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

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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 & 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.

*Keywords*: Trematoda; Spirorchiidae; Life cycle; Vermetidae; Conservation; Sea turtles, Transmission
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 embolized 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, 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 pre-hatching 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 LS5 (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 5x 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 fragment were trimmed to match the shortest sequence in each alignment.

Bayesian inference and Maximum Likelihood analyses of the 28S rDNA dataset were
conducted to explore distinctions and relationships among these taxa. Bayesian inference
analysis was performed using MrBayes version 3.2.6 (Ronquist et al., 2012) and Maximum
Likelihood analysis was performed using RAxML version 8.2.8 (Stamatakis, 2014), run on
the CIPRES portal (Miller et al., 2010). The software jModelTest version 2.1.10 (Darriba et
al., 2012) was used to estimate the best nucleotide substitution model for the dataset.
Bayesian inference and Maximum Likelihood analyses were conducted using the GTR+I
model predicted as the best estimators by the Akaike Information Criterion (AIC) in
jModelTest. Nodal support in the Maximum Likelihood analysis was estimated by performing 100 bootstrap pseudoreplicates. Bayesian inference analysis was run over 10,000,000 generations (ngen = 10000000) with two runs each containing four simultaneous Markov Chain Monte Carlo (MCMC) chains (nchains = 4) and every 1000th tree saved (samplefreq = 1000). Bayesian inference analysis used the following parameters: nst = 6, rates = invgamma, ngammacat = 4, and the priors parameters of the combined dataset were set to ratepr = variable. Samples of substitution model parameters, and tree and branch lengths were summarised using the parameters ‘sump burnin = 3000’ and ‘sumt burnin = 3000’. These ‘burnin’ parameters were chosen because the log likelihood scores ‘stabilised’ well before 3,000,000 replicates in the Bayesian inference analysis.

3. Results

3.1. Morphological analysis

The only gastropod found in the holding tanks in the Oceanogràfic Rehabilitation Centre was identified by Dr Rüdiger Bieler of the Chicago Field Museum, Illinois, USA as *Thylaeodus cf. rugulosus* (Monterosato, 1878) (Caenogastropoda, Vermetidae) (Fig. 1A). This vermetid species occurs in the western Mediterranean and the central Atlantic (Bieler, 1995). We lodged five voucher specimens in the Chicago Field Museum (FMNH 344699-700).

We found infections of spirorchiid sporocysts in 38 of the 45 *T. cf. rugulosus* individuals dissected (infection prevalence 84%). Large and apparently fully developed ocellate furcocercous cercariae were detected in just three infections; most of the sporocysts had only immature cercariae which could be recognised as such by the presence of eye-spots and developing tails.
3.2. Description of intramolluscan stages

Sporocysts in digestive gland of *T. cf. rugulosus*. Body tubular, containing numerous developing cercariae but no more than two close to fully developed. Maximum length observed 1432 µm.

Cercaria (Figs. 1B, C) (based on single largest recovered specimen). Body 348 × 101 µm. Tegumental spines on body and tail, minute. Dorsal fin-fold not detected. Eye-spots prominent, 159 µm from anterior extremity. Anterior organ 128 × 76 µm; head gland present, prominent. Penetration glands in two groups of three with bodies filling posterior extremity. Ventral sucker 260 µm from anterior extremity, 49 µm long. Tail 974 µm long in total, maximum width 76 µm. Furcae 260 × 37 µm, surrounded by distinct fin-fold terminally. Permanent preparations of cercariae and sporocysts have been lodged in the Queensland Museum, Brisbane, Australia (Nos G 235497- G 235509).

