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Elucidation of the first definitively identified life cycle for a marine turtle blood fluke (Trematoda: Spirorchiidae) enables informed control ☆

Thomas H. Cribb [a,*], Jose L. Crespo-Picazo [b], Scott C. Cutmore [a], Brian A. Stacy [c], Phoebe A. Chapman [d], Daniel García-Párraga [b]

[a] The University of Queensland, School of Biological Sciences, St Lucia, Queensland 4072, Australia
[b] Fundación Oceanogràfic, Veterinary Services & Research Department, Avanqua Oceanogràfic-Ágora, C/ Eduardo Primo Yúfera 1B, 46013 Valencia, Spain
[c] National Marine Fisheries Service, Office of Protected Resources and University of Florida, College of Veterinary Medicine, Gainesville, FL, USA
[d] The University of Queensland, Veterinary Marine Animal Research, Teaching and Investigation Unit, School of Veterinary Science, Gatton, Queensland 4343, Australia

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ABSTRACT

Blood flukes of the family Spirorchiidae are significant pathogens of both free-ranging and captive marine turtles. Despite a significant proportion of marine turtle mortality being attributable to spirorchiid infections, details of their life cycles remain almost entirely unknown. Here we report on the molecular elucidation of the complete life cycle of a marine spirorchiid, identified as *Amphiorchis* sp., infecting vermetid gastropods and captive bred neonate *Caretta caretta* in the Oceanogràfic Aquarium, in Valencia, Spain. Specimens of a vermetid gastropod, *Thylaeodus* cf. *rugulosus* (Monterosato, 1878), collected from the aquarium filtration system housing diseased *C. caretta*, were infected with sporocysts and cercariae consistent with the family Spirorchiidae. We generated rDNA sequence data (internal transcribed spacer 2 (ITS2) and partial 28S rDNA) from infections from the vermetid which were identical to sequences generated from eggs from the serosa of the intestine of neonate *C. caretta*, and an adult spirorchiid from the liver of a *C. caretta* from Florida, USA. Given the reliability of these markers in the delineation of trematode species, we consider all three stages to represent the same species and tentatively identify it as a species of *Amphiorchis* Price, 1934. The source of infection at the Oceanogràfic Foundation Rehabilitation Centre, Valencia, Spain, is inferred to be an adult *C. caretta* from the western Mediterranean being rehabilitated in the same facility. Phylogenetic analysis suggests that this *Amphiorchis* sp. is closely related to other spirorchiids of marine turtles (species of *Carettacola* Manter and Larson, 1950, *Hapalotrema* Looss, 1899 and *Learedius* Price, 1934). We discuss implications of the present findings for the control of spirorchiidiasis in captivity, for the better understanding of epidemiology in wild individuals, and the elucidation of further life cycles.

1. Introduction

The Spirorchiidae Stunkard, 1921 is an assemblage of 20 genera of blood flukes which parasitise the circulatory system of freshwater and marine turtles globally (Platt, 2002; Roberts et al., 2016). Spirorchiids have recently come to prominence as they have been widely shown to cause extensive tissue injury and mortality in marine turtles (Glazebrook et al., 1981; Gordon et al., 1998; Flint et al., 2010; Stacy et al., 2010a). As with blood fluke infections in other taxa, disease in turtles results from both inflammation and vascular injury caused by the trematodes and embolised ova, which affect a variety of tissues (Gordon et al., 1998; Stacy et al., 2010a).

Despite their significance as a frequent cause of disease in some marine turtles, lack of knowledge of their life cycles hampers progress in our understanding of the epidemiology of marine spirorchiids. Life cycles are known for five freshwater spirorchiid species, all of which infect pulmonate (heterobranch) gastropods as first intermediate hosts (Wall, 1941a,b, 1951; Pieper, 1953; Holliman and Fisher, 1968; Turner and Corkum, 1977). Numerous other putative but unidentified freshwater spirorchiid cercariae are known from both pulmonate and caenogastropod gastropods. In stark contrast, there exists just a single report of a marine turtle spirorchiid life cycle, that of Stacy et al. (2010b) who reported a spirorchiid in a fissurellid (vetigastropod) limpet from Florida,

☆ Nucleotide sequence data reported in this paper are available in GenBank under the accession numbers: KX987107–KX987112.

