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DESCRIPTION OF TWO NEW GILL MYXOZOANS FROM SMALLMOUTH (*MICROPTERUS DOLOMIEU*) AND LARGEMOUTH (*MICROPTERUS SALMOIDES*) BASS

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ABSTRACT: Two previously undescribed species of myxozoan parasites were observed in the gills of bass inhabiting the Potomac and James River basins. They are described using morphological characteristics and small-subunit (SSU) rDNA gene sequences. Both were taxonomically identified as new species of *Myxobolus*; *Myxobolus branchiarum* n. sp. was found exclusively in smallmouth bass, and *Myxobolus micropterii* n. sp. was found in largemouth and smallmouth bass. Small, spherical, white plasmodia of *M. branchiarum* from smallmouth bass were observed grossly in the gills; these plasmodia had an average length of 320.3 μm and width of 246.1 μm. The development of the plasmodia is intralamellar in the secondary lamellae of the gills. Mature spores were pyriform in shape with a length of 12.8 ± 1.4 (8.1–15.1) μm and width of 6.9 ± 1.1 (4.0–9.0) μm. Analysis of SSU rDNA identified *M. branchiarum* in a sister-group to 3 species of *Henneguya*, although morphologically caudal appendages were absent. *Myxobolus micropterii* observed in the gills of largemouth and smallmouth bass had larger, ovoid, cream-colored plasmodia with an average length of 568.1 μm and width of 148.1 μm. The cysts developed at the distal end of the gill filament within the primary lamellae. The mature spores were ovoid in shape with a length of 10.8 ± 0.7 (9.2–12.2) μm and width of 10.6 ± 0.6 (9.0–11.8) μm. SSU rDNA analysis placed *M. micropterii* in a sister group with *Henneguya lobosa* and *Myxobolus oliveirai*. The highest prevalence of *M. branchiarum* was observed in the gills of bass collected from the Cowpasture River (50.9%). Prevalence was 44.6% in bass from the Potomac River and only 4.3% in bass collected from the Shenandoah River. A seasonal study of *M. branchiarum*, which included both infected and uninfected smallmouth bass, determined that a significantly higher intensity was observed in the spring than in the summer ($P < 0.001$) or fall ($P = 0.004$). In an analysis excluding uninfected bass, a higher intensity was observed in the spring than in the summer ($P = 0.001$) or fall ($P = 0.008$). Prevalence and seasonal differences were not determined for *M. micropterii*.

Since 2002, spring mortalities, primarily of adult smallmouth bass (*Micropterus dolomieu*), of varying severity have been observed in a number of tributaries of the Potomac River. Small white cysts were observed on the gills of fish sampled during the mortality events and during additional seasonal studies conducted in 2004 in the South Branch Potomac River and in 2006 in the Shenandoah River (Potomac drainage) and Cowpasture River (James drainage). Histological analyses indicated that these cysts were plasmodia containing myxozoan parasites. Myxozoan plasmodia were also sampled from the gills of adult largemouth bass (*Micropterus salmoides*) during a low dissolved oxygen mortality event at a West Virginia State fish hatchery located in the Ohio River drainage in 2006. The same types of oval-shaped plasmodia were also later found on the tips of gill filaments from smallmouth bass in the South Branch Potomac and Shenandoah Rivers. No other species of myxozoans were isolated from the gills.

More than 19 genera of myxozoans are known to parasitize freshwater fishes in North America (Hoffman, 1999). The genus *Myxobolus* Butschli, 1882, includes approximately 800 species worldwide and is the most commonly observed myxozoan to parasitize fishes (Eiras et al., 2005). *Henneguya* Thélohan, 1892, is the second largest group of freshwater myxozoans that infect fish, and this genus is closely related to *Myxobolus* (Eiras, 2002). Most species in the 2 genera are histozoic and have traditionally been identified by the species of fish they infect, infection site, and spore morphology. A distinguishing diagnostic feature of *Henneguya* is the presence of caudal projections (Lom and Dyková, 2006). Species of both genera are commonly observed in the gills of many species of fish. Tissue tropism (epithelium, connective tissue, cartilage, or vascular) and affinity within the gills for specific infection sites (intralamellar, interlamellar, or gill arch) are important characteristics for species identification (Hoffman, 1999; Molnár, 2002).

To date, no *Henneguya* species and only 5 species of *Myxobolus* (3 from largemouth bass and 2 from smallmouth bass) have been identified in the black basses. *Myxobilatus mictospora* in the urinary bladder was originally named *Henneguya mictospora*, but was later redescribed as belonging to *Myxobilatus* (Kudo, 1920; Booker and Current, 1981). Only *Myxobolus microcystis* in gill lamellae (Price and Mellen, 1980) and *Myxobolus cartilaginis* in the cartilage of the gill arches (Hoffman et al., 1965) have been observed from gill tissue of largemouth bass.

The phylogenetic classification of myxozoan species has been questioned due to a lack of appropriate data, inadequate descriptions, and plasticity of spore morphology (Andree et al., 1999; Salim and Desser, 2000). An example of this inadequacy is demonstrated by *Myxobolus exiguus*, which has tissue tropism and morphology analogous to 5 different species (Bahri and Marques, 1996). The use of molecular techniques, specifically SSU and LSU rDNA (small- and large-subunit ribosomal DNA), has eliminated phenotypic plasticity as a source of diagnostic uncertainty and improved accuracy in species identifications (Salim and Desser, 2000; Eszterbauer, 2004; Whipps et al., 2004; Fiala, 2006).

The goal of the present study was to characterize 2 previously undescribed species of *Myxobolus*. An extensive literary review of both *Myxobolus* and *Henneguya* genera did not produce any species similar to those found in this study. Morphological characteristics and SSU rDNA gene sequences were used to describe these species and discriminate their identity from previously identified myxozoans. A seasonal intensity analysis of *Myxobolus branchiarum*, which infects the gills of smallmouth bass, was also conducted.

MATERIALS AND METHODS

Fish collection and field processing

Approximately 20 smallmouth bass were collected during the spring, summer, and fall of 2004 from the South Branch Potomac River and in

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TABLE I. Primers used for amplification and sequencing of *Myxobolus* from the gills of smallmouth and largemouth bass. The primer 18R was coupled with primers HEN2146F, 3F, and MYX472F as 3 individual sets.

Primer	Sequence (5′-3′)	Reference
MYXF	ACC GTG GGA AAT CTA GAG CTA	Iwanowicz et al. (2008)
MYXR	GTT CCA TGC TAT YAA CAT TCA A	Iwanowicz et al. (2008)
HEN2146F	ACC GTG GGA AAT CTA GAG CTA ATA C	This paper
MYXGEN3F	GGA CTA ACR AAT GCG AAG GCA	Jirku et al. (2006)
MYX472F	AGC AGG CGC GCA AAT TAC CCA ATC C	This paper
18R	CTA CGG AAA CCT TGT TAC G	Whipps et al. (2004)

2006 from the Shenandoah (a tributary of the Potomac) and Cowpasture (a tributary of the James) Rivers. Smallmouth bass were collected by electroshocking, or they were netted if found floating moribund. The bass were killed by submersion into a lethal dose of tricaine methanesulfonate (MS-222, Argent Finquel®, Redmond, Washington), and any gross abnormalities were noted. Gills were macroscopically examined for the presence of plasmodia, and samples were placed in phosphate-buffered saline (PBS) for wet mount preparations or in Z-Fix™ (Anatech Ltd., Battle Creek, Michigan) for preservation and subsequent histological evaluation. Samples of moribund largemouth bass from a West Virginia State fish hatchery were sent on ice to the U.S. Fish and Wildlife Service Fish Health Center in Lamar, Pennsylvania in 2006. Tissues preserved in 10% neutral buffered formalin were processed for histological evaluation.

Morphological and histological analyses

Morphological descriptions were made by examining tissue sections and wet mount slides according to the guidelines set forth by Lom and Arthur (1989). Wet mounts of smallmouth bass gills were prepared in PBS. Wet mounts of largemouth bass gills samples were prepared using a sample fixed in 96% alcohol. Plasmodia were viewed intact, or compressed to release the contents. Slides were viewed with a Nikon Eclipse E600 microscope, images were captured with a Hitachi HV-C20 CCD camera, and spores and plasmodia were measured with Image-Pro Plus 3.0 (Media Cybernetics, Silver Spring, Maryland) image analysis software. Spores were air dried and stained with Giemsa to enhance spore staining and Lugol's iodine solution to identify the presence of an iodinophilous vacuole. All measurements are in μm, unless otherwise indicated.

Gill tissue from smallmouth and largemouth bass was fixed for at least 24 hr in 10% Z-Fix or 10% neutral buffered formalin. Tissues were routinely processed for histology, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin, or Giemsa (Luna, 1992). For electron microscopy, smallmouth bass gill tissue samples were fixed for 1 hr at room temperature in 2% glutaraldehyde in 0.1 M phosphate buffer, transferred to fresh fixative, and stored at 4 C. Samples were immediately shipped overnight to the Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Canada, for processing. Tissue was washed in 0.1 M phosphate buffer and transferred to 1% osmium tetroxide in phosphate buffer for 1 hr at room temperature. After osmium fixation, the tissue was dehydrated through a series of ethanol grades to propylene oxide. Next, the tissue was infiltrated with Spurr's resin (Canemco-Marivac, St. Laurent, Quebec, Canada) and propylene oxide in a ratio of 1:1 and then 3:1 for 2 hr each and in pure Spurr's resin overnight in a vacuum desiccator. Finally, tissues were embedded in pure Spurr's resin at 60 C for 24 hr. Semithin sections (0.5 mm) were cut from the gill tissue. The sections were stained with 1% toluidine blue in 1% sodium tetraborate solution and viewed with a light microscope to identify plasmodia. Ultrathin sections (90 nm) were prepared and retrieved onto copper super grids (200 mesh) and stained with uranyl acetate and Sato's lead stain. The sections were examined and photographed using a Hitachi H7500 transmission electron microscope operated at 80 kV.

DNA sequencing and analysis

Gill clips with plasmodia from bass were stored in 96% ethanol for DNA extraction. Genomic DNA was extracted using the DNeasy® Tissue Kit (Qiagen Inc., Valencia, California) according to the manufacture's protocol. The 18S rDNA gene cluster was targeted for amplification using PCR primers that targeted myxozoan sequences and excluded host sequences (Table I). Conditions for PCR reactions for the 4 primer sets are as follows. Smallmouth bass DNA samples were amplified with the MYXF-MYXR, HEN2146F/18R, 3F-18R, and MYX472F-18R primer sets and denatured at 95 C for 3 min. This was followed by 35 cycles of 95 C for 30 sec, 52 C for 35 sec, and 72 C for 90 sec. Products were subjected to a final extension at 72 C for 10 min and held at 4 C prior to sequencing for all PCR reactions. PCR products were analyzed and cleaned with QIAquick® PCR Purification Kit (Qiagen). Forward and reverse PCR primers were used as sequencing primers for the other respective reactions prior to sequencing.

Largemouth bass DNA samples were amplified with the MYXF-MYXR primer set. Amplicons were cloned into pCR®2.1 TOPO (Invitrogen, Carlsbad, California), and the resulting plasmids were used to transform Top 10®Competent Cells (Invitrogen). Plasmid DNA was extracted with QIAprep® Spin Miniprep Kit (Qiagen) and sequenced.

Myxozoan SSU rDNA sequences were acquired from the NCBI database and aligned with ClustalW v. 1.4 (Thompson et al., 1994). Phylogenetic analysis included the 23 most similar sequences to *Myxobolus branchiarum* and *M. micropterii*. *Kudoa thrysites* was used as an outgroup. Full sequences from the GenBank database were used for analysis (missing data and gaps were assigned as appropriate). Bayesian analysis was conducted using MrBayes 3.1.1 software (Ronquist and Huelsenbeck, 2003). Bayesian inference of phylogeny was conducted using the general time reversible (GTR+I+G) model based on the most appropriate DNA substitution model as calculated by MrModeltest v. 2.2 (Nylander, 2004). Parameters for the Bayesian analysis included 1,000,000 generations of Markov chain Monte Carlo sampling sampled once every 100 generations. A consensus phylogram was constructed based on the trees sampled in the asymptotic phase of the Bayesian analysis (burnin = 100, trees used for consensus = 600). The consensus phylogram was edited in Dendroscope v. 2.7.4 (Huson et al., 2007).

A sequence identity matrix was calculated for *M. micropterii* and *M. branchiarum* with the closest *Henneguya* spp. and *Myxobolus* spp. sequence matches identified using a low-complexity, E = 0.01 NCBI BLAST search (Table II). The resulting sequences were degapped and realigned in a pairwise fashion for all possible combinations using ClustalW v. 1.4 (Thompson et al., 1994). Sequence identities were determined for these pairwise alignments with the BioEdit Sequence Alignment Editor v. 7.0.1 (Hall, 1999).

Seasonal observations

Prevalence and seasonal intensity of *Myxobolus branchiarum* were quantified from smallmouth bass collected in 2004 from the South Branch of the Potomac River and in 2006 from the Shenandoah and Cowpasture Rivers. Histological sections of gills were examined for prevalence and qualitatively scored based on plasmodia intensity. Intensity values were determined based on seasonality and seasonality of prevalence. Intensities of parasite infections were scored as the number of plasmodia observed on 1 histological cross section of gill as follows: 0 for no plasmodia, 1 for 1–10 plasmodia, 2 for 11–20 plasmodia, 3 for 21–50 plasmodia, and 4 for 51 or more plasmodia. The intensity scores were analyzed with an ANOVA, followed by a Tukey test in the program R to determine seasonal differences (R Development Core Team, 2009). Prevalence and seasonal intensity of *Myxobolus micropterii* were not determined due to the low level of infection in smallmouth bass and a small sample size of largemouth bass.

TABLE II. Sequence identity (%) between nucleotide sequences of *Myxobolus branchiarum* and *Myxobolus micropterii* and 5 closely related myxozoan species. Bold font has been used to identify the 2 new species.

	Sequence identity matrix						
Sequence	*Myxobolus oliveirai* (HM754633)	*Henneguya* sp. (EU732599)	*Henneguya creplini* (EU732598)	*Henneguya lobosa* (EU732600)	*Henneguya doori* (U37549)	***Myxobolus branchiarum*** (JF714994)	***Myxobolus micropterii*** (JF714885)
Myxobolus oliveirai	ID	0.842	0.828	0.829	0.800	**0.821**	**0.823**
Henneguya sp.	0.842	ID	0.925	0.823	0.886	**0.867**	**0.825**
Henneguya creplini	0.828	0.925	ID	0.823	0.924	**0.845**	**0.824**
Henneguya lobosa	0.829	0.823	0.823	ID	0.795	**0.789**	**0.816**
Henneguya doori	0.800	0.886	0.924	0.795	ID	**0.813**	**0.783**
Myxobolus branchiarum	**0.821**	**0.867**	**0.845**	**0.789**	**0.813**	**ID**	**0.817**
Myxobolus micropterii	**0.823**	**0.825**	**0.824**	**0.816**	**0.783**	**0.817**	**ID**

DESCRIPTION

Myxobolus branchiarum n. sp.

(Figs. 1A, D, 2)

Diagnosis: White, subspherical-shaped plasmodia; length 320.3 ± 15.7, range 210.8–423.9. Width 246.1 ± 18.9, range 150.0–420.8 (n = 15). Spores pyriform in shape (Fig. 1A). Sutural ridge on posterior end. Sutural folds absent (Table III). Spore length 12.8 ± 0.1, range 8.1–15.1. Width 6.9 ± 0.1, range 4.0–9.0; thickness 5.7 ± 0.1, range of 4.8–7.1. Length-to-width ratio of spore 1.9. Spores with 2 polar capsules of about equal size. Polar filament length 59.2 ± 2.8, range 31.8–92.4; coiled 8–9 times and perpendicular to longitudinal axis of capsules (Fig. 2). Iodinophilous vacuole absent. All spore and plasmodia measurements made with PBS preserved gill samples.

Taxonomic summary

Type host: Smallmouth bass (*Micropterus dolomieu*).

Site of infection: Secondary lamellae of the gills.

Type locality: Potomac River (West Virginia and Maryland).

Additional type localities: Shenandoah River and Cowpasture River (Virginia).

Prevalence: One hundred of 224 (44.6%) fish infected from the Potomac River (2004), 29 of 57 (50.9%) fish infected from the Cowpasture River (2006), and 7 of 154 (4.3%) fish infected from the Shenandoah River (2006).

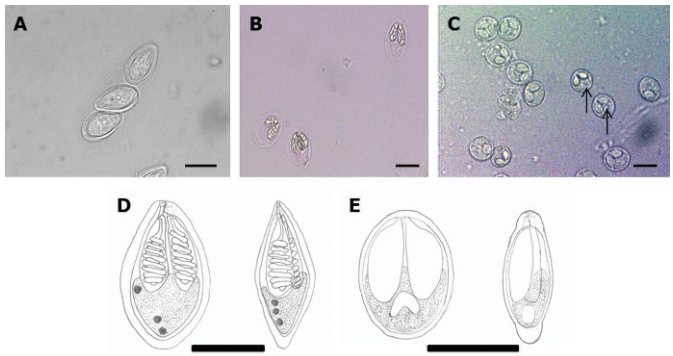

FIGURE 1. Three types of *Myxobolus* spp. known to parasitize the gills of smallmouth (*Micropterus dolomieu*) and largemouth (*Micropterus salmoides*) bass. (**A**) *Myxobolus branchiarum* from the secondary lamellae of the gills of smallmouth bass from the Potomac River Basin. (**B**) *Myxobolus microcystis* from the gills of largemouth bass (Price and Mellen, 1980). (**C**) *Myxobolus micropterii* from the primary lamellae of the gills of largemouth bass from a West Virginia State fish hatchery and the Potomac and Shenandoah Rivers. Note the presence of an iodinophilous vacuole (arrows). Line drawings of *Myxobolus branchiarum* (**D**) and *Myxobolus micropterii* (**E**) are also shown. Scale bars = 10 μm.

Type specimens: USNPC Nos. 104898 and 104899, U.S. National Parasite Collection, Beltsville, Maryland.

Etymology: The species is named after the site of infection.

GenBank accession number: JF714994.

Remarks

Myxobolus branchiarum resembles numerous other pyriform-shaped species of *Myxobolus* that exhibit tropism for the gills of fish. *Myxobolus microcystis*, previously identified in largemouth bass, shares similar morphology to that of *M. branchiarum* (Price and Mellen, 1980). The syntype of *Myxobolus microcystis* (USNPC No. 075230, U.S. National Parasite Collection, Beltsville, Maryland) was compared to *M. branchiarum*, and 2 characteristics were clearly different. *Myxobolus microcystis* (Fig. 1B) has a slightly less pyriform body shape than *M. branchiarum* (Fig. 1A) and possesses an iodinophilous vacuole. *Myxobolus pseudokoi* (Li and Desser, 1985), *Myxobolus longisporus* (Nie and Li, 1992), *Myxobolus ampullicapsulatus* (Zhao et al., 2008), and *Myxobolus bilobus* (Cone et al., 2005) are gill parasites that share similar morphological features to *M. branchiarum*, but differ in size of spore and polar capsules, and host specificity.

Myxobolus micropterii n. sp.

(Fig. 1C, E)

Diagnosis: White, oval-shaped plasmodia; length 568.1 ± 106.2, range 334.2–954.7. Width 148.1 ± 10.7, range 110.9–189.1 (n = 6); length and width measured from histology prepared slides. Spores subspherical (Fig. 1C). Sutural ridge on posterior end. Sutural folds absent. Following measurements from alcohol-preserved gill clipping (Table III). Spore length 10.8 ± 0.1, range 9.2–12.2. Width 10.6 ± 0.1, range 9.0–11.8. Length-to-width ratio of spore 1.0; thickness 6.8 ± 0.1, range 5.3–8.7. Spores with 2 polar capsules of about equal size. Polar filaments unable to

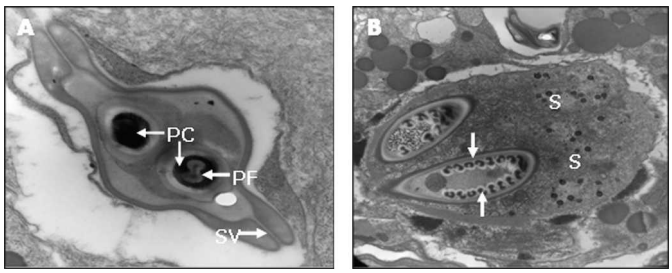

FIGURE 2. Electron micrographs of *Myxobolus branchiarum* from the secondary lamellae of the gills of smallmouth bass (*Micropterus dolomieu*). (**A**) Coronal view of *M. branchiarum*. Note the sutures with the 2 spore valves (SV) and 2 polar capsules (PC) and polar filament (PF), ×50,000. (**B**) Top view of *M. branchiarum* illustrating the 2 polar capsules with polar filaments (arrows) and numerous sporoplasmosomes within the sporoplasm (S), ×15,000.

TABLE III. Mature spore measurements of *Myxobolus branchiarum* and *Myxobolus micropterii* from the gills of smallmouth and largemouth bass from the Potomac River Basin.

Measurements (μm)	Mean ± SE	Minimum	Maximum
Myxobolus branchiarum (PBS)			
Plasmodia (n = 15)			
Length	320.3 ± 15.7	210.8	423.9
Width	246.1 ± 18.9	150.0	420.8
Spore			
Length (n = 101)	12.82 ± 0.14	8.07	15.14
Width (n = 98)	6.86 ± 0.11	4.01	8.97
Thickness (n = 55)	5.67 ± 0.08	4.75	7.14
Polar filament length (n = 32)	59.25 ± 2.77	31.79	92.42
Myxobolus micropterii (96% alcohol)			
Plasmodia (n = 6)			
Length	568.05 ± 106.22	334.20	954.70
Width	148.10 ± 10.73	110.90	189.10
Spore (n = 59)			
Length	10.77 ± 0.09	9.16	12.23
Width	10.62 ± 0.08	9.01	11.78
Thickness	6.77 ± 0.08	5.28	8.65

be viewed in capsules. Spore measurements from gill clippings preserved in water with average length 11.0–13.0, width 10–12, and thickness 7–8. Polar capsule length 4–5, width 2–3. Polar filament length 35–40 and perpendicular to longitudinal axis of capsules. Iodinophilous vacuole present.

Taxonomic summary

Type host: Largemouth bass (*Micropterus salmoides*).
Additional type host: Smallmouth bass (*Micropterus dolomieu*).
Site of infection: Gill filaments.
Type locality: State fish hatchery (West Virginia) in Ohio River drainage.
Additional type localities: Potomac River (West Virginia) and Shenandoah River (Virginia).
Type specimens: USNPC No. 104900, U.S. National Parasite Collection, Beltsville, Maryland.
Etymology: The species is named after the original type hosts.
GenBank accession number: JF714995.

Remarks

Species that share a number of morphological features with *Myxobolus micropterii* include *Myxobolus rotundus* from common bream *Abramis brama* (Molnár et al., 2009), *Myxobolus lamellus* from white sucker *Catostomus commersoni* (Grinham and Cone, 1990), and *Myxobolus oliveirai* from *Brycon hilarii* (Milanin et al., 2010). *Myxobolus rotundus* has an iodinophilous vacuole, develops in the secondary gill lamellae, and has a slightly more pyriform shape than *M. micropterii* and possesses an intercapsular appendix. *Myxobolus lamellus* is slightly longer than *M. micropterii*, develops within the secondary gill lamellae, and does not possess an iodinophilous vacuole. There are differences in spore shape and size and host species of *M. micropterii* and *M. oliveirai*. The spores of *M. oliveirai* are pear shaped and slightly larger than the spores of *M. micropterii* and infect *Brycon hilarii*. However, the plasmodia of *M. micropterii* and *M. oliveirai* are similar. Plasmodia from both species develop in the distal portion of the gill filament and exhibit asynchronous development of pansporoblasts and spores.

DNA sequencing

A combination of primer sets enabled sequencing in both directions of 1653 base pairs (bp) of SSU rDNA of *Myxobolus branchiarum*. Primer sets

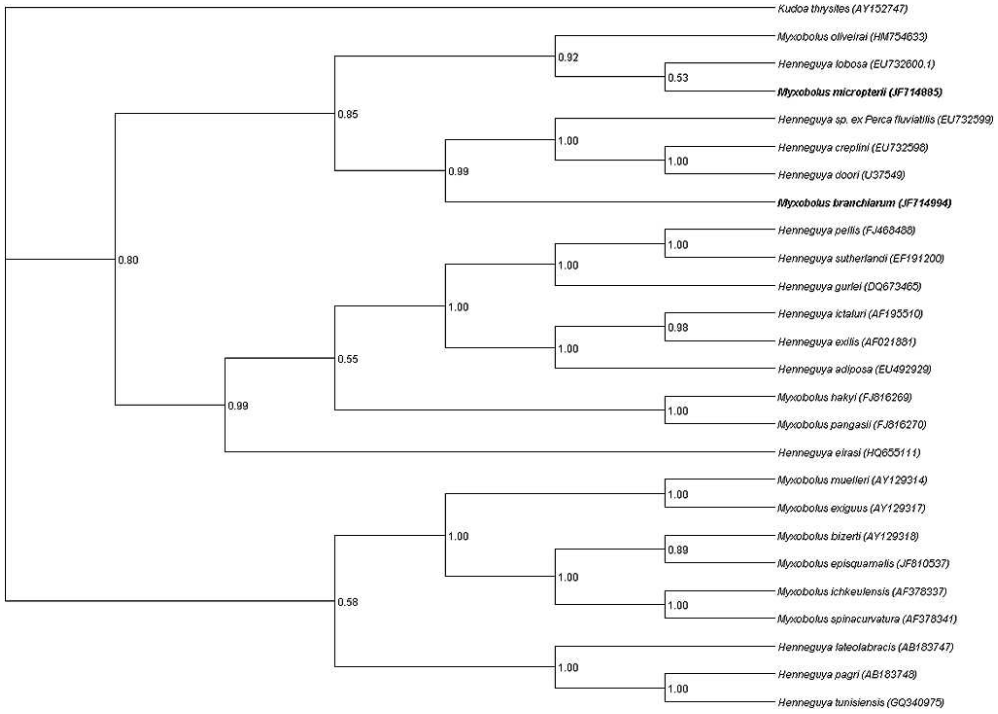

FIGURE 3. Phylogenetic tree from the Bayesian inference analysis of myxozoan SSU rDNA. *Myxobolus micropterii* was placed in a sister-group to *Myxobolus oliveirai*, and *Henneguya lobosa* and *Myxobolus branchiarum* were placed in a sister-group to *Henneguya doori*, *Henneguya* sp. ex *Perca fluviatilis*, and *Henneguya creplini*.

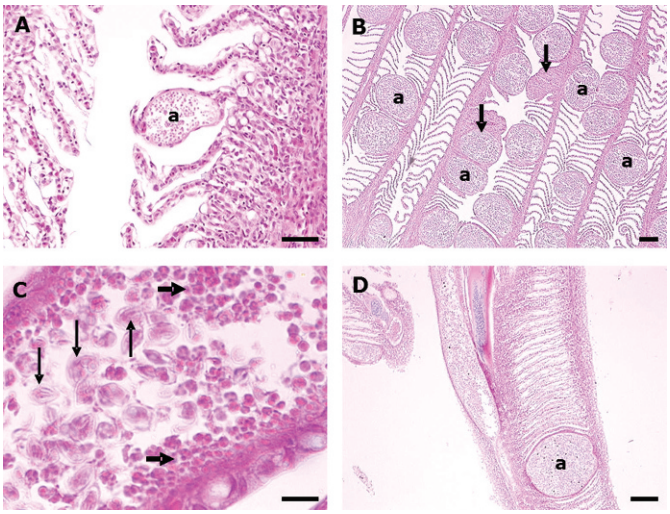

FIGURE 4. Infection of *Myxobolus branchiarum* in the secondary lamellae of the gills of smallmouth bass (*Micropterus dolomieu*). (**A**) Plasmodia of *Myxobolus branchiarum* develop as intralamellar cysts (a). Scale bar = 30 μm. (**B**) Heavily infected gill with numerous myxozoan plasmodia (a), as well as areas of epithelial hyperplasia and inflammation. Scale bar = 100 μm. (**C**) Plasmodium containing mature spores (thin arrows) and pansporoblasts (thick arrows). Scale bar = 10 μm. (**D**) Mixed infection of *M. branchiarum* (a) and *Myxobolus micropterii* (b) from the South Branch Potomac River. Scale bar = 50 μm. Hematoxylin and eosin stain.

MYXF-MYXR, HEN2146F-18R, 3F-18R, and Myx472F-18R amplified a single product per amplification of 639, 795, 891, and 881 bp, respectively. Phylogenetic analysis placed *M. branchiarum* in a sister-group with *Henneguya doori*, *Henneguya* sp. ex *Perca fluviatilis*, and *Henneguya creplini* (posterior probability = 0.99 by Bayesian analysis). The primer set MYXF-MYXR amplified a single product amplification product of 732 bp of *M. micropterii* from largemouth bass. Phylogenetic analysis placed *M. micropterii* in a sister-group with *M. oliveirai* and *Henneguya lobosa* (posterior probability = 0.92 by Bayesian analysis) (Fig. 3).

Histopathology

The plasmodia of *Myxobolus branchiarum* develop as intralamellar cysts in individual secondary lamellae, either under the epithelial layer or within the capillary vessel (Fig. 4A). Hypertrophy and hyperplasia of the epithelial and goblet cells around the cysts are common and are often resolved as the plasmodia mature. In some cases, an increase in eosinophilic granular cells, necrosis, and gill epithelial lifting were observed. In high-intensity infections, the plasmodia appeared to be interlamellar, as the developing parasites totally occupied the infected secondary lamellae (Fig. 4B). The spores develop asynchronously within the plasmodia, with the pansporoblasts located along the periphery of the plasmodia and the mature spores more centrally located (Fig. 4C). Early- and late-stage plasmodia were observed in the same gill sample. Mixed infections of the 2 new species were observed in the gills of a few smallmouth bass from the South Branch Potomac River (Fig. 4D). Microvilli that extend from the plasmodia into the host tissue are evident in electron microscopy images (Fig. 5).

The plasmodia of *Myxobolus micropterii* develop in the distal portion of the gill filaments under the epithelial layer adjacent to the filament cartilage near the capillary vessel (Fig. 6A). The spores develop asynchronously within the plasmodia, with the mature spores centrally located and the pansporoblasts located peripherally within the plasmodia (Fig. 6B). Secondary lamellae around the plasmodia were absent. Eosinophilic granular cells were often observed at the site of infection in addition to a decrease in goblet cells, which were occasionally necrotic.

Seasonal changes in abundance

Seasonal intensity differences of *Myxobolus branchiarum* plasmodia were observed in the gills of smallmouth bass from the South Branch Potomac River and from the Cowpasture River. Plasmodia intensity was highest in the spring, lowest in the summer, and intermediate in the fall. In the analysis including both infected and uninfected bass, significant differences were observed between the spring and summer ($P < 0.001$), summer and fall ($P < 0.001$), and fall and spring ($P = 0.004$) (Fig. 7A). In the analysis of only infected bass, significant differences were observed between the spring and summer ($P = 0.001$) and spring and fall ($P = 0.008$) but not between the summer and fall (Fig. 7B). Overall, there were considerably more mature spores in the plasmodia during the spring. Immature stages in the plasmodia were observed in high numbers during the fall. *Myxobolus branchiarum* was infrequent in smallmouth bass collected from the Shenandoah River, and no significant seasonal differences were observed.

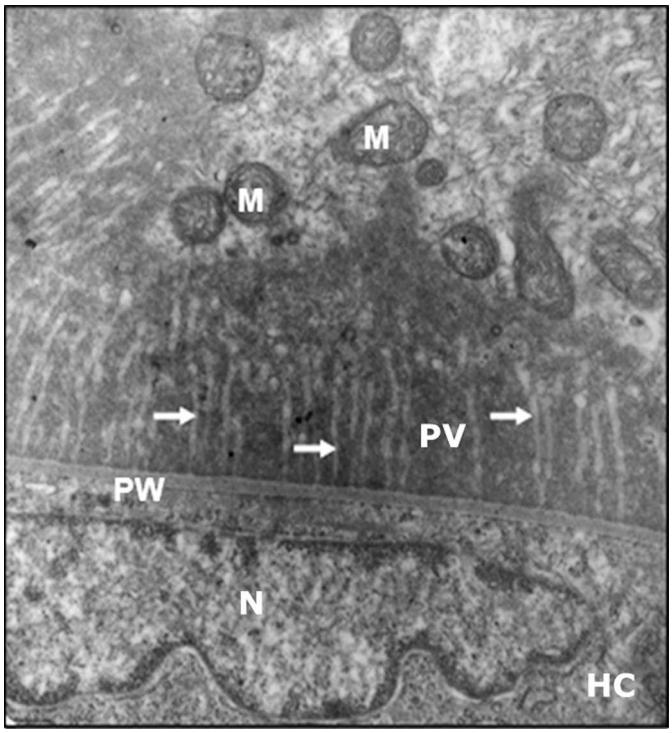

FIGURE 5. Electron micrograph of the plasmodia of *Myxobolus branchiarum* from the secondary lamellae of the gills of smallmouth bass (*Micropterus dolomieu*). The plasmodium wall (PW) consists of 2 membranes, and the inner membrane forms an area of pinocytic canals (arrows), which supply nutrients from the host cell (HC) tissue to the plasmodia. Pinocytic vesicles (PV) surround the pinocytic canals. Mitochondria (M) and the nucleus of the host cell (N) are also apparent. ×30,000.

DISCUSSION

The small white plasmodia observed in the gills of smallmouth bass from the Potomac River and its tributaries during spring mortality events and from a largemouth bass mortality in a West Virginia State fish hatchery were found to be related to the presence of 2, previously undescribed, species of *Myxobolus.* They share morphological features to other species of *Myxobolus*, but they are the first known to infect the gills of smallmouth bass. A mixed infection of the 2 species in the same smallmouth bass gill sample was occasionally observed in samples from the South Branch Potomac River. Smallmouth bass are a prized and highly sought after sport fish, so their health and conservation are economically important. Hence, it is surprising that these species have not been previously seen. It is possible, however, that they

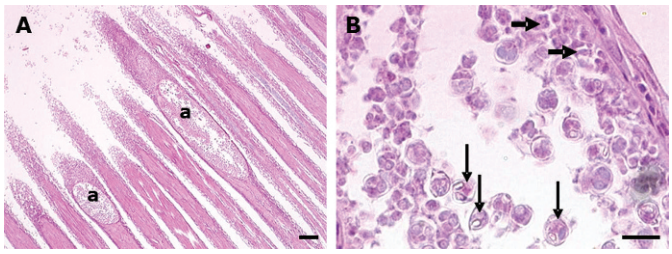

FIGURE 6. *Myxobolus micropterii* in the gills of largemouth bass (*Micropterus salmoides*). (**A**) Plasmodia (arrows) were observed on the tips of the gill filaments. Scale bar = 100 μm. (**B**) Mature spores (thin arrows) and pansporoblasts (thick arrows) were observed within plasmodia. Scale bar = 10 μm.

have been recently introduced to the Potomac and James River basins or that there is an increased prevalence or intensity due to environmental factors affecting the abundance of the unknown invertebrate intermediate host.

Based on the comparison of morphological and molecular analyses to other *Myxobolus* spp., we described the 2 new species here as *Myxobolus branchiarum*, after its location in the host, and *Myxobolus micropterii*, after the host species. The classical identification of *Myxobolus* spp. is based on spore morphology, tissue tropism, and host specificity (Lom and Arthur, 1989). No species of *Myxobolus* that infect smallmouth bass gills has been described, and only 2, *M. cartilaginis* (Hoffman et al., 1965) and *M. microcystis* (Price and Mellen, 1980), have been described from largemouth bass. While molecular data are not available for the latter 2 species, they differ morphologically from the new species described.

Furthermore, based on gene homology, *Myxobolus branchiarum* clusters with *H. doori*, *H. creplini*, and *H.* sp. ex *Perca fluviatilis* (Fig. 3). These 3 species of *Henneguya* infect the gills of perch (*Perca flavescens* and *Perca fluviatilis*) and pike-perch (*Sander lucioperca*) (Cone, 1994; Molnár, 1998). *Myxobolus micropterii* also clusters with *Henneguya lobosa*, which infects the gills of pike (*Esox lucius*) (Hoffman, 1999). Classically, the genera *Myxobolus* and *Henneguya* are morphologically distinguished based on the presence or absence of caudal appendages (Davis, 1944). However, this characteristic has been challenged because some *Myxobolus* spp., such as *Myxobolus mississippiensis* and *Myxobolus turpisrotundus*, occasionally possess an irregular caudal appendage (Cone and Overstreet, 1997; Liu et al., 2010). Additionally, for some species, the phylogenetic separation of *Myxobolus* and *Henneguya* is not supported based on SSU rDNA and LSU rDNA analysis (Liu et al., 2010). Moreover, Smothers et al. (1994) determined that *Myxobolus* and *Henneguya* are paraphyletic and that some *Myxobolus* spp. are phylogenetically related to species of *Henneguya*.

Myxobolus micropterii is morphologically similar to *M. rotundus* and *M. lamellus* (Grinham and Cone, 1990; Molnár et al., 2009), and all 3 species exhibit tropism for the gills. *Myxobolus micropterii* is more similar to *Myxobolus* in morphology and, unlike *M. branchiarum*, has a phylogenetic relationship with a *Myxobolus* sp. and *Myxobolus oliveirai* (Andree et al., 1999; Lom and Dyková, 2006). In a study by Andree et al. (1999), *Myxobolus* spp. correlated best by tissue tropism based on SSU rDNA sequence. The SSU rDNA nucleotide sequence of *M. micropterii* shares homology with *M. oliveirai*, and both species exhibit tropism for the gill filament (Milanin et al., 2010).

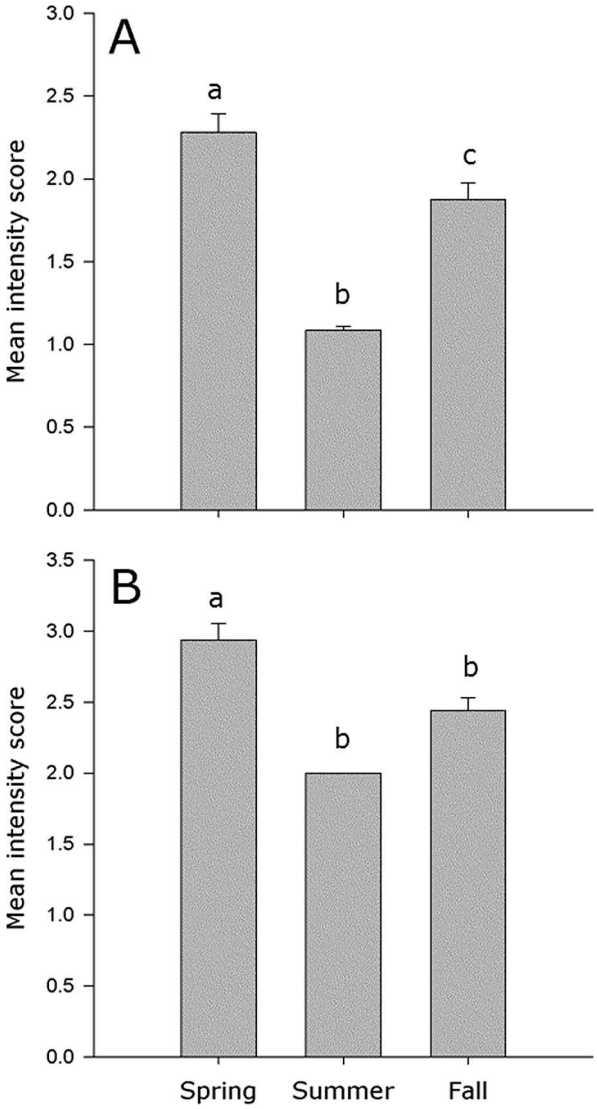

FIGURE 7. Seasonal comparison of mean intensity scores ± standard error of *Myxobolus branchiarum* from the gills of smallmouth bass collