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DETECTION OF SPIRORCHIID TREMATODES IN GASTROPOD TISSUES BY POLYMERASE CHAIN REACTION: PRELIMINARY IDENTIFICATION OF AN INTERMEDIATE HOST OF *LEAREDIUS LEAREDI*

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ABSTRACT: Marine spirorchiid trematodes are associated with morbidity and mortality in sea turtles worldwide. The intermediate hosts remain unknown, and discovery efforts are hindered by the large number and great diversity of potential hosts within sea turtle habitats, as well the potential for low prevalence and overdispersion. A high-throughput DNA extraction and polymerase chain reaction–based method was developed to detect the internal transcribed spacer 2 (ITS2) region of the ribosomal gene of 2 spirorchiid genera, *Learedius* and *Hapalotrema*, within pooled samples of gastropod tissues. A model system consisting of freshwater snail (*Pomacea bridgesii*) tissues and DNA extracts spiked with adult *Learedius learedi* and known quantities of spirorchiid DNA was used to develop and test the technique. Threshold of detection was found to be equivalent to an early prepatent infection within 1.5 g of gastropod tissue. This technique was used to screen approximately 25 species of marine gastropods at a captive facility where green turtles (*Chelonia mydas*) become infected by *L. learedi*. The parasite was detected in a sample of knobby keyhole limpet (*Fissurella nodosa*), thus providing the first evidence of an intermediate host for a marine spirorchiid trematode. This technique has many potential applications in trematode life cycle discovery studies.

Spirorchiid trematodes are implicated as a cause of morbidity and mortality of sea turtles worldwide, yet the life cycles of these parasites remain unknown (Glazebrook and Campbell, 1990; Gordon et al., 1998; Work et al., 2005; Jacobson et al., 2006). The only life cycles that have been established in this group are for species in the freshwater genera *Enterohaematotrema*, *Spirorchis*, and *Vasotrema* (Smith, 1997). Knowledge of parasite life cycles is essential to the understanding of virtually every aspect of host-parasite interaction, including epidemiology of infections and influences on prevalence and disease in the host. Discovery of marine spirorchiid intermediate hosts is complicated by the diversity of habitats utilized by sea turtles, the diversity of gastropods within those habitats, and the potential for low prevalence and overdispersion among intermediate hosts (Rohde, 1993). There have been only 2 previously reported attempts to discover marine spirorchiid intermediate hosts, both of which were unsuccessful (Greiner et al., 1980; Dailey et al., 1992).

Classical parasitological methods for intermediate host studies rely on monitoring individual snails for cercariae emergence and microdissection to detect trematode infections. These methods can be time consuming, do not detect early infections, and require that investigators distinguish the many digenean taxa that may infect targeted gastropods. With no advance knowledge to focus surveys, the effort required to investigate life cycles in the marine system is daunting in terms of the number of different marine gastropod species and the numbers of individuals that must be screened. A combined molecular and morphological approach to spirorchiid life cycle investigation offers many potential advantages, including relatively rapid screening of large numbers of gastropods, greater sensitivity, and specificity.

The present study describes the development and field application of a high-throughput DNA extraction technique and polymerase chain reaction (PCR) protocol for sensitive and specific detection of species in 2 spirochiid genera, *Learedius* and *Hapalotrema*, within gastropod tissues. Using this method, a variety of gastropod species was screened at a facility where green turtles (*Chelonia mydas*) are known to acquire *Learedius learedi* infections. *Learedius learedi* was detected in a pooled organ sample of an intertidal keyhole limpet, *Fissurella nodosa*, thus providing the first evidence of a marine spirorchiid intermediate host. The findings from the present study hopefully will provide the means for the necessary confirmatory studies and facilitate discovery efforts in other regions.