* Corresponding author. Fax: +61 7 3365 4699.
E-mail address: T.Cribb@uq.edu.au (T.H. Cribb).

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USA. However, this infection was detected by molecular analysis alone; the parasite stages were not observed by microscopy.

Caretta caretta (the loggerhead turtle) is considered conservation-dependent globally, including the Mediterranean population (Ullmann and Stachowitsch, 2015). In support of the population, the Oceanogràfic Foundation in Valencia, Spain runs a head-starting program in which cohorts of juvenile *C. caretta* are incubated and reared from eggs translocated from nearby Mediterranean beaches. The facility also rehabilitates sick, bycaught and injured juvenile and adult specimens of *C. caretta* from the nearby Mediterranean coast for eventual reintroduction. Adult and juvenile turtles are always held in separate tanks, but the water passing through the tanks is shared and recirculated. From January 2015 many captive-hatched juvenile turtles began exhibiting debilitation and wasting disease that was characterised by weight loss, malabsorption, anorexia, gastrointestinal stasis and occasionally neurological signs of incoordination or swirling behaviour. Upon necropsy and subsequent histopathology, spirorchiid eggs were found in several tissues and organs, and were especially evident, even macroscopically, within the serosa of the intestine. Individual *C. caretta* that survived longest in the facility harboured the greatest numbers of eggs. Given that the water supply of the aquarium facility is semi-closed and pretreated, that prehatching transmission of spirorchiids is unknown, and the evidence of increasing egg loads in tissues with age despite consecutive deworming treatment (Jacobson et al., 2003), it was concluded that transmission was occurring within the facility. These circumstances created an opportunity to identify the intermediate host within the filtration system. Here we report the elucidation of the intermediate host of the spirorchiid parasite using novel ribosomal data and detail the morphology of the stages associated with it.

2. Materials and methods

2.1. Morphological specimens

Examination of the aquarium system for potential intermediate hosts in September 2015 revealed the presence of just one gastropod species, a sessile form that was attached to piping delivering and draining water from the turtle holding tanks. We dissected specimens of this gastropod species under a stereomicroscope in a solution of 0.85% saline. When observed, asexual stages and cercariae of trematodes were fixed either in near boiling saline and then transferred immediately to 80% alcohol, or directly in cold alcohol for samples for molecular analysis.

Specimens for morphological analysis were subsequently washed in fresh water, stained with Mayer's haematoxylin, destained in a solution of 1.0% HCl and neutralised in 0.5% ammonium hydroxide solution. Specimens were then dehydrated through a graded ethanol series, cleared in methyl salicylate and mounted in Canada balsam. Measurements were made using an Olympus SC50 digital camera mounted on an Olympus BX-53 compound microscope using cellSens Standard imaging software. Measurements are given in µm and, where length is followed by breadth, the two measurements are separated by '×'.

2.2. Molecular sequencing

Total genomic DNA was extracted using phenol/chloroform extraction techniques (Sambrook and Russell, 2001). We amplified the partial D1-D3 fragment of the 28S nuclear rDNA region using the primers LSU5 (5′-TAG GTC GAC CCG CTG AAY TTA AGC A-3′; Littlewood, 1994) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′; Snyder and Tkach, 2001) and the internal transcribed spacer 2 (ITS2) region using the primers 3S (3S: 5′-GGT ACC GGT GGA TCA CGT GGC TAG TG-3′; Morgan and Blair, 1995) and ITS2.2 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′; Cribb et al., 1998). The 28S rDNA region has proven informative in phylogenetic analysis of blood flukes and the ITS2 rDNA region has proven effective in species discrimination (Blasco-Costa et al., 2016).

