J. Parasitol., 98(4), 2012, pp. 873–875

Phylogenetic Position of *Creptotrema funduli* in the Allocreadiidae Based on Partial 28S rDNA Sequences

Stephen S. Curran, Eric E. Pulis, Dennis O. Hugg, Jessica P. Brown, Lynnae C. Manuel, and Robin M. Overstreet, The University of Southern Mississippi, Department of Coastal Sciences, 703 East Beach Drive, Ocean Springs, Mississippi 39564. *e-mail: stephen.curran@usm.edu*

ABSTRACT: The infrequently reported allocreadiid digenean *Creptotrema funduli* Mueller, 1934 is documented from the blackstripe topminnow, *Fundulus notatus* (Cyprinodontiformes: Fundulidae), in the headwaters of the Biloxi River, Harrison County, Mississippi. Specimens from Mississippi were compared with the type material from *Fundulus diaphanus menona* from Oneida Lake, New York, and no substantial difference was found. A fragment of ribosomal DNA, comprising a short portion of the 3′ end of 18S nuclear rDNA gene, internal transcribed spacer (ITS) genes (including ITS1, 5.8S, and ITS2), and the 5′ end of the 28S gene including variable domains D1–D3 was sequenced for the species. A portion of the 28S rDNA gene from *C. funduli*, plus similar fragments from 8 other allocreadiids and the callodistomatid *Prosthenhystera* sp., were aligned and subjected to maximum likelihood and Bayesian inference analyses. Resulting phylogenetic trees were derived from the analyses and used to estimate the relationship of *Creptotrema* Travassos, Artigas, and Pereira, 1928 with other allocreadiids. *Creptotrema* was found to be closely related to *Megalogonia* Surber, 1928 and 3 Neotropical genera, i.e., *Wallinia* Pearse, 1920, *Creptotrematina* Yamaguti, 1954, and *Auriculostoma* Scholz, Aguirre-Macedo, and Choudhury, 2004. No molecular data were available for species in *Creptotrema* prior to this study, so the ITS1, 5.8S, and ITS2 genes have been made available for comparative studies involving neotropical species in the genus.

Creptotrema funduli Mueller, 1934 (Figs. 1, 2) is a digenean belonging in the Allocreadiidae Looss, 1901. *Creptotrema* Travassos, Artigas, and Pereira, 1928 includes *C. funduli* from northern North America, *Creptotrema agonostomi* Salgado-Maldonado, Cabañas-Carranza, and Caspeta-Mandujano, 1998 from Mexico, and 6 other species indigenous to South America (Curran 2008). In the present study, *C. funduli* is reported from a new host, the blackstripe topminnow, *Fundulus notatus* (Rafinesque, 1820) (Cyprinodontiformes), in the upper reaches of the Biloxi River, Harrison County, Mississippi. Specimens from Mississippi were compared critically with the type material, and nuclear ribosomal genes were sequenced from extracted genomic DNA (from Mississippi material). We investigated the phylogenetic relationship of *C. funduli* within the Allocreadiidae based on maximum likelihood (ML) and Bayesian inference (BI) analyses using partial sequences of the 28S rDNA gene. The data produced are based on new genetic material plus sequences downloaded from GenBank.

Thirty individuals of the blackstripe topminnow were collected from a single site on the Big Biloxi River in Harrison County, Mississippi (30°34.093′N, 89°07.842′W) from 2 May through 1 September 2011. Fish were transported live to our laboratory where they were killed and examined for parasites within 12 hr. Forty-seven specimens of *C. funduli* were collected from the intestine of 18 fish (prevalence of infection 60%). Mean intensity of infections was 2.6 worms per infected fish. Live worms were rinsed in saline solution (7.5 ppt), examined using a dissecting microscope, and either transferred to 95% ethanol (specimens destined for genomic DNA extraction) or killed with hot tap water and transferred to a 10% neutral buffered formalin solution (specimens destined for vouchering). Formalin-fixed specimens were rinsed in distilled water and stained using aqueous Van Cleave's hematoxylin following the procedure by Curran (2008).

Five vouchers were deposited in the United States National Parasite Collection, Beltsville, Maryland (USNPC Nos. 105238–105241), and 5 vouchers were deposited at The University of Southern Mississippi's (USM) Gulf Coast Research Laboratory (GCRL) Museum, Ocean Springs, Mississippi (GCRL Museum Nos. 3069–3073). Genomic DNA was extracted individually from 6 ethanol-fixed specimens using a Qiagen DNeasy extraction kit (Qiagen Inc., Valencia California) following the instructions provided. DNA fragments measuring approximately 2,500 base pairs long, comprising a short portion of 3′ end of 18S nuclear rDNA gene, internal transcribed spacer (ITS) genes (ITS1, 5.8S, and ITS2), and the 5′ end of the 28S gene including variable domains D1–D3 were amplified from each DNA sample by polymerase chain reaction (PCR) using the forward primer ITSF (5′-CGCCCGTCGCTACTACCGATTG-3′) and the reverse primer 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′). Amplified PCR

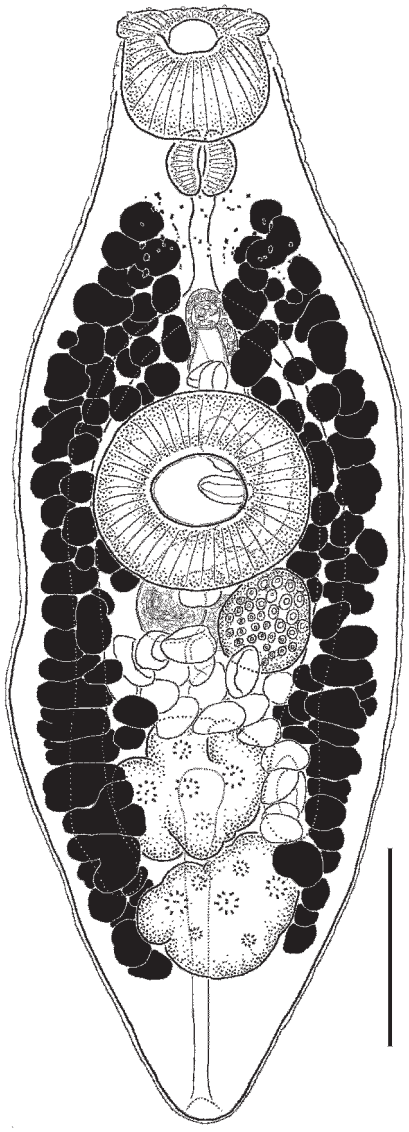

FIGURE 1. *Creptotrema funduli* Mueller, 1934. Ventral view of whole-mount collected from *Fundulus notatus* in the Biloxi River, Harrison County, Mississippi. Scale bar = 200 μm.

DOI: 10.1645/GE-3066.1

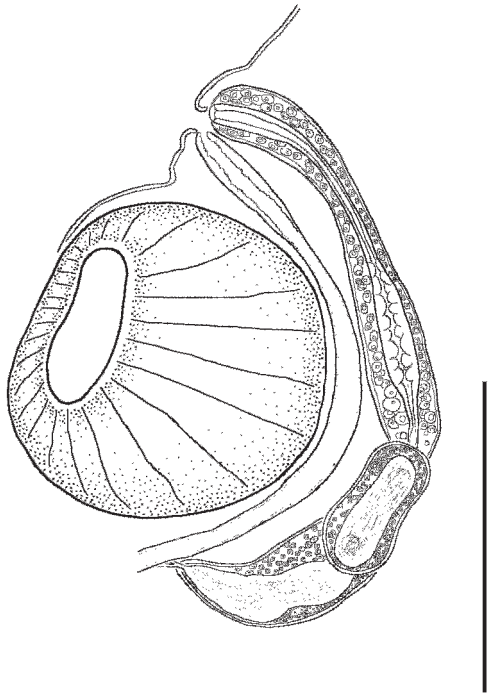

FIGURE 2. *Creptotrema funduli* Mueller, 1934. Terminal genitalia of laterally mounted specimen from *Fundulus notatus* in the Biloxi River, Harrison County, Mississippi. Scale bar = 200 μm.