MATERIALS AND METHODS

Detection of *L. learedi* and *Hapalotrema* species by PCR

The ITS2 was sequenced from voucher specimens of *L. learedi* (GenBank GU937892), *Hapalotrema mistroides* (GenBank GU937893), *Hapalotrema pambanensis* (GenBank GU937894), and *Hapalotrema postorchis* (GenBank GU937895), using custom consensus primers designed from comparative alignments of schistosomes and other digenean taxa. These genera were selected because of their implications for sea turtle health based on necropsy of stranded turtles in Florida (data not shown). The forward primer SPIR1 (5′-GAGGGTCGGCTTAT-TATCTATCA-3′) and outer reverse primer SPIR2 (5′-TCACATCT-GATCCGAGGTCA-3′) were complementary to the 3′-end of the 5.8s gene and 5′-end of the 28s gene, respectively. Based on these sequences, a series of primers were designed and tested; 3 primers ultimately were selected for use in detection studies in a hemi-nested format. The first round of PCR used the consensus primers SPIR1 and SPIR2 for amplification of digenean ITS2. The second round used SPIR1 with a specific reverse primer, HLC4 (5′-GCAGCAACTCAACCTGR-TAAACC-3′), designed to amplify the ITS2 of *L. learedi* and 3 *Hapalotrema* species. The Taqman PCR kit (Qiagen, Valencia, California) was used for all reactions, which were performed in a 20 µl reaction volume according to standard protocol in a thermal cycler (PCR Sprint, Thermo Hybaid, Franklin, Massachusetts). Reaction conditions for the first reaction included initial denaturation at 95 C for 5 min, then 45 cycles of denaturation at 95 C for 60 sec; annealing at 50 C for 45 sec, and DNA extension at 72 C for 120 sec, followed by a final extension step at 72 C for 10 min. A higher annealing temperature of 56 C was used for the second reaction.

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TABLE I. Protocol for extraction of DNA from pooled gastropod samples.

Gastropod preparation and tissue lysis
1. Remove the hepatopancreas (digestive gland) and gonad
2. Pool these tissues to a combined wet weight of 1.5 g
3. Place wet tissue into an aluminum foil pouch and freeze in liquid nitrogen for 20 sec
4. Crush tissues and place in 10 ml of CTAB lysis buffer with 75 µl of proteinase K (Qiagen)
5. Incubate overnight at 55 C
DNA extraction
1. Add 12 ml of chloroform:isamyl alcohol (24:1) and mix by gentle inversion
2. Centrifuge at 7,700 *g* for 30 min
3. Transfer aqueous phase to clean tube and repeat chloroform extraction
Precipitation
1. Add Na acetate (pH 5.4) to aqueous phase to obtain a final concentration of 0.3 M
2. Add 2 volumes of 100% ethanol and mix by swirling
3. Centrifuge at 17,000 *g* for 1 hr
4. Discard supernatant and wash with 70% ethanol
5. Centrifuge at 17,000 *g* for 30 min
6. Dry pellet and resuspend in 1 ml of TE (pH 8.0)
DNA clean-up (pigment removal)
1. Add aliquot of DNA to QIAquick spin column
2. Dilute eluted DNA into TE for PCR

The PCR products were resolved in 1% agarose gels, and direct sequencing identified all bands of interest. The expected amplicon size for the spirorchiid-specific primers was 175 base pairs. Trematode amplicons produced by the conserved primers typically were between 300 and 500 base pairs. The bands were excised and purified using the QIAquick gel extraction kit (Qiagen). Direct sequencing was performed using the Big-Dye Terminator Kit (Perkin-Elmer, Branchburg, New Jersey) and analyzed on ABI 377 automated DNA sequencers at the University of Florida Center for Mammalian Genetics DNA Sequencing Facilities.