PCR for both the 28S and ITS2 regions was performed with a total volume of 20 µl consisting of 5 µl of 5× MyTaq Reaction Buffer (Bioline, United Kingdom), 0.75 µl of each primer (10 pmols), 0.25 µl of Taq polymerase (Bioline MyTaq™ DNA Polymerase) and 2 µl of DNA template (approximately 10 ng), made up to 20 µl with Invitrogen™ (United States) ultraPURE™ distilled water. Amplification was carried out on an MJ Research (United States) PTC-150 thermocycler. The following profile was used to amplify the 28S region: an initial 95 °C denaturation for 4 min, followed by 30 cycles of 95 °C denaturation for 1 min, 56 °C annealing for 1 min, 72 °C extension for 2 min, followed by a single cycle of 95 °C denaturation for 1 min, 55 °C annealing for 45 s and a final 72 °C extension for 4 min. The following profile was used to amplify the ITS2 region: an initial single cycle of 95 °C denaturation for 3 min, 45 °C annealing for 2 min, 72 °C extension for 90 s, followed by four cycles of 95 °C denaturation for 45 s, 50 °C annealing for 45 s, 72 °C extension for 90 s, followed by 30 cycles of 95 °C denaturation for 20 s, 52 °C annealing for 20 s, 72 °C extension for 90 s, followed by a final 72 °C extension for 5 min. Amplified DNA was purified using a Bioline ISOLATE II PCR and Gel Kit according to the manufacturer's protocol. Cycle sequencing of purified DNA was carried out using ABI Big Dye™ v.3.1 chemistry following the manufacturer's recommendations, using the same primers used for PCR amplification as well as the additional 28S primers 300F (5′-CAA GTA CCG TGA GGG AAA GTT G-3′; Littlewood et al., 2000), ECD2 (5′-CCT TGG TCC GTG TTT CAA GAC GGG-3′; Littlewood et al., 1997) and 1200R (5′-GCA TAG TTC ACC ATC TTT CGG-3′; Lockyer et al., 2003a). Cycle sequencing was carried out at the Australian Genome Research Facility.

We extracted DNA from eggs found in the serosa of the small intestine of infected *C. caretta* using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). The extraction protocol was in accordance with the manufacturer's instructions, with the exception that 1 g of Silica/Zirconia 0.5 mm beads (Daintree Scientific, Tasmania, Australia) was utilised to disrupt the eggs in a Biospec (United States) Mini-Beadbeater 16 for 3 min prior to extraction, and the final elution was made in 100 µl of elution buffer as opposed to the recommended 200 µl. PCRs for egg extractions were carried out using primers L3F and L2R for 28S, and IF1 and IR1 for ITS2 (Chapman et al., 2015). Reactions and cycling conditions were as described by Chapman et al. (2015). PCR products were visualised on a 1% agarose gel and submitted to the Animal Genetics Laboratory (School of Veterinary Science, University of Queensland, Gatton, Australia) for purification and sequencing using the same primers as for PCR.

Sequencher™ version 4.5 (GeneCodes Corp., United States) was used to assemble and edit contiguous sequences, and the start and the end of the ITS2 rDNA region were determined by annotation through the ITS2 Database (Koetschan et al., 2012) using the 'Metazoa' model.

2.3. Phylogenetic analysis

Partial 28S rDNA sequences generated during this study were aligned with those of species of Schistosomatoidea available on GenBank using MUSCLE version 3.7 (Edgar, 2004) with ClustalW sequence weighting and UPGMA clustering for iterations 1 and 2 (Table 1). The resultant alignments were refined by eye using MESQUITE (Mesquite: a modular system for evolutionary analysis. Version 2.72 http://mesquiteproject.org) and the ends of each

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