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TARGETED rDNA SEQUENCE DETERMINATION FROM GEOGRAPHICALLY ISOLATED POPULATIONS OF *PROTEROMETRA MACROSTOMA* (TREMATODA: AZYGIIDAE)

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ABSTRACT. *Proterometra macrostoma*, a digenetic trematode, was described to have eight morphological variants from cercariae specimens collected from North Elkhorn Creek, Scott County, Kentucky. We have identified and characterized a new population of *P. macrostoma* collected from Clear Creek, Wayne County, Indiana, through examining shed cercariae. The objectives of this study were to conduct rDNA sequence comparisons among individual cercarial phenotypic variants from both populations to (1) determine the partial 28S and complete internal transcribed spacer 2 (ITS2) ribosomal DNA sequence for *P. macrostoma*, (2) demonstrate that Clear Creek samples are indeed *P. macrostoma*, and (3) phylogenetically place *P. macrostoma* among related trematodes. First, we identified and collected *Pleurocera semicarinata livescens* from Clear Creek and monitored these gastropod intermediate hosts for patent trematode infections. Of the four different species of trematodes observed, the characteristic furcocystocercous *P. macrostoma* cercariae were identified. Examination of the cercariae demonstrated phenotypic variation in spined and/or unspined papillae as established in the prior North Elkhorn Creek study. Genomic DNA was isolated and purified from individual cercariae, representing different morphological variants, in both populations. PCR was successful in amplifying across three rDNA loci (partial 5.8S, complete ITS2, and partial 28S) and multiple recombinant clones were independently sequenced. This study represents the first sequence determination for these rDNA regions in *P. macrostoma*. Subsequent rDNA sequence analysis confirmed 100% identity independent of population or phenotypic variant and phylogenetic analyses placed *P. macrostoma* within a monophyletic clade of the *Proterometra* genus. Ongoing specimen acquisition, sequence analysis, and phenetic studies should be conducted to further resolve *Proterometra* phylogeny.

Keywords: Parasitology, trematode, cercaria, rDNA, Wayne County

INTRODUCTION

Trematodes are a globally diverse and highly successful class of flatworm. *Proterometra macrostoma* is a digenetic trematode requiring a freshwater gastropod intermediate host and ingestion of the shed cercariae by a centrarchid fish, the definitive host (Dickerman 1934, 1945; Uglem & Aliff 1984). The geographic distribution of *P. macrostoma* comprises at least 12 states within the Great Lakes and Mississippi River drainages (Riley & Uglem 1995). In this study, we identify a putative population of *P. macrostoma* in Clear Creek (CC), Wayne County, Indiana.

Prior studies have categorized *P. macrostoma* into variant strains based on the presence and organization of spined papillae on the anterior and middle sections of the characteristic furcocystocercous cercariae (Dickerman 1945; Riley & Uglem 1995). In particular, Riley & Uglem (1995) recognized eight strains (I-VIII) of *P. macrostoma* from multiple locations, and initially characterized from North Elkhorn Creek (NEC), Scott County, Kentucky (38.18333°N; -84.48861°W). In addition, these putative strains demonstrated contrasting (1) shedding rates that seemingly coincided with the presence/absence of migrant centrarchid hosts, (2) cercarial swimming patterns within the water column, and (3) infectivity into varied sunfish definitive hosts (Riley & Uglem 1995).

A logical progression of this work, explicit from the Riley & Uglem (1995) study, called for DNA sequence analysis to elucidate the conspecific nature of morphological variants within this population. Additionally, Womble et al. (2016) highlight the need for additional *Proterometra* rDNA sequence to be elucidated, particularly *P. macrostoma*, in order to resolve sequence-constrained azygiid phylogenies. To date, the only *P. macrostoma* DNA sequences available in Gen-

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Bank consist of partial sequences of the 18S small subunit ribosomal RNA and cytochrome oxidase subunit 1 genes (Van Steenkiste et al. 2015).