Test model for gastropod DNA extraction and PCR

Multiple methods of tissue lysis and DNA extraction were tested to devise a method by which DNA could be extracted from relatively large quantities of gastropod tissue. Specimens of albino mystery snails (*Pomacea bridgesii*) initially were used to test extraction methods so that pigment would not interfere with DNA yield determination by spectrophotometry. The protocol that best fulfilled our requirements in terms of sample volume, available equipment, DNA yield, and purity began with removal of gastropods from the shell and separation of the hepatopancreas and gonad. Only these organs were included in the DNA extraction, with the exception of very small gastropods (less than 3 mm in greatest dimension) from which all soft tissue was processed. Gastropod tissues were combined to a total wet weight of 1.5 g. Wet weight rather than individual numbers defined methods so that the technique would be applicable to gastropods of different sizes. The complete tissue lysis and DNA extraction protocol is given in Table I. The lysis buffer was modified from Winnepenninckx et al. (1993) and consisted of 2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, and 100 mM TrisCl, buffered to a pH of 8.0. Included in this protocol is an additional step to remove melanin, a PCR inhibitor, which is abundant within the hepatopancreas and co-purifies with nucleic acid. Multiple methods, including serial dilution of DNA, were tested, and the best result in terms of PCR amplification and signal strength was obtained by using the QIAquik PCR purification kit (Qiagen) on DNA extracts prior to PCR. Detection of spirorchiid ITS2 using DNA extracts was attempted using several different quantities of template DNA (10, 50, 100, 500, and 1,000 ng) from snail tissues that were extracted with a single adult *L. learedi*.

The threshold of detection for the gastropod DNA extraction and PCR technique was tested by spiking *Pomacea bridgesii* (wild-type) total extracts with known quantities of spirorchiid DNA. Detection was measured using embryonated eggs (*H. mistroides*) as biological units. Eggs were obtained from the tissues of dead stranded turtles and counted into a 1.5-ml tube using a dissecting microscope and microcapillary tubes. To ensure rupture of eggs and complete DNA extraction, eggs were placed into 180 µl of deionized water, frozen at −80 C, thawed, and then sonicated for 1 min. These steps were repeated 3 times, which resulted in obvious rupture of approximately 90% of the eggs. Thus, a 10% error rate was considered in egg DNA extractions, and 110 eggs were included to obtain DNA from a target of 100. After sonication, DNA was extracted using the DNeasy kit (Qiagen). Total gastropod DNA extracts were spiked with 1 to 10 egg equivalents of spirorchiid DNA prior to treatment for removal of pigment.

Gastropod collections and screening

Gastropods were collected from the coastal habitat adjoining the Cayman Turtle Farm, Limited (CTFL), Grand Cayman Island, British West Indies during October 2006, July 2007, and April 2008 under permits granted by the Marine Conservation Board of the Grand Cayman Islands. All collections were performed by snorkeling or under SCUBA. Most of the collected gastropods were stored frozen at −80 C until extraction of DNA and PCR were performed. A subset of specimens was screened by microdissection. Preparation of gastropods and DNA extraction was performed in a separate laboratory from PCR. DNA from *H. mistroides* (30 fg of template DNA) was used as a positive control in lieu of *L. leardi* DNA to allow identification of any cross-contamination of PCR samples. Any suspected positive results were re-examined by repeating the pigment removal step using an additional aliquot of the original DNA extract and PCR. All amplicons of appropriate size or interest were sequenced for confirmation. Select examples of amplicons obtained from the first round of PCR (consensus trematode primers) were sequenced to confirm detection of non-spirorchiid trematodes as a quality control measure for the pooling and DNA extraction techniques.

RESULTS

Gastropod DNA extraction and spirorchiid detection by PCR

The gastropod DNA extraction technique using albino *P. bridgesii* yielded between 1.85 and 1.87 mg of DNA from 1.5 g of starting material. Spectrophotometric analyses of 100-fold dilutions of DNA yielded 260/280 ratios of 1.73 to 1.76, indicating removal of most contaminants. Snail tissues extracted with an adult *L. learedi* yielded single bright bands by PCR using all template DNA quantities, whereas *P. bridgesii* tissues without added *L. learedi* were PCR negative. Based on these results, 100 ng of template DNA was used in each PCR reaction. Further testing of *P. bridgesii* spiked with various amounts of DNA from embryonated eggs found that the DNA equivalent of 1 egg could be detected. This level of sensitivity is comparable the earliest possible stage of infection in gastropods.

Field collections and screening of gastropods

The CTFL has a population of approximately 10,000 *C. mydas*. Preliminary field studies indicated greater than 98% prevalence of infection by *L. learedi* based on necropsy of harvested turtles (B. Stacy, unpubl. obs.). The facility has a flow-through filtration system whereby large volumes of seawater are pumped into the tanks via an open intake channel, and the unprocessed effluent is discharged approximately 215 m from the intake area. There is no significant gastropod colonization of turtle tanks; thus any intermediate hosts likely are within adjoining coastal habitat. The coastal habitat consists of a rocky limestone shore and hard

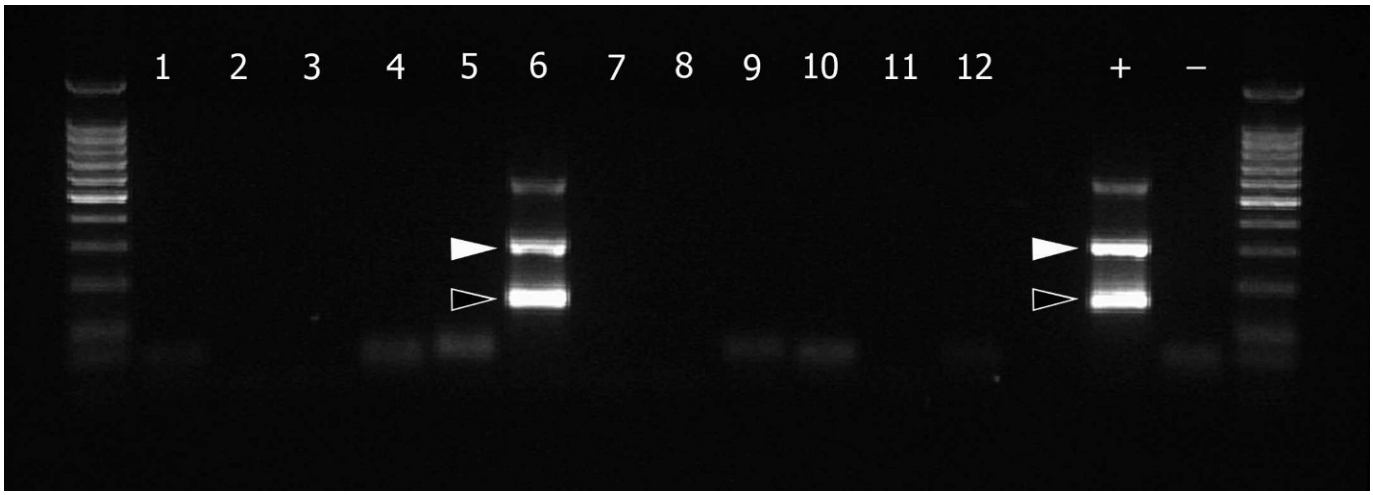

FIGURE 1. Gel electrophoresis of products obtained from PCR amplification of the trematode ITS2 from DNA extracted from pooled hepatopancreas and gonad samples of knobby keyhole limpet (*Fissurella nodosa*). The brightest bands in the ladder lanes are 500 base pairs. The bright bands in lane 6 reflect amplification of the complete ITS2 (300 bp, white arrowhead) using consensus primers (round 1) and the smaller 175 bp amplicon (black arrowhead) indicates specific amplification of the 5′ region of the ITS2 (round 2). Products of similar size are present in the positive control lane (+), which is *Hapalotrema mistroides* DNA. Direct sequencing of the products excised from lane 6 confirmed the sequence to be *Learedius learedi* ITS2.

bottom that descends relatively rapidly to a mini-wall (15- to 18-m deep) and then to a shear main wall. The rocky limestone shore includes abundant tide pools, and the subtidal zone is extensively pocketed by burrows of rock-boring urchins (*Echinometra lucunter*). Collected gastropods included a variety of tidal, intertidal, and subtidal species. Almost all gastropods were collected within 5 m of the shoreline, although the surveyed habitat extended from the tidal pools within the iron shore to the nearshore reef system. A single night collection was performed, but did not yield significantly different species from daytime collections, with the exception of 2 cowry species, *Cypraea acicularis* and *Cypraea cinerea*.

