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Short Communication

Molecular evidence of undescribed *Ceratonova* sp. (Cnidaria: Myxosporea) in the freshwater polychaete, *Manayunkia speciosa*, from western Lake Erie

David M. Malakauskas [a,*], R. Benjamin Snipes [a], Ann M. Thompson [a], Donald W. Schloesser [b]

[a] Francis Marion University, Department of Biology, P.O. Box 100547, Florence, SC 29502, USA
[b] U.S. Geological Survey, Great Lakes Science Center, 1451 Green Road, Ann Arbor, MI 48105, USA

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ABSTRACT

We used PCR to screen pooled individuals of *Manayunkia speciosa* from western Lake Erie, Michigan, USA for myxosporean parasites. Amplicons from positive PCRs were sequenced and showed a *Ceratonova* species in an estimated 1.1% (95% CI = 0.46%, 1.8%) of *M. speciosa* individuals. We sequenced 18S, ITS1, 5.8S, ITS2 and most of the 28S rDNA regions of this *Ceratonova* sp., and part of the protein-coding EF2 gene. Phylogenetic analyses of ribosomal and EF2 sequences showed the Lake Erie *Ceratonova* sp. is most similar to, but genetically distinct from, *Ceratonova shasta*. Marked interspecific polymorphism in all genes examined, including the ITS barcoding genes, along with geographic location suggests this is an undescribed *Ceratonova* species. COI sequences showed *M. speciosa* individuals in Michigan and California are the same species. These findings represent a third parasite in the genus *Ceratonova* potentially hosted by *M. speciosa*.

1. Introduction

The Myxozoa compose a parasitic cnidarian subphylum that exhibits complex life cycles. These parasites require a definitive annelid or bryozoan host, and a vertebrate intermediate host, which is typically a fish (Bartošová et al., 2009). While Myxozoa are common in aquatic systems, some cause substantial mortality in economically-valuable wild and cultured fishes, making them a concern to natural resource managers (Malakauskas and Wilzbach, 2012; Malakauskas et al., 2013). For example, *Ceratonova shasta* Noble causes annual mortality of up to 40% in juvenile salmonids in the main stem of the lower Klamath River, Oregon-California, USA (Foott et al., 1999, 2004). Therefore identification of *Ceratonova* populations is important because they may play a role in fish population dynamics.

Traditionally, spore morphology has been used to identify and describe myxozoan species (Shulman, 1966). However, spore morphology is inconsistent throughout parasite life cycles, and cannot be used to differentiate between cryptic species (e.g., Hartigan et al., 2011). Some molecular markers, including 18S, 28S and EF2 genes have been successfully used to evaluate higher level phylogenetic relationships, but have limited ability to differentiate individual species (Bartošová et al., 2009; Atkinson et al., 2015); however, recent studies have successfully used ribosomal ITS regions to distinguish between myxozoan species (Atkinson et al., 2015).

In this study, we used a PCR-based assay to screen for the presence of *Ceratonova* in the Laurentian Great Lakes. Herein, we present the first evidence of an undescribed *Ceratonova* species found in samples of the polychaete, *Manayunkia speciosa* Leidy, from western Lake Erie, Michigan, USA.

2. Methods

2.1. Specimen collection

M. speciosa individuals from western Lake Erie, Michigan, USA were collected with an Ekman sampler (42.033333°N, 83.177778°W; Schloesser, 2013). Samples from the Klamath River (41.84925°N, 122.571167°W) and Crystal Lake (40.933900°N, 121.549596°W), California, USA were collected during previous studies on the biology of *M. speciosa* (Malakauskas and Wilzbach, 2012; Willson et al., 2010). Samples were rinsed through sieves, decanted, preserved in 95% ethanol (Schloesser, 2013), and taken to the laboratory where they were examined using a variable-

* Corresponding author.
E-mail addresses: dmalakauskas@fmarion.edu (D.M. Malakauskas), rsnipes2382@g.fmarion.edu (R.B. Snipes), athompson4804@g.fmarion.edu (A.M. Thompson), dschloesser@usgs.gov (D.W. Schloesser).

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power dissecting microscope for the presence of *M. speciosa* individuals. An intestinal scraping from a Chinook salmon (*Oncorhynchus tshawytscha* Walbaum) containing *C. shasta* myxospores was provided by S. Foott, U.S. Fish and Wildlife Service, California-Nevada Fish Health Center, Anderson, California, USA.

2.2. DNA extraction

Specimens for molecular characterization of the Lake Erie *Ceratonova* isolate and prevalence assays were pooled into 200 groups of 5 individuals prior to DNA extraction. *C. shasta* myxospores were isolated from intestinal debris using centrifugation in a 55% glucose solution (USFWS and AFS-FHS, 2014) prior to DNA extraction. DNA extractions were performed following Malakauskas et al. (2015). PCR inhibitors were removed from DNA from California *M. speciosa* specimens using the OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, California) following manufacturer's instructions.

2.3. PCR and sequencing

PCR was performed using standard methods. Initial PCR screening of specimens used primers ERIB1 (Barta et al., 1997) and ACT1r (Hallett and Diamant, 2001), however amplification of the Lake Erie isolate was inconsistent with these primers. Therefore, we sequenced our first successful amplicon and used it to design a nested assay with primers ERIB1 and Cer18S1774R and primers Cer18S704F and Cer18S1468R to determine *Ceratonova* prevalence in *M. speciosa* (Supplementary Data A). A dilution series to evaluate assay sensitivity showed this assay detected one spore per pool of five *M. speciosa* individuals.

Primers ERIB1, Cer18S1774R, Cer18S1469F, NC13R (Gasser et al., 1993), NLF160, NLF1050, NLR1260, NLR3284 (Bartošová et al., 2009) were used to amplify 18S, ITS1, 5.8S, ITS2, and 28S regions of rDNA (present study; Barta et al., 1997; Bartošová et al., 2009; Gasser et al., 1993; Machida and Knowlton, 2012). As only an incomplete ITS2 sequence was available for *C. shasta* (http://www.ncbi.nlm.nih.gov/), we amplified this region using primers Cer18S1469F andCer28SR1. Primers CerEF2F and CerEF2R and EF2int2F, designed using a *C. shasta* EF2 sequence or taken from the literature (present study; Bartošová et al., 2013), were used to amplify a portion of elongation factor 2 (EF2). Sequences for listed primers are present in Supplementary Data B. Sequenced amplicons were used to design additional primers (not shown) for sequencing when needed.

Cytochrome *c* oxidase, subunit I (COI) barcoding genes were amplified in *M. speciosa* using primers LCO 1490 and HCO 2198 (Folmer et al., 1994).

Amplicons used to characterize *Ceratonova* spp. and *M. speciosa* were PCR-purified using the UltraClean PCR Clean-Up kit (MO BIO Laboratories, Carlsbad, California) and sent to the Georgia Genomics Facility at the University of Georgia (Athens, Georgia) for sequencing. Identities of *Ceratonova* amplicons from pooled-prevalence assays were confirmed by restriction digest with *Hin*dIII (New England BioLabs, Ipswich, Massachusetts).

2.4. Data analysis

Boundaries of 18S and ITS1 regions were found using data in Whipps et al. (2004). Boundaries for the 5.8S, ITS2 and 28S were found using a 5.8S database (Cullings and Vogler, 1998) and the ITS2 annotation online tool (http://its2-old.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/index.pl?annotator), using the Meta-

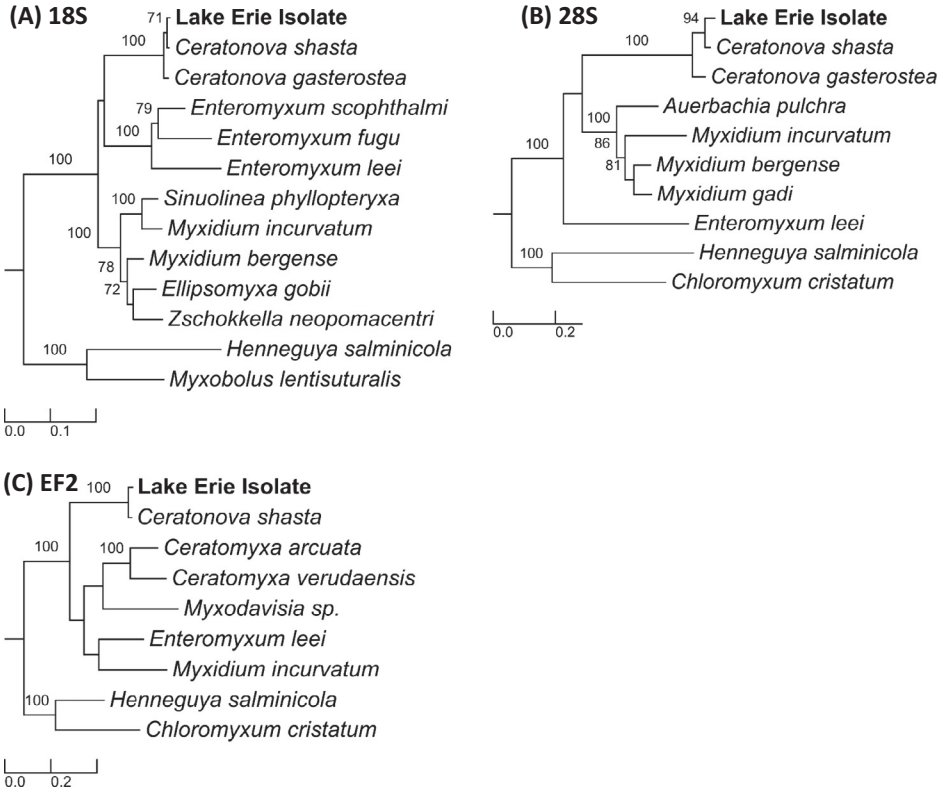

Fig. 1. Maximum likelihood (ML) analysis of DNA sequences. Trees were tested with 1000 bootstrap replicates. Only support values of 70 or greater are shown. Scale bars represent substitutions per nucleotide. (A) Analysis of 18S rooted on *Henneguya salminicola* and *Myxobolus lentisuturalis*. (B) Analysis of 28S rooted on *H. salminicola* and *Chloromyxum cristatum*. (C) Analysis of EF2 rooted on *H. salminicola* and *C. cristatum*.