Sequence analyses of the 28S ribosomal large subunit gene and the internal transcribed spacer 2 (ITS2) region have been used in crafting numerous trematode phylogenies and species identifications, including *Proterometra* (Olson et al. 2003; Womble et al. 2015, 2016). The objectives of this study were to use targeted rDNA sequence analysis to (1) determine the 28S ribosomal large subunit and ITS2 rDNA sequences for *P. macrostoma*, (2) verify the identity of recently isolated CC samples of *P. macrostoma*, and (3) use targeted rDNA sequencing to investigate the strain variants of *P. macrostoma* among CC and NEC parasite populations. Our novel findings would provide insight into the intraspecies phylogeny and taxonomy of these geographically separated populations of trematodes and in doing so validate their phylogenetic relationships among other digeneans. Thus, this study would provide the first report examining the relationship between rDNA sequence and *P. macrostoma* phenotypic variants as justified by Riley & Uglem (1995).

MATERIALS AND METHODS

Study sites and specimen acquisition.—In June 2014, our research team was the first to identify *Pleurocera semicarinata livescens* (formerly *Elimia semicarinata*), a known intermediate host of *P. macrostoma*, in Clear Creek (CC), Wayne County, Indiana and purportedly the same intermediate host species examined in the original NEC report (Riley & Uglem 1995). CC is a tributary of the Whitewater River in Wayne County, Indiana. In June 2015 and 2016, over 300 *Pleurocera semicarinata livescens* snails were collected from CC (39.81851°N; -84.91737°W) and subsequently taxonomically verified (R.T. Dillon, pers. comm.). Individual snails were maintained in 40 ml of filtered creek water at 18° C with a 12 hr light/12 hr dark photoperiod and surveyed for patent infections via cercarial shedding. Live individual cercariae were collected and immediately processed for genomic DNA isolation and purification. Infected and uninfected snails were properly returned to the original collection site. To date, four species of trematode cercariae have been observed emerging from this new population including the characteristic furcocystocercous cercariae of *P. macrostoma*. This new population of putative *P. macrostoma* exhibits the spined and unspined variant cercarial phenotypes as described in NEC. NEC is a tributary of the Ohio River in Scott County, Kentucky. Freshly emerged and isopropanol-preserved NEC (38.18333°N; -84.48861°W) *P. macrostoma* cercariae, shed from *Pleurocera semicarinata livescens* snails, were provided by Dr. Ronald Rosen. Recently shed (< 24 hr) cercariae were imaged using an EVOS FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA) and categorized into strains based on Riley & Uglem (1995).

Identification of this new CC population allowed for preliminary comparative studies with the previously described NEC *P. macrostoma* population (Riley & Uglem 1995; Rosen et al. 2013). Phenotypic *P. macrostoma* variants, based on the pattern or absence of spined papillae, were confirmed in both NEC and CC cercariae shed from *Pleurocera semicarinata livescens* snails. First, in June 2015, snails (n = 118) were collected from NEC and 37% were infected with *P. macrostoma*. Phenotypic variants I, III, IV, V, and VIII were classified from these NEC samples based on Riley & Uglem (1995). In June 2016, the new population of putative *P. macrostoma* in CC was identified (Fig. 1) and shed cercariae were classified as type IV and VI variants. The characteristic large furcocystocercous cercariae demonstrated dark emergence patterns typical of *P. macrostoma* (Lewis et al. 1989). While the frequency of total trematode infections from CC was 37% (from > 300 snails sampled), only 3.5% were infected with *P. macrostoma*. Trematode coinfections in the CC population were relatively abundant which might validate the lower *P. macrostoma* incidence. This reduced overall infectivity has been witnessed in prior trematode co-exposure studies. Both reduced overall infectivity in the intermediate host and cercarial productivity was observed in a schistosome model system with co-exposure of parasite strains (Thiele & Minchella 2013).