A total of 4,083 gastropods from the CTFL was screened by PCR (Table II). A single positive PCR result was obtained from a pooled sample of 13 individual knobby keyhole limpets (*F. nodosa*), an intertidal limpet species, which were collected in July 2007 (Figs. 1, 2). Purification and dilution of DNA (pigment removal), PCR, and sequence confirmation were repeated 2 additional times, and amplicons were identified as *L. learedi* ITS2. A total of 550 *F. nodosa* was examined, including 413 that were collected during April 2008 in efforts targeting this species after the original positive result was obtained (137 were in the initial collection). No additional positive results were detected. Cercariae development was observed in 2.3% (10/438) of *F. nodosa* examined by microdissection, which included 25 individuals in July 2007 and 413 from April 2008. All of these cercariae were cotylomicrocercous, and the sequence obtained using the consensus trematode primers indicated greatest homology with an unidentified opocoelid species, which is consistent with the observed cercaria morphology. No bifurcate spirorchiid cercariae were observed.

Of the 528 gastropods of other species examined by microdissection, cercariae development was observed only in 4 (0.76%). Some gastropods were screened only by microdissection because of small sample numbers, small specimen size, or other logistical reasons. These species included *Bursa rhodostoma*, *Bursa* sp., *Conus mus*, *Diodora minuta*, *C. acicularis*, *C. cinerea*, *Mitra barbadensis*, *Nertia tesselata*, *Pisania pusio*, *Plicopurpura patula*, *Pollia auritula*, *Stramonita rustica*, *Strictospira* sp., and *Talparia cinerea*. Cercariae development was observed in *Fissurella barbadensis*, *Diodora listeri*, *Nodilittorina dilatata*, and *Cenchritas muricatus*. None of these cercariae exhibited morphology consistent with spirorchiid trematodes. Additional non-spirorchiid trematode infections (unidentified taxa) were detected in *Nodilittorina augustior*, *Nodilittorina mespillum*, *Nerita peloronta*, and *Cerithium littoratum* by consensus trematode PCR.

DISCUSSION

The internal transcribed spacer 2 (ITS2) of the ribosomal gene was selected as the target for detection of spirorchiid DNA because of high copy number and sufficient variation to promote specific detection and identification. The ribosomal gene, which includes the ITS2 targeted by the PCR technique, is present in the genome as numerous tandem repeats; thus, each parasite cell contains many copies for detection by PCR. In addition, Nolan and Cribb (2005) demonstrated the utility of ribosomal DNA in discriminating digenean species in an extensive review of ITS studies. With appropriate primer design, we have found the ITS2 to be a useful target for specific detection of spirorchiids, as well as broader detection of other digenean trematodes within hosts that is limited only by the available comparative genetic reference data in current databases.

Ideally, gastropods exposed to infectious miracidia with known time points of infection would have been used to define sensitivity; however, this was not possible given that none of the life cycles has been discovered for any marine spirorchiids. The use of a captive-propagated freshwater gastropod in this study spiked with parasite material was a useful surrogate for validating the technique. The threshold of targeted spirorchiid detection was equivalent to the earliest possible prepatent infection based on our ability to detect a single egg equivalent in 1.5 g of host tissue.

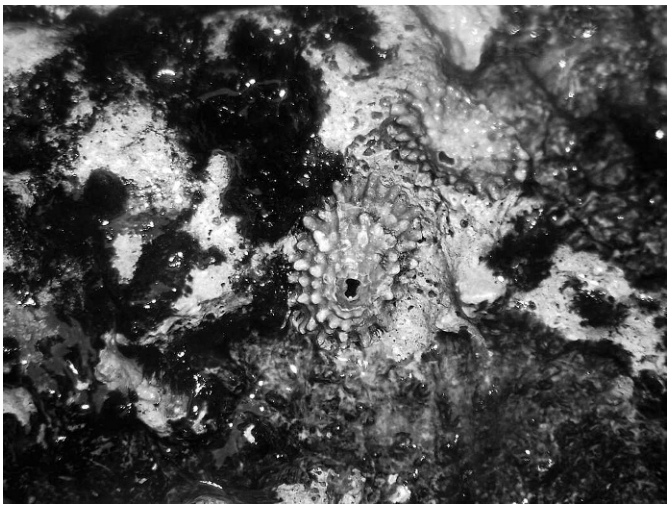

FIGURE 2. Two knobby keyhole limpets (*F. nodosa*) within the intertidal zone at the Cayman Turtle Farm, Limited, Grand Cayman Island, British West Indies.

Although this measure of sensitivity is only an approximation, it is adequate for most applications in life cycle discovery, especially when there are a large number of potential host species to be examined. Naturally infected gastropods likely will include individuals in which host organs are extensively effaced by developing parasites; therefore, the amount of parasite material present will exceed that of an embryonated egg by many orders of magnitude.

Co-purification of melanin and nucleic acid created problems early in the study. The unconventional use of a commercial spin-column kit may have resulted in inconsistencies in yield and increased the cost of the technique; however, this method proved superior to serial dilution in the development phase of the study. More refined methods for removal of melanin undoubtedly can be developed. Nonetheless, the utility of this high through-put molecular screening technique was evidenced by successful detection of digenean infections in several of the gastropod species examined. These results are very promising for future spirorchiid studies, as well as investigations of life cycles of other trematodes.

The detection *L. learedi* in the limpet *F. nodosa* is the first evidence of an intermediate host for a marine spirorchiid. Unfortunately, we were not able to demonstrate spirorchiid cercariae in subsequent studies, despite the gross examination of over 400 individual *F. nodosa*. A total of 137 *F. nodosa* was screened in the initial collection that included the positive sample; thus, prevalence among the collected limpets was as low as less than 1% (assuming only 1 limpet was positive in the pooled sample). This level of infection is consistent with low prevalence observed in other primary intermediate host populations, despite high prevalence in definitive hosts. High fecundity of asexual stages, i.e., abundant production of cercariae, as well as prolonged survival of infected intermediate hosts, may result in comparatively higher prevalence in definitive hosts. Holliman (1971) reported a prevalence of 0.11% for the freshwater spirorchiid *Spirorchis parvus* and hypothesized that high cercariae output rather than abundance of infected snails maintains this spirorchiid life cycle. Gastropod hosts of freshwater spirorchiids have been documented to produce as many as 100 cercariae per day and live as long as several months (Pieper, 1953; Goodchild and Fried, 1963; Holliman, 1971). Daily cercariae output in the hundreds has been documented in the intermediate hosts of some *Schistosoma* species (Marquardt et al., 2000). If prevalence of *L. learedi* infection was as low as 0.1% in *F. nodosa*, approximately 2,995 individuals would have to be screened to obtain a 95% chance of detecting a single infected gastropod (Cannon, 2001).

There are other factors that may affect prevalence and distribution of parasitism within intermediate hosts. Although the prevalence of *L. learedi* infection is high in harvest-age *C. mydas* (3- to 4-yr-old) at the CTFL, only 10% of infected turtles had eggs detected in the feces; accordingly, eggs were consistently detected in very low numbers and were difficult to find in positive samples (B. Stacy, unpubl. obs.). Therefore, *L. learedi* may be cycling at a relatively low level of abundance in terms of fecal output of eggs into in the system. Another consideration is that the distribution of infected *F. nodosa* extends, or is more concentrated, outside of the study area, which was limited to boundaries of the CTFL under the collection permit. The flow of the effluent discharge carrying embryonated eggs is highly variable and depends on the prevailing current. Thus, eggs of *L. learedi* may be broadly distributed within the coastal habitat outside of the CTFL. Finally, variation in seasonal prevalence also may have affected detection in follow-up studies. Low seasonal prevalence, including periods where parasites were undetectable, has been documented in intermediate hosts of freshwater spirorchiids (Fernandez and Esch, 1991; Rosen et al., 1994).

The possibility of laboratory contamination as an explanation for the positive PCR result is remote. No additional positive PCR results for *L. learedi* were detected in another 13,729 gastropods (570 pooled samples) screened during this study and other concurrent studies. Furthermore, dissection of gastropods and DNA extraction were performed in a laboratory separate from the PCR laboratory, and *H. mistroides* DNA rather than *L. learedi* was used as a positive PCR control to facilitate recognition of any contamination. Another plausible, although seemingly improbable, scenario other than infection is that the positive *F. nodosa* ingested the eggs, but was not actually infected. *Fissurella nodosa* is an intertidal species and feeds on algae. It is plausible that eggs adhered to the algae were ingested. The intensity of the positive PCR result, however, was much stronger than observed in the detection model studies when small quantities, such as egg equivalents, were introduced. Also, none of the other limpet species or other gastropod species that also feed on algae yielded a positive result. Our conclusion is that the positive PCR result supports that *F. nodosa* is an intermediate host for *L. learedi*, although confirmatory studies are needed.

We recommend that limpets, especially fissurellids, be included among gastropods examined in future life cycle discovery efforts. To our knowledge, the only trematodes previously reported in fissurellids are *Proctoeces* spp. (Balboa et al., 2001). Although there appears to be general overlap between distribution of fissurellids and documented localities of *Learedius learedi*, as well as other spirorchiids, the same is true for other globally widespread gastropod taxa. Information on the worldwide occurrence of marine spirorchiid species is lacking, thus any comparison with distribution of specific gastropods is not supported at this time.

TABLE II. Marine gastropods collected and screened for spirorchiid trematode infection from the Cayman Turtle Farm, Limited, Grand Cayman Island, British West Indies.

Class Gastropoda	Number
Clade Vetigastropoda	
Fissurellidae	
Diodora listeri	43
Fissurella nodosa	550
Fissurella fascicularis	106
Fissurella barbadensis	40
Hemitoma octoradiata	38
Unidentified limpet species	184
Trochidae	
Tegula lividomaculata	11
Tegula fasciata	16
Turbinidae	
Lithopoma caelatum	56
Clade Neritomorpha	
Neritidae	
Nerita versicolor	81
Nerita peloranta	46
Neritina virginea	200
Puperita pupa	276
Clade Caenogastropoda	
Cerithiidae	
Cerithium eburneum	22
Cerithium littoratum	75
Cerithiopsidae	
Cerithiopsis sp.	146
Littorinidae	
Cenchritis muricatus	203
Nodilittorina angustior	247
Nodilittorina dilatata	273
Nodilittorina mespillum	317
Nodilittorina ziczac	360
Buccinidae	
Engina turbinella	115
Columbellidae	
Columbella mercatoria	310
Coralliophilidae	
Coralliophila abbreviata	10
Fasciolariidae	
Leucozonia nassa	292
Muricidae	
Thais deltoidea	66

The results of the present study provide a useful molecular tool for life cycle investigations with proven application in field studies and the first evidence of the identity of a gastropod intermediate host of a marine spirorchiid. It is a useful exercise to retrospectively assess whether this approach truly had advantages over classical screening methods or a combination of micro-dissection and molecular characterization. In addition to the previously discussed advantages of increased sensitivity and specificity, logistical considerations led to selection of a molecular technique. Foremost, only a limited amount of time could be spent at the field location, and much of this effort was expended on collections; thus thorough examination of all gastropods would not have been possible. Recognition of developing trematodes was difficult following freezing, which was essential for preservation and transportation. Furthermore, micro-dissection of all specimens may or may not have yielded clear evidence of spirorchiid cercariae in the positive sample, depending on the stage of infection. We did not foresee the difficulties in substantiating the molecular data in follow-up studies. Application of molecular-based screening in other studies obviously will have to consider the relative merits and feasibility of different methods and situations. Although our findings are regarded as preliminary, we feel that molecular screening was a reasonable approach in this instance given the limited amount of advanced information with which to guide discovery efforts and other constraints. These findings may be used to guide the necessary follow-up studies and to overcome many of the significant difficulties of life cycle discovery in the marine environment.

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