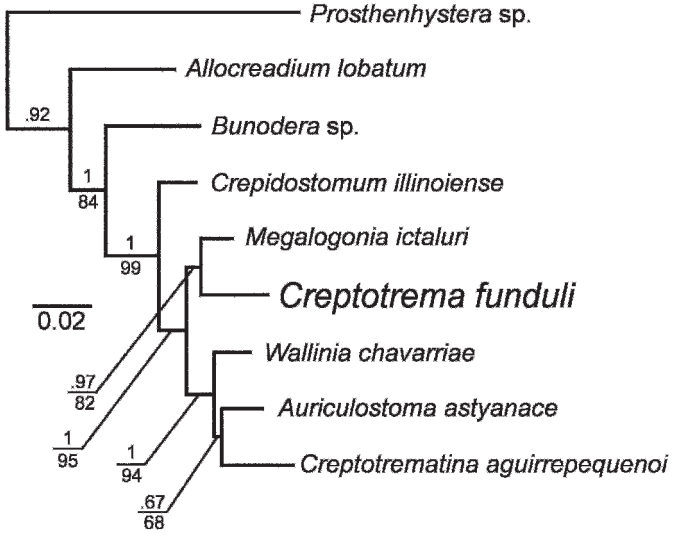

FIGURE 3. Phylogram of relationships among some genera of the Allocreadiidae based on Bayesian inference analysis of 28S rDNA. Posterior probabilities are shown above the nodes and bootstrap support values are shown below. The phylogram is mid-point rooted. Scale bar = 0.02% sequence variance.

products were gel-purified with 1.2% agarose gel in 1× TAE buffer. Samples of PCR product were cut from the gel, and samples were extracted and purified using Qiagen QIAquick™ columns according to the manufacturer's instructions. Purified products were again amplified using ITSF and 1500R primers plus 900F (5′-CCGTCTTGAAACACGGAC-CAAG-3′), 300F (5′-CAAGTACCGTGAGGGAAAGTTG-3′), 300R (5′-CAACTTTCCCTCACGGTACTTG-3′), digl2 (5′-AAGCATATCAC-TAAGCGG-3′), digl2R (5′-CCGCTTAGTGATATGCTT-3′), ECD2 (5′-CTTGGTCCGTGTTTCAAGACGGG-3′), and d58R (5′-GTCGATG-TTCAAAGCAGTATGC-3′). Purified reactions were then sequenced using an Applied Biosystems (Carlsbad, California) 3130™ Genetic Analyzer automated DNA sequencer with Applied Biosystems BigDye™ terminator chemistry. Continuous sequences were assembled and edited using Sequencher® version 5.0 sequence analysis software (Gene Codes Corporation, Ann Arbor, Michigan). Sequences were aligned with the Clustal_W module in BioEdit version 7.0.9 (Hall, 1999) and further edited by eye. Sequences from all 6 isolates of *C. funduli* were identical.

A representative sequence was submitted to GenBank under accession number JQ425256. All sequences measured 2,627 bases long and consisted of a fragment of the 3′ end of 18S nuclear rDNA gene (28 bases), ITS1 (750 bases), 5.8S (152 bases), ITS2 (289 bases), and a fragment of the 5′ end of the 28S gene including variable domains D1–D3 (1,380 bases). The interface between the ITS2 and 28S fragment was located using the Internal Transcribed Spacer 2 Ribosomal Database (Keller et al., 2009). The partial 28S rDNA sequence from *C. funduli* was then aligned with comparable sequences obtained from GenBank as follows: *Allocreadium lobatum* Wallin, 1909 (EF032693); *Auriculostoma astyanace* Scholz, Aguirre-Macedo, and Choudhury, 2004 (HQ833707); *Bunodera* sp. (HQ833704); *Crepidostomum illinoiense* Faust, 1918 (HQ833705); *Creptotrematina aguirrepequenoi* Jiménez-Guzmán, 1973 (HQ833708); *Megalogonia ictaluri* Surber, 1928 (EF032694); and *Wallinia chavarriae* Choudhury, Hartvigsen-Davardin, and Brooks, 2002 (HQ833703). The alignment was trimmed on each end to the length of the shortest sequence. The resulting alignment was 1,236 bases with gaps. Phylogenetic analysis of the data was performed using BI with MrBayes version 3.1.2 software (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The best nucleotide substitution model was estimated with jModeltest 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008), based on Akaike Information Criterion, as general time reversible with estimates of invariant sites and gamma-distribution among-site rate variation (GTR+I+T).

The following model parameters were used in MrBayes: nst = 6, rates = invgamma, ngen = 1,000,000, samplefreq = 100. The first 2,500 trees were discarded as "burn-in". The burn-in value was 2,500, estimated by plotting the log-probabilities against generation and visualizing plateau in parameter values, (sump burnin = 2,500). Nodal support was estimated by posterior probabilities using the sumt command (Huelsenbeck et al., 2001). The analysis was mid-point rooted, treating *Prosthenhystera* sp. (Callodistomidae Odhner, 1910) (GenBank EF032690) as part of the ingroup. ML analysis using heuristic search options was also conducted using PAUP* 4.0b10 (Swofford, 2002) with the analysis rooted by *Prosthenhystera* sp. The resulting best tree showed identical topology to that derived by BI. Nodal support for the ML-derived tree was inferred by bootstrap analysis using 1,000 replicates, with each replicate terminating after 100 addition-sequence replicates. The BI derived tree is presented as a mid-point rooted phylogram with posterior probability values presented above bootstrap values on each branch (Fig. 3).