DNA extraction and PCR amplification.—Total genomic DNA from individual *P. macrostoma* cercariae was extracted and purified using a DNeasy Blood and Tissue Kit according to the manufacturer's (Qiagen, Valencia, CA) instructions. The elution volume was reduced (50ul) and eluted twice to increase the final concentration of the genomic DNA. Three universal trematode primer sets

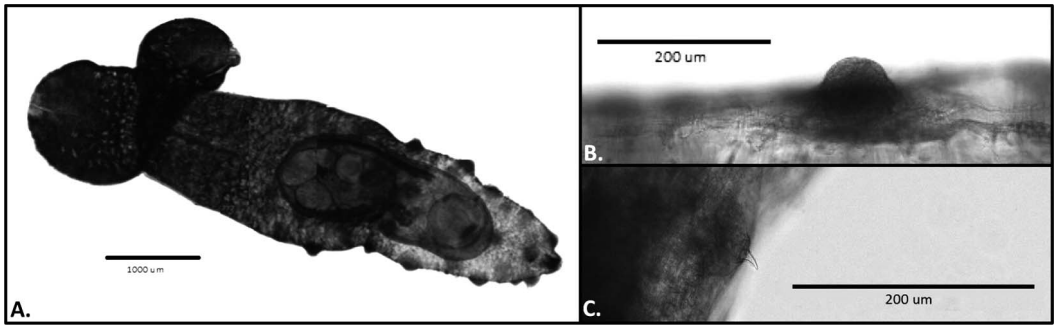

Figure 1.—Microscopic image of the furcocystocercous *Proterometra macrostoma* cercaria isolated from Clear Creek, Richmond, Indiana. A. Composite image of representative variant type IV cercaria. B. Higher magnification image indicating smooth, unspined papillae characteristic for the anterior and middle third sections of type IV cercariae. C. Higher magnification of spined papillae characteristic of the middle third section of type IV cercariae. Respective scale bars (μm) are indicated in each panel.

targeting the rDNA region (Fig. 2) of the *P. macrostoma* genome were used in this study: (1) LSU5 5′-TAGGTCGACCCGCTGAAYTTAAGCA-3′) and 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′); (2) 3S (5′-GGTACCGGTGGATCACTCGGCTCGTG-3′) and A28 (5′-GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3′); and (3) OphetF1 (5′-CTCGGCTCGTGTGTCGATGA-3′) and OphetR1 (5′-GCATGCARTTCAGCGGGTA-3′). The LSU5/1500R primer set was used for initial confirmation of *P. macrostoma* species identification producing an amplicon within the 28S large ribosomal locus. This region and this primer set was previously used in the formation of robust trematode phylogenies (Barker et al. 1993; Olson et al. 2003). PCR cycling parameters for the LSU5/1500R primer set were as follows: 3 min at 94° C; 35 total cycles of 94° C for 30 sec, 56° C for 30 sec, 72° C for 2 min; and a 7 min extension at 72° C. The 3S/A28 and OphetF1/OphetR1 primer sets amplify the internal transcribed spacer 2 (ITS2) region of rDNA and have been influential in determining several helminth phylogenies (Bowles et al. 1995; Womble et al. 2015, 2016; Sherrard-Smith et al. 2016). PCR cycling parameters for the ITS2 primer sets were as follows: 5 min at 95° C; 35 total cycles of 94° C for 30 sec, 53° C for 1 min, and 72° C for 1 min; and a 7 min extension at 72° C. Appropriate positive and negative PCR controls were used throughout the study to safeguard resulting sequence accuracy and to account for potential PCR errors. All PCRs were performed using the premixed 2× PCR Master Mix (Promega, Madison, WI) and all amplicons were sepa-

