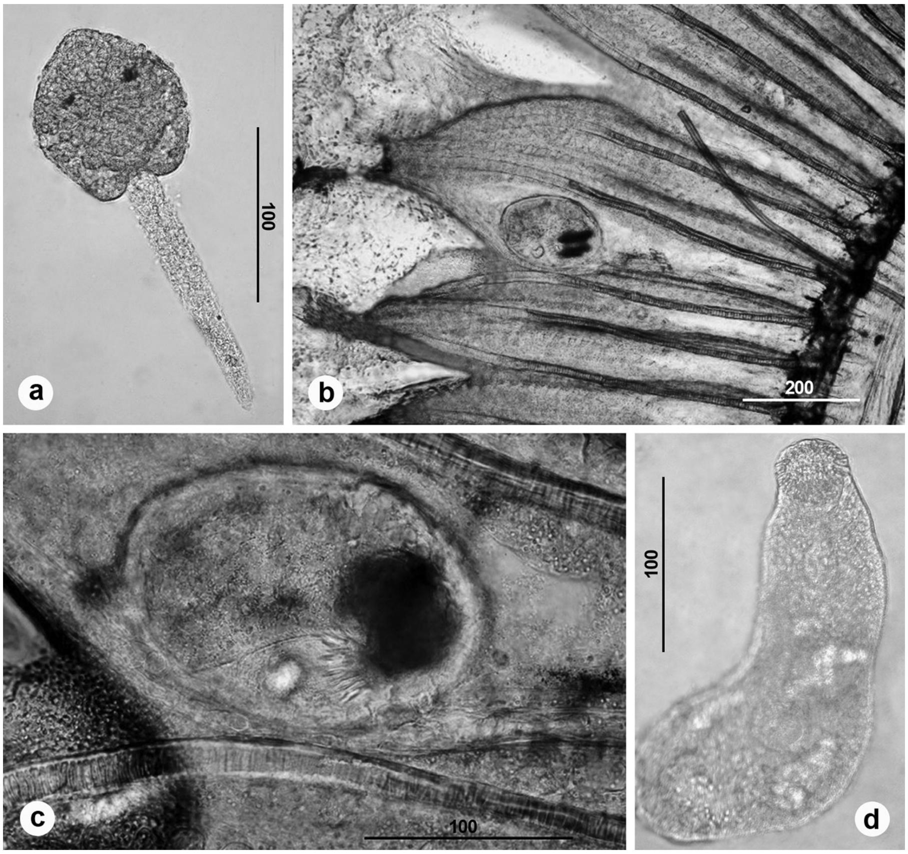

Figure 1. ***Centrocestus formosanus*** **(a) Cercaria obtained from Melanoides tuberculata collected in Parque do Ingá, Maringá. (b) Gills of** ***Poecilia reticulata*** **infected with metacercaria. (c) Metacercaria obtained from the gills of** ***Poecilia reticulata*****. (d) Metacercaria out of the cyst. Obs.: scale in µm. 413x387mm (95 x 95 DPI).**

formosanus with 100% similarity when compared to sequences obtained from GenBank (a total of 8 sequences of *C. formosanus*), using BLASTn tool, considering the partial fragment obtained in this study. Of the sequences data compared with those generated by our study, four were obtained during the adult stage of development (KY075663, KY075664, KY075665 and HQ874609), three during the cercaria stage of development (MG738251, KY369153 and KY351633), and one sequence during the metacercaria stage of development (KY369154) (Supplementary Material - Table SI).

DISCUSSION

Our study is the first record of *C. formosanus* in the southern region of Brazil parasitizing *M. tuberculata* and poeciliid fish. In Brazil, metacercariae of this parasite were recorded in *Poecilia reticulata*, *Australoheros facetus* (Jenyns, 1842) and *Oreochromis niloticus* (Linnaeus, 1758) (both Cichlidae), all in Minas Gerais State (Pinto & Melo 2012a, b, Pinto et al. 2014). In other countries, *C. formosanus* was identified in 40 species of fish which can act as an intermediate hosts (Martin 1958, Salgado-Maldonado et al. 1995). Various possible intermediate hosts can be economically important, for example, *Ctenopharyngodon idellus* Valenciennes, 1844 (Cyprinidae), *Cyprinus carpio* Linnaeus, 1758 (Cyprinidae), *Ictalurus punctatus* (Rafinesque, 1818) (Ictaluridae), *Hypophthalmichthys molitrix* (Valenciennes, 1844) (Cyprinidae), *Megalobrana amblycephala* (Yih, 1955) (Cyprinidae), *Mylopharyngodon piceus* (Richardson, 1846) (Cyprinidae), *Oreochromis aureus* (Steindachner, 1864) (Cichlidae) (Scholz & Salgado-Maldonado 2000). Fish parasitized by *C. formosanus* may have their development affected, showing fitness reduction or even lethal effects, due to histological modifications which result in hyperplasia, hyperemia and congestion (Martin 1958, Vélez-Hernández et al. 1998, Mitchell et al. 2000, Piazza et al. 2006, Tolley-Jordan & Chadwick 2012, Pinto et al. 2014). Thus, the presence of these parasites in fish farms requires financial and management efforts, including spending on specialists and products used to control the infection, causing economic loss (Mitchell et al. 2005, Tavechio et al. 2009).

Fishes obtained in our study (Poeciliidae) were introduced to control mosquitoes' vectors of different diseases (Araújo et al. 2009) and are invasive species, as well as *M. tuberculata* Invasive species can facilitate the introduction of non-specific parasites resulting in economic losses, besides the threatens to the conservation, as diseases in different fish species (Huston et al. 2014, McDermott et al. 2015, Sumuduni et al. 2018).

These fishes are not commonly consumed by the local population, because of their reduced size and usefulness as aquarium fish. However, different birds' species can eat them and be infected by metacercariae, contributing to the development of the parasite to the adult stage, transmitting the eggs of this parasite to other fish through infected feces (Thatcher 1993, Takemoto et al. 2004). Digenean transmission can also occur during birds migration, promoting the dispersion of infectious diseases in aquatic ecosystems (Rząd & Busse 2015, Sánchez et al. 2018) indicating that aquatic and wading birds could be the main animals transmitting Trematodes (Fredensborg et al. 2006).

Some birds were already recorded infected with *C. formosanus*, as *Bubulcus ibis coromandus* Boddaert, 1783 (Ardeidae), *Butorides striata* Linnaeus, 1758 (Ardeidae) and *Nycticorax nycticorax* Linnaeus, 1758 (Ardeidae) (Pinto & Melo 2010). The monitoring data of the Parque do Ingá (Maringá 2007) recorded *Butorides striata*, and could be a candidate to the definitive host of *C. formosanus* in this environment, and this species presents a migratory behavior (Brum et al. 2016). Among the species occurring in the Parque do Ingá, future investigations are necessary considering the feeding habits of different birds, as *Aramides saracura* Spix, 1825 (Rallidae), *Ardea alba* Linnaeus, 1758 (Ardeidae), *Egretta thula* Molina, 1782 (Ardeidae), *Megaceryle torquata* Linnaeus, 1766 (Alcedinidae), *Nannopterum brasilianus* Gmelin, 1789 (Phalacrocoracidae) and *Pitangus sulphuratus* Linnaeus, 1766 (Tyrannidae), since eventually they could serve as host of *C. formosanus*.

In addition to harming fish in natural and aquaculture environments, *C. formosanus* also is considered zoonotic, using humans as a definitive host (Chai et al. 2013). Infections were recorded in humans that consume raw fish meat in various countries, as Taiwan (Nishigori 1924), China (Chen et al. 1991), Vietnam (De & Le 2011) and Lao People's Democratic Republic (Chai et al. 2013). However, these studies did not show the pathogenicity degree in their hosts (Chai et al. 2013). Nevertheless, experimental infections using mice as a model and other *Centrocestus* species showed intestinal alterations including villous atrophy and hyperplastic crypt (Hong et al. 1997).

The presence of *C. formosanus* in Maringá city supports the importance of studies about the parasitic fauna in fish, especially species presenting zoonotic potential. Additionally, the prevalence shown in our results could correspond to more infected hosts, so the introduction of species such as *C. formosanus* and *M. tuberculata* may have impacts on local fauna; *M. tuberculata* was the first time recorded in this environment too. In this sense, using an integrative taxonomy approach (Dayrat 2005) enables solving life cycles of digenean species, particularly those presenting health risks and potentially zoonotic, besides allowing the correct identification of parasites using morphological and molecular approaches.

Acknowledgments

The authors acknowledge the support and infrastructure of the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura of the Universidade Estadual de Maringá (NUPÉLIA–UEM) and the financial support of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (446150/2014-2) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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SUPPLEMENTARY MATERIAL

Table SI

How to cite

CICCHETO JRM, STABILE BHM, FABRIN TMC, DE OLIVEIRA AV, TAKEMOTO RM & DA GRAÇA RJ. 2021. The first record of *Centrocestus formosanus* (Trematoda: Heterophyidae) in southern Brazil supported by molecular data. An Acad Bras Cienc 93: e20191017. DOI 10.1590/0001-3765202120191017.

Manuscript received on August 29, 2019;
accepted for publication on April 13, 2020

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Juliana R. M. Ciccheto and Rodrigo J. da Graça collected the data. Juliana R. M. Ciccheto and Bruno H. M. Stabile wrote the paper. Thomaz M. C. Fabrin, Bruno H. M. Stabile and Rodrigo J. da Graça performed the molecular analyzes and interpreted the data. Alessandra V. de Oliveira and Ricardo M. Takemoto provided revisions to scientific content of manuscript, access to research components, and funding. Rodrigo J. da Graça designed the work.