3.3. Molecular analysis

ITS2 and partial 28S rDNA sequences were generated from asexual trematode stages dissected from three individual *T. cf. rugulosus* and from spirorchiid eggs taken from the serosa of the intestine of a necropsied neonate *C. caretta* from the Oceanogràfic. Sequences from the intestinal eggs and the stages infecting vermetid gastropods were identical for both markers. The new sequence data were compared with that available from GenBank and with a library of unpublished sequences from marine turtles from off Florida, USA held by one of the authors. No matches were identified from GenBank, but a perfect match (for both markers) was found between samples from Valencia and an adult spirorchiid collected from *C. caretta* from Florida. The voucher specimen relating to the Florida infection has been deposited in the United States National Museum as no. 1422020. In the combination of
unfused intestinal caeca, two testes with the cirrus-sac and genital pore between, and slender body with the vitelline follicles extending to the caecal extremities, the specimen is consistent with the genus *Amphiorchis* Price, 1934. On this basis, we identify the form from the Valencia rehabilitation centre as *Amphiorchis* sp. We refrain from attempting to identify the specimen relative to the five recognised species of *Amphiorchis* because its poor condition renders this unreliable.

Preliminary Bayesian inference and Maximum Likelihood analysis of the partial 28S rDNA alignment of a wide range of Schistosomatoidea produced a topology (essentially identical in both analyses) in which species of marine genera of Aporocotylidae formed a well-supported clade sister to all spirorchiids and schistosomatids. The Spirorchiidae was paraphyletic, forming one clade of freshwater spirorchiids and a second strongly supported clade of four marine spirorchiids (including the current form, *Amphiorchis* sp.). The clade of marine spirorchiids was sister to schistosomatids. These results are broadly consistent with previous analyses (Snyder, 2004; Orelis-Ribeiro et al., 2014; Pinto et al., 2015) and are therefore not reproduced here. In light of these preliminary results we realigned and analysed a reduced data set comprising just sequences of marine spirorchiids (including *Amphiorchis* sp.), using a range of schistosomatids as the outgroup. Maximum Likelihood and Bayesian inference analyses of this reduced dataset resulted in phylograms with identical topologies (Fig. 2), in which the marine spirorchiids formed a well-supported clade to the exclusion of the schistosomatids. Within the marine spirorchiid clade, *Carettacola hawaiiensis* Dailey, Past & Balazs, 1991 was basal to the remaining taxa and *Amphiorchis* sp. was sister to *Hapalotrema pambanensis* Mehrotra, 1973 (senior syn. of *Hapalotrema mehraii* Rao, 1976 according to Chapman et al. (2015)) and *Learedius learedi* Price, 1934.
4. Discussion

The genetic match between sequences derived from infections from the vermetid gastropod *T. cf. rugulosus*, from eggs from the intestine of neonate *C. caretta* at the Oceanogràfic, and from adult worms from *C. caretta* from Florida allows us to conclude that the life cycle of this species has been demonstrated.

The voucher specimen from *C. caretta* from Florida is consistent with the genus *Amphiorchis*, but is too poorly preserved to allow reliable identification to species. Notably, no species of *Amphiorchis* is known from *C. caretta*; all five described *Amphiorchis* spp. are reported from either *Chelonia mydas* or *Eretmochelys imbricata* (Price, 1934; Oguro, 1938; Simha, 1970; Fischthal and Acholonu, 1976; Gupta and Mehrotra, 1981; Werneck and Silva, 2013). The present form may thus prove to be a new species. Our phylogenetic analyses suggest that the present *Amphiorchis* sp. is closely related to species of *Hapalotrema* Looss, 1899, *Learediús* Price, 1934 and *Carettacola* Manter & Larson, 1950. These four genera are morphologically similar, and recent molecular analysis by Chapman et al. (2015) found that the single species of *Learediús* nested within *Hapalotrema*. Further molecular characterization of species of these genera is needed.

Despite the absence of previous reports of species of *Amphiorchis* from *C. caretta*, this species hosts a rich fauna of spirorchiids from five genera (Luhman, 1935; Manter and Larson, 1950; Jacobson et al., 2006; Stacy et al., 2010a; Chen et al., 2012). Strikingly, as far as we can detect, spirorchiids have not previously been reported from *C. caretta* nor other turtle species from the Mediterranean despite several surveys (e.g. Manfredi et al., 1998; Gracan et al., 2012). However, it is likely this population does harbour spirorchiids, given that the ultimate source of the infection of *Amphiorchis* sp. in the neonate turtles housed at the Oceanogràfic rescue centre is inferred to have been mature turtles taken from the western
Mediterranean. *Caretta caretta* from the western Mediterranean are part of a trans-Atlantic population, genetically distinguishable from the eastern Mediterranean population (e.g. Carreras et al., 2011). Possibly there is a distinction in the parasites of these populations, which might explain the absence of spirorchiids in previous reports of parasites of some Mediterranean *C. caretta*.