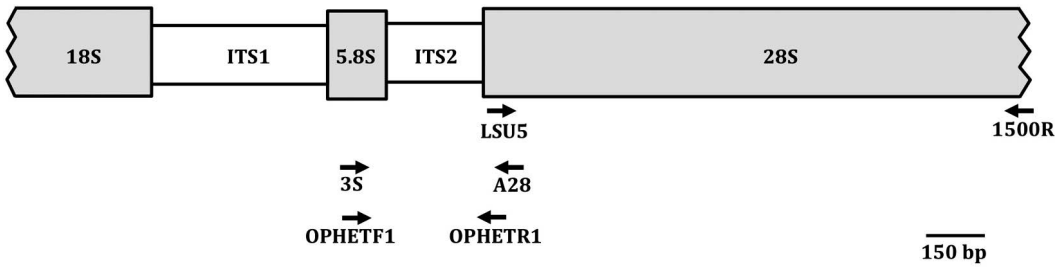

Figure 2.—Partial schematic of the *Proterometra macrostoma* rDNA locus and location of universal trematode primer sets used in this study. Positions of the three primer sets are indicated by name directly below directional arrows. Schematic is to scale (1 cm = 150 bp) with the exception of arrow lengths. Internal transcribed spacer 1, upstream from the 18S region, is not shown. To assist with scaling, the 18S and 28S regions have been truncated as indicated (jagged edges). Best estimations of the ITS1 and 5.8S region sizes were based on prior trematode rDNA studies.

rated and visualized on a 1% agarose gel stained with SYBR Safe.

Recombinant cloning, sequencing, and sequence determination.—Remaining PCR amplicons were cloned using the TOPO TA Cloning™ Kit for Sequencing (pCR™4-TOPO™ Vector and One Shot™ TOP10 Chemically Competent *E. coli*) following manufacturer's (Thermo Fisher Scientific, Waltham, MA) protocols. Multiple recombinant colonies were selected from each cloning reaction and independently grown overnight in 4 ml of LB broth plus ampicillin. Recombinant plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Size and purity of recombinant clones was verified by *Eco*RI restriction digest and gel electrophoresis. Approximately 700 ng per sample of purified recombinant plasmid DNA was sequenced at the Yale University DNA Analysis Facility on Science Hill using M13 Forward and Reverse sequencing primers. The plasmid vector allowed for sequencing calls to have settled prior to calling the amplicon bases allowing for sequencing precision at the PCR product termini. Recombinant sequences were trimmed, aligned (ClustalW and Geneious Alignment) using default settings, and analyzed using Geneious (v.10.2.3). Consensus sequences were determined from sequencing both strands of independent recombinant clones. Consensus sequences have been deposited into GenBank with the accession numbers MF927953-MF927958.

Phylogenetic analysis.—Phylogenetic analysis was independently performed on the partial 28S rDNA and the ITS2 rDNA sequence. Since the rDNA sequences of *P. macrostoma* from each variant and population shared 100% sequence identity, only the consensus was used in subsequent analyses. First, the overlapping sequences from the 3S/28 and LSU5/1500R amplicons allowed for the formation of a 1,758 bp contig. NCBI BLASTn of this contig retrieved related azygiid rDNA sequences for subsequent phylogenetic analysis: *Azygia longa* (KC985234.1), *Proterometra* sp. (KC985237.1), and *Otodistomum cestoides* (AY222187.1). Together with our *P. macrostoma* contig and *Diplodiscus mehrai,* the designated outgroup, these sequences were trimmed to between 1,259–1,262 bp which represented the approximate 28S rDNA region amplified by the LSU5/1500R primer set. These five sequences were aligned using the MUSCLE algorithm (Geneious v.10.2.3) set to eight for the maximum number of iterations (Edgar 2004; Kearse et al. 2012). The alignment was exported to MEGA6.06 and assessed for optimal phylogenetic test using the Find Best DNA/Protein Models (ML) analytic (Tamura et al. 2013). The general time reversible plus gamma (GTR+G) yielded the lowest BIC (7102.431). A phylogenetic tree was constructed using the GTR+G model on the MrBayes plugin in Geneious v.10.2.3 (Huelsenbeck & Ronquist 2001).

Based on this scarcity of total 28S rDNA sequence data from related taxa, the ITS2 region alone was subject to phylogenetic analysis and followed Womble et al. (2016) using trimmed *Azygia longa* (KT808319.1), *Leuceruthrus micropteri* (KT808320.1), *Proterometra ariasae* (KT808317.1), *Proterometra epholkos* (KM503118.1), *P. macrostoma* (MF927955), and *Transversotrema borboleta* (JF412524.1), set as the outgroup. Briefly, the ITS2 region was aligned (ClustalW, default conditions, in MEGA6.06), manually reviewed/edited, and subject to Kimura's 2-parameter and gamma distributed (K2+G) model (Kimura 1980). The K2+G model had yielded the lowest BIC (2268.782) from the best model predictor in MEGA6.06.