For the most part, morphological observations and measurements of key features from specimens of *C. funduli* from Mississippi (n = 18) conformed to the diagnosis for the genus (see Caira and Bogéa, 2005) and to measurements given in the original description for the species (Mueller, 1934). Our specimens from Mississippi were slightly larger overall, ranging from 730–1,375 μm long by 236–421 μm wide, compared with 685–1,000 μm long by 274–300 μm wide (Mueller, 1934). In addition, the pharynx was generally slightly larger in the Mississippi specimens, ranging from 48–77 μm long by 57–71 μm wide compared with an average diameter of 55 μm in the original description (Mueller, 1934). Mueller (1934) presented line drawings of specimens with an oral sucker-to-ventral sucker-width ratio of 1:1.4–1.5. We observed the oral sucker-to-ventral sucker-width ratios from the syntypes (USNPC No. 32543) (n = 7) to range from 1:1.2–1.5. The same ratio from Mississippi specimens (n = 18) was 1:1.2–1.4. In both the generic diagnosis by Caira and Bogéa (2005) and in the original description, the margin of the testes is reported to be smooth (=entire), but we observed that the testicular margins were somewhat irregular (with lobes extending in length less than half their width) in 13 specimens from Mississippi (all larger than 1,011 μm in total body length), and we observed the uterus encroaching on the posterior testis in 3 of the larger specimens from Mississippi. Remnants of eyespots are expected in adults of all allocreadiid species, but they were not previously reported for *C. funduli*. We observed prominent remnants of eyespots in the forebody of the syntypes deposited by Mueller (1934) and in all Mississippi specimens. The slight differences between the Mississippi specimens and the original series should be attributed to the relatively

TABLE I. Reports of adult specimens of *Creptotrema funduli*, host species (Order: Family), locality, and museum specimens deposited.

Author	Host	Locality	Museum specimens
Mueller, 1934	*Fundulus diaphanus menona* Jordan & Copeland, 1877 (Cryprinodontiformes)	Oneida Lake, New York	7 Syntypes USNPC No. 32543
Bangham, 1937	*Culea inconstans* (Kirtland, 1840) (Gasterosteiformes)	Wayne County, Ohio	None
Bangham, 1940	*Fundulus chrysotus* (Günther, 1866) (Cryprinodontiformes)	Charlotte County, Florida	None
Bangham, 1955	*Umbra limi* Kirtland, 1840) (Esociformes)	Lake Huron	None
Wiles, 1975	*Fundulus diaphanus diaphanus* (Cryprinodontiformes)	Nova Scotia, Canada	None
Dechtiar and Christie, 1988	*U. limi*	Ontario, Canada	National Museum of Natural Sciences, Ottawa, Canada*
Dechtiar et al., 1988	*U. limi*	Ontario, Canada	National Museum of Natural Sciences, Ottawa, Canada*
Szalai and Dick, 1991	*Perca flavescens* (Mitchill, 1814) (Perciformes)	Manitoba, Canada	None

* Parasites from this study were temporarily held at University of Guelph, Ontario, Canada pending final deposition at the National Museum of Natural Sciences, Ottawa, Canada.

small sample sizes from which data has been obtained thus far for the species.

The tree we produced agrees in topology with previously published estimates of phylogeny for the Allocreadiidae (see Curran et al., 2011 and references therein). In the present analyses, *Creptotrema* was most closely related to the North American genus *Megalogonia* Surber, 1928, a genus represented by a single species, *M. ictaluri*, in ictalurid catfishes (Siluriformes) (Fig. 3). If it is assumed that *C. funduli* and the 7 other species in the genus represent a natural group, the present tree and the previously mentioned ones suggest that South American allocreadiids are paraphyletic and probably have ancestors with North American origins.

Mueller (1934) described *C. funduli* from *Fundulus diaphanus menona* Jordan and Copeland, 1877 (Cyprinodontiformes) from Oneida Lake, New York. The species has since been reported on a number of occasions from various fishes throughout North America. Reports of adults collected are presented in Table I. The other North American species, *C. agonostomi*, occurs in the Balsas catfish, *Ictalurus balsanus* Jordan and Snyder, 1900 (Siluriformes) and in the mountain mullet, *Agonostomus mugilicola* (Bancroft, 1834) (Mugiliformes) in Mexico (Salgado-Maldonado et al., 1998). Interestingly, *Creptotrema* is not known from Central America, but 6 species are present in South America where they parasitize indigenous pimelodids and headstanding catfishes (Siluriformes), anostomids (Characiformes), at least 1 cichlid (Perciformes), and 1 toad (Anura) (see Curran, 2008). It is apparent that species of *Creptotrema* utilize definitive hosts from a wide range of vertebrate orders.