Our studies provide direct evidence that marine spirorchiids can be transmitted in closed-system aquaria. Notably, the *Amphiorchis* specimens collected from Florida, which were genetically identical to the specimens from Valencia, was also from a captive juvenile *C. caretta*. This individual had been raised in an aquarium from hatching and was believed to have acquired fatal spirorchiidiasis in captivity (unpublished observations). Glazebrook and Campbell (1990) reported spirorchiids in hatchling-raised individuals of both *C. mydas* and *E. imbricatus* in Queensland, Australia. Furthermore, the study of Stacy et al. (2010b) was based on material from a Cayman Island turtle farm where transmission of *L. learedi* occurs (Greiner et al., 1980). Given the threatened status of many marine turtles and the frequent rearing of young turtles and rehabilitation of older turtles in aquarium facilities, our work demonstrates the need for vigilance to ensure that pathogenic spirorchiid infections are not transmitted within these systems. Elimination of vermetids is now one clear approach to the control of spirorchiidiasis in turtle hatchery and rehabilitation centres. Notably, many vermetids produce non-planktonic “crawl-away juveniles” (e.g. Calvo et al., 1998), and this reproductive habit may make them especially liable to multiply in marine aquaria.

We do not know whether *T. cf. rugulosus* is the only host for the present *Amphiorchis* sp., however, given the general pattern of specificity of trematodes for molluscan intermediate hosts (Cribb et al., 2001), it seems unlikely that the host range is wider than a range of vermetids. As far as we can determine, no spirorchiid has been reported from more than a single gastropod family. A potentially parallel system may be exemplified by an
Aporocotylid (fish blood fluke), *Cardicola forsteri* Cribb, Daintith & Munday, 2000. The tuna hosts of this species are highly mobile marine animals that, similar to marine turtles, travel large distances. This trematode infects different polychaete intermediate hosts in Australia and Japan (Cribb et al., 2011; Shirakashi et al., 2016). Thus, further examination of vermetids in other geographical locations may well expand the intermediate host range of this *Amphiorchis* sp..

There is extensive literature relating to the life cycles of spirorchiids, but almost all of it relates to freshwater species. A series of classical and often highly detailed experiment-based studies characterised life cycles for five freshwater species from North America – *Spirorchis artericola* (Ward, 1921), *Spirorchis elephantis* (Cort, 1918), *Spirorchis parvus* (Stunkard, 1923), *Spirorchis scripta* Stunkard, 1923 and *Vasotrema robustum* (Stunkard, 1928) by Wall (1941a, b, 1951), Pieper (1953), Holliman and Fisher (1968), and Turner and Corkum (1977). All these species infect Planorbidae (“Pulmonata”: Planorboidea). The cercariae of these species are morphologically similar and were recognised by Wall (1941b) as a distinct group among furcocercous cercariae, united by a range of features including being large, distomate, and having a distinctive arrangement of eye-spots. No complete new life cycle has been described for a spirorchiid for many years. However, five further spirorchiid cercariae have recently been characterised as such by molecular analyses (Brant et al., 2006; Kraus et al., 2014; Pinto et al., 2015). Table 2 summarises the reports of these and the five classically elucidated cycles. There are also descriptions of several other cercariae likely to be spirorchiids (e.g. Nasir and Diaz, 1973; Bayssade-Dufour et al., 1989). The status of these species as spirorchiids cannot be considered certain (in the absence of experimental or molecular data) and they do not modify our understanding of the apparent intermediate host range of the Spirorchiidae. Before the present study, the only report of marine spirorchiid asexual stages was that by Stacy et al. (2010b), who generated sequences...
consistent with the marine spirorchiid *L. learedi* from samples from the tissues of a fissurellid limpet, *Fissurella nodosa*, collected from a marine turtle mariculture facility. Notably, the approach did not lead to observation of the parasitic stages and vermetids were not among the potential host taxa examined. The details of this report are included in Table 2.

The limited material available for examination in the present study did not allow the detailed observations on the anatomy of the intramolluscan stages made by earlier studies (e.g. Wall, 1941a, b, 1951). However, the present specimens are broadly consistent with previously described spirorchiid cercariae. All typical features of previously described spirorchiid cercariae were present – substantial size, well-developed eye-spots and ventral sucker, penetration glands occupying space posterior to eye-spots, and a well-developed head organ. We did not detect a dorsal finfold or “cuticular crest” in the present form. In this, it resembles the cercariae of *S. artericola, S. elegans, S. elephantis* and *S. scripta* which lack finfolds, rather than those of *V. robustum* and *S. parvus* which have them. Overall, it appears that the morphology of spirorchiid cercariae is conservative.

The new data reported here makes the distribution of intermediate hosts of spirorchiids especially striking. Freshwater species overwhelmingly infect planorbids (Heterobranchia: “Pulmonata”), with just a single species known from an ampullariid (Caenogastropoda) from South America. The two reported marine infections are from gastropods from separate subclasses, a caenogastropod (present study) and a vetigastropod (Stacy et al., 2010b). Although understanding of the deep branches of gastropod evolution remains unstable (e.g. Aktipis and Giribet, 2010), it is clear that the Heterobranchia, Caenogastropoda and Vetigastropoda represent deeply distinct and ancient lineages within the Gastropoda. It is surprising that *L. learedi* and the present form (*Amphiorchis* sp.), which are evidently closely related, have intermediate hosts that are deeply phylogenetically distant. Such a host distribution implies dramatic host-switching. However, there is a precedent for
such a contrast in that African and Asian species of *Schistosoma* Weinland, 1858, which represent distinct clades in the same genus (Lockyer et al., 2003b), infect gastropods as phylogenetically distinct as caenogastropods and vetigastropods.

The identification of a vermetid as an intermediate host for marine spirorchiids has clear implications for the search for further intermediate hosts of marine spirorchiids. Stacy et al. (2010b) observed that this search is challenging due to the immense range of potential hosts and because infections might be rare or localised. We consider that it now makes sense to focus future studies on fissurellid limpets and vermetids. In this context, we note that both families of gastropods have been surveyed for trematode infections previously and shown to be infected. Fissurellids are known to be infected by a hemiurid and an opecoelid (Cable, 1956, 1963). Vermetids are known to be infected by an echinostomatid, a hemiurid, a mesometrid, a microphallid and a possible opecoelid (Prévôt, 1969, 1971a, b; Jousson and Bartoli, 1999). Despite these reports, we suspect that the nature of these gastropod families, especially the particularly unconventional Vermetidae, has led to them being less studied than many other groups of gastropods. In some parts of the world, vermetids occur in such large numbers that they comprise reef-building organisms (Shier, 1969; Safriel, 1975). Should the vermetid species involved prove to be intermediate hosts for spirorchiids, then such localities may be especially important in spirorchiid transmission.

The sessile habit of the Vermetidae raises the intriguing possibility that they could attach to the carapaces of turtles so that the intermediate hosts of spirorchiids are carried around with the turtles; this idea was suggested by Frazier et al. (1985) before any marine spirorchiid life cycle was known. There is extensive literature on the epibiotic fauna of turtle carapaces (e.g. Frazier et al., 1985; Kitsos et al., 2005; Pfaller et al., 2008; Domenech et al., 2015). However, apart from a few references to unidentified ‘tubeworms’ by Frazier et al. (1985), which might refer to several kinds of animals, we have not detected any reports of
Vermetidae on turtles. Vermetids are easily confused with some groups of polychaetes (especially Serpulidae) to the extent that some species of the two groups were described in the wrong phylum. We think that vermetids may well occur on turtles, and such infestation is worthy of a specific search.

The present study is just the second to report information on marine spirorchiid life cycles, following that of Stacy et al. (2010b), and the first evidence of sporocysts and cercariae formation in a marine intermediate host. The intermediate hosts identified in the two studies represent completely unrelated gastropods. Additionally, this is the first known report of spirorchiid infection in sea turtles in the Mediterranean Sea. Thus, despite recent progress, we do not yet have a broad understanding of the transmission of marine spirorchiiids. Increased knowledge is bound to prove valuable in explaining the epidemiology of spirorchiiids over the complex life cycles of their hosts, which incorporate pelagic, inshore and migratory phases. Also, as seen here, improved life cycle knowledge can enhance the well-being of captive turtle populations.

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praziquantel after oral administration of single and multiple doses in loggerhead sea

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IV: interactive taxon sampling for internal transcribed spacer 2 based phylogenies.

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Fig. 1. Life cycle of *Amphiorchis* sp. (A) *Thylaeodus cf. rugulosus* (Vermetidae), the intermediate host of *Amphiorchis* sp ex *Caretta caretta*. Scale bar = 3 mm. (B) Whole cercaria of *Amphiorchis* sp. ex *T. cf. rugulosus*. Scale bar = 100 µm. (C) Cercarial body of *Amphiorchis* sp. Scale bar = 50 µm.

Fig. 2. Relationships of marine Spirorchidae, with Schistosomatidae as outgroup, based on Maximum Likelihood and Bayesian inference analysis of the partial 28S rDNA dataset. GenBank accession numbers are indicated in parentheses. Nodal support values for each node are indicated as posterior probabilities (Bayesian inference) above the node and bootstrap support (Maximum Likelihood) below the node. Support values < 70 are not shown. Sequences generated in this study are in bold.
Fig. 1
Fig. 2

- Amphiorchis sp. sporocyst (KX987111)
- Amphiorchis sp. adult (KX987109)
  - Hapalotrema pambanense (AY604708)
  - Learedius learedi (AY604707)
  - Carettacola hawaiensis (AY604709)
  - Ornithobilharzia canaliculata (AY157248)
  - Austrobilharzia variglandis (AY157250)
  - Bivitellobilharzia naiiri (JQ975005)
    - Schistosoma mansoni (Z46503)
    - Schistosoma japonicum (Z46504)
    - Dendritobilharzia pulverulenta (AY157241)
    - Heterobilharzia americana (AY157246)
    - Schistosomatium douthitti (AY157247)
Table 1 28S rDNA sequences from GenBank analysed in this study.

<table>
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<tr>
<th>Species</th>
<th>Host</th>
<th>Locality</th>
<th>GenBank Accession #</th>
<th>Reference</th>
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<td><em>Hapalotrema mehra</em></td>
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<td><em>Ornithobilharzia canaliculata</em></td>
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<td>Littlewood and Johnston (1995)</td>
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Table 2. Hosts of spirorchiid intermediate hosts demonstrated as such either experimentally or by molecular analysis.

<table>
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<th>Species</th>
<th>Host</th>
<th>Family</th>
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<td>Experimental</td>
<td>Wall (1941a)</td>
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<td>Experimental</td>
<td>Wall (1941b)</td>
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<td>Experimental</td>
<td>Wall (1951)</td>
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<td>Planorbidae</td>
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<td>Pieper (1953)</td>
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<td><em>Helisoma aniceps</em></td>
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<td>Goodchild and Kirk (1960)</td>
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Mol., molecular studies.
Highlights

• Spirorchiidae blood flukes are significant pathogens of marine turtles but their life cycles remain almost entirely unknown.

• We show that Amphiorchis sp. of Caretta caretta infects a vermetid gastropod, Thylaeodus cf. rugulosus.

• We matched DNA from infections from the vermetid, eggs from neonate C. caretta, and an adult spirorchiid from C. caretta.

• The Amphiorchis sp. is closely related to marine turtle spirorchiid of the genera Carettacola, Hapalotrema and Learedius.

• The findings enable control of spirorchiidiasis in captivity and better understanding of epidemiology in wild individuals.
Graphical abstract