RESULTS AND DISCUSSION

Identification of *P. macrostoma* and the variant phenotypes in the NEC and CC populations was imperative to meet the objectives of this study. The subsequent targeted rDNA sequence elucidation allowed for: (1) the first determination of the partial 28S ribosomal large subunit and complete ITS2 rDNA sequences for *P. macrostoma*, (2) sequence comparisons among the CC and NEC variants, and (3) the phylogenetically grouping of *P. macrostoma* among CC and NEC parasite populations and other azygiid trematodes.

The universal LSU5/1500R primer sets (Fig. 2) yielded amplicons for all samples in the expected size range (1,392 bp) within the 28S ribosomal large subunit gene locus. No difference in band size was evident following agarose gel imaging. PCR fragments were subsequently cloned and sequenced yielding an identical 1,346 bp primer trimmed sequence independent of the strain or study site. The resulting sequence represents an

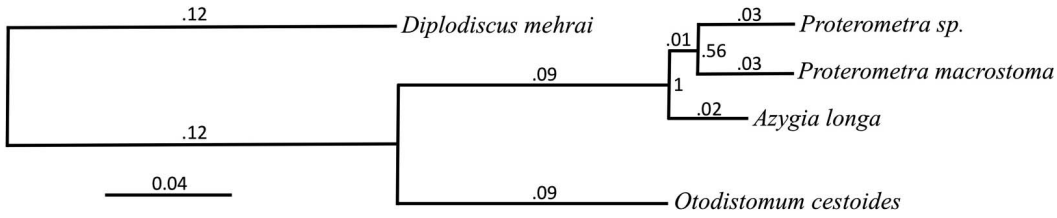

Figure 3.—Bayesian phylogenetic tree of *Proterometra macrostoma* among other digenean trematodes based on partial 28S rDNA. Posterior probabilities are placed to the right of nodes and substitutions per site are indicated above each branch. GenBank sequence accessions used are as follows: *Proterometra* sp. (KC985237.1), *Proterometra macrostoma* (contig of MF927953/MF927955 or MF927954/MF927956), *Azygia longa* (KC985234.1), *Otodistomum cestoides* (AY222187.1), and *Diplodiscus mehrai* (KX506857.1). *Diplodiscus mehrai* was assigned as the outgroup because of its phylogenetic relationship to the Azygiidae. The total characters used in the inference were 1,268. A scale bar is indicated.

estimated 35% of the entire 28S large ribosomal subunit rDNA (Lockyer et al. 2003; Blair 2006). Consensus alignments for each strain were readily achieved and used to resolve the rare ambiguous bases. NCBI nucleotide BLAST results of the consensus sequence produced two 95% identity matches: *Azygia longa* 28S ribosomal RNA gene, partial sequence (GenBank ID: KC985234.1) and *Proterometra* sp. SSC-2013 28S ribosomal RNA gene, partial sequence (GenBank ID: KC985237.1) (Calhoun et al. 2013). The relatedness of these taxa demonstrated novel authentication of the partial 28S large subunit rDNA sequence for *P. macrostoma*. The 1,346 bp sequence was 100% identical between sites (CC and NEC) and morphological variants (CC: IV, and VI; NEC (III, IV, V, VIII). For each site, the consensus *P. macrostoma* partial 28S large subunit rDNA sequence has been submitted to GenBank (MF927953 and MF927954).

To further validate the rDNA conservation of the two populations and morphological variants, the ITS2 region was selected for amplification and targeted sequencing. In prior studies, ITS2 sequence analysis has been used as a phylogenetic determinant of trematode species delineation (Morgan & Blair 1998; Nolan & Cribb 2005) , including a novel species, *Proterometra ariasae* (Womble et al. 2016). The A28/S3 and OPHETF1/OPHETR1 primer sets (Fig. 2) successfully amplified products of 476 bp and 423 bp respectively, independent of population or morphological variant. ITS2 sequence comparisons yielded 100% identity regardless of population or phenotypic variant. ITS2 sequences for variants IV and VI from CC and variants IV and VIII from NEC were used in the analysis. Resulting ITS2 consensus sequences have been submitted to GenBank (MF927955- MF927958). BLAST comparisons of consensus sequences yielded a top hit with 96% identity to *Proterometra ariasae* isolate MW-PS-134 5.8S ribosomal RNA gene, partial sequence; internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (KT808318.1) (Womble et al., 2016). These findings strongly indicate that both populations are indeed *P. macrostoma*. The fact that the phenotypic variants, independent of population, lack DNA polymorphisms suggests genetic conservation among morphological strains.

The Bayesian phylogenetic inference representing partial 28S rDNA resulted in the placement of *P. macrostoma* within a monophyletic clade with the related taxa *Proterometra sp.* (KC985237.1) (Fig. 3). This *Proterometra* clade then formed a sister clade with the azygiid fluke, *Azygia longa* (KC985237.1). Even with the limited number of comparative 28S rDNA sequences available, this represents the first phylogenetic comparison able to include *P. macrostoma*. The ITS2 phylogenetic analysis placed *P. macrostoma* within a monophyletic clade exclusive to the *Proterometra* genus (Fig. 4). These findings were consistent with and provide additional support for the Womble et al. (2016) phylogenetic models.

In summary, the perfect sequence conservation of the 1,758 bp rDNA region among morphological variants and geographically separated populations (CC and NEC) makes the case against classifying the *P. macrostoma* variant strains based on rDNA sequence alone. Ultimately, this study provides novel and relatively extensive baseline rDNA sequence information for *P. macrostoma*, identifies a new Indiana population of *P. macrostoma*, and suggests the morphological

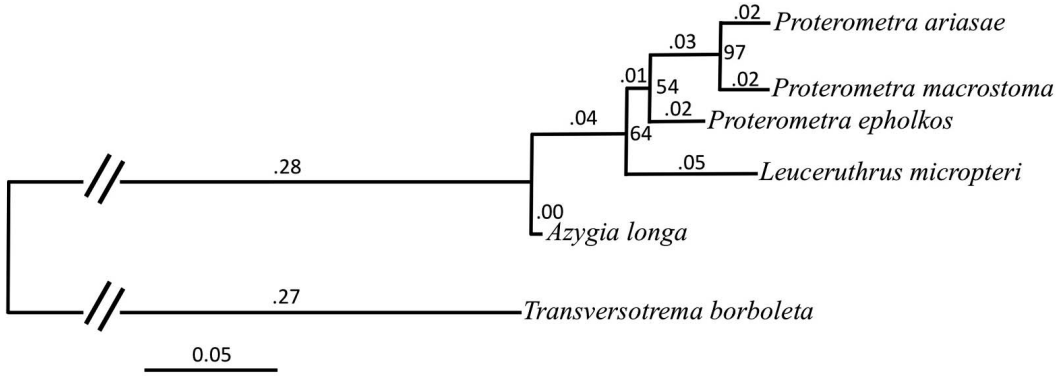

Figure 4.—Maximum Likelihood phylogenetic tree placing *Proterometra macrostoma* among other azygiid trematodes inferred from internal transcribed spacer 2 (ITS2) rDNA sequences. The percentage of trees yielding clustered taxa is indicated to the right of nodes and substitutions per site are indicated above each branch. GenBank sequence accessions used are as follows: *Proterometra ariasae* (KT808317.1), *Proterometra macrostoma* (MF927955), *Proterometra epholkos* (KM503118.1), *Leuceruthrus micropteri* (KT808320.1), *Azygia longa* (KT808319.1), and *Transversotrema borboleta* (JF412524.1). *Transversotrema borboleta* was assigned as the outgroup because of its phylogenetic relationship to the Azygiidae. The total characters used in the inference were 361. A scale bar is indicated.

phenotypes, present in both populations, lack sequence variation within the rDNA region examined. Our findings also contribute to a developing *Proterometra* phylogeny. Future phenetic studies to examine spined and unspined papillae structure and gene expression/regulation in another Indiana population are warranted and could provide additional insight into this system (Krist 2000).

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