We are grateful for the help with molecular techniques provided by Jean A. Jovonovich and Janet Wright (both from the University of Southern Mississippi [USM]), and we thank the students in the 2011 USM Summer Field Program for assisting with collections. Eric P. Hoberg and Patricia A. Pilitt (USNPC) provided the loan of the type material for *C. funduli*. This material is based on work supported by the National Science Foundation under grant no. 0529684 and USDC, NOAA award no. NA08NOS4730322.

LITERATURE CITED

BANGHAM, R. V. 1937. Parasites of Wayne County (Ohio) stream fish. Transactions of the American Fisheries Society **66:** 357–358.

———. 1940. Parasites of fresh-water fish of southern Florida. Proceedings of the Florida Academy of Sciences **5:** 289–307.

———. 1955. Studies on fish parasites of Lake Huron and Manitoulin Island. American Midland Naturalist **53:** 184–194.

CAIRA, J. N., AND T. BOGÉA. 2005. Family Allocreadiidae Looss, 1902. *In* Keys to the Trematoda, Vol. II, A. Jones, R. A. Bray, and D. I. Gibson (eds.). CABI Publishing and the Natural History Museum, London, U.K., p. 417–436.

CURRAN, S. S. 2008. Two new species of *Creptotrema* (Digenea: Allocreadiidae) from South America. Revista Mexicana de Biodiversidad **79:** 15S–21S.

———, V. V. TKACH, AND R. M. OVERSTREET. 2011. Phylogenetic affinities of *Auriculostoma* (Digenea: Allocreadiidae), with descriptions of two new species. Journal of Parasitology **97:** 661–670.

DECHTIAR, A. O., AND W. J. CHRISTIE. 1988. Survey of the parasite fauna of Lake Ontario fishes. *In* Parasites of fishes in the Canadian waters of the Great Lakes, S. J. Nepszy (ed.). Great Lakes Fisheries Commission Technical Report 51, p. 66–95.

———, J. J. COLLINS, AND J. A. RECKAHN. 1988. Survey of the parasite fauna of Lake Huron fishes. *In* Parasites of fishes in the Canadian waters of the Great Lakes, S. J. Nepszy (ed.). Great Lakes Fisheries Commission Technical Report 51, p. 19–48.

GUINDON, S., AND O. GASCUEL. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology **52:** 696–704.

HALL, T. 1999. BioEdit version 7.0.9 (updated 2007). Ibis Biosciences, Carlsbad, California.

HUELSENBECK, J. P., AND F. RONQUIST. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics **17:** 754–755.

———, ———, R. NIELSEN, AND J. P. BOLLBACK. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science **294:** 2310–2314.

KELLER, A., T. SCHLEICHER, J. SCHULTZ, T. MÜLLER, T. DANDEKAR, AND M. WOLF. 2009. 5.8S–28S rRNA interaction and HMM-based ITS2 annotation. Gene **430:** 50–57.

MUELLER, J. F. 1934. Two new trematodes from Oneida Lake fishes. Transactions of the American Microscopical Society **53:** 231–236.

POSADA, D. 2008. jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution **25:** 1253–1256.

RONQUIST, F., AND J. P. HUELSENBECK. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:** 1572–1574.

SALGADO-MALDONADO, G., G. CABAÑAS-CARRANZA, AND J. M. CASPETA-MANDUJANO. 1998. *Creptotrema agonostomi* n. sp. (Trematoda: Allocreadiidae) from the intestine of freshwater fish of Mexico. Journal of Parasitology **82:** 431–434.

SWOFFORD, D. L. 2002. PAUP* version 4.0b10: Phylogenetic analysis using parsimony (and other methods). Sinauer Associates, Sunderland, Massachusetts.

SZALAI, A. J., AND T. A. DICK. 1991. Role of predation and parasitism in growth and mortality of yellow perch in Dauphin Lake, Manitoba. Transactions of the American Fisheries Society **120:** 739–751.

WILES, M. 1975. Parasites of *Fundulus diaphanous* (LeSueur) (Pisces: Cyprinodontidae) in certain Nova Scotian freshwaters. Canadian Journal of Zoology **53:** 1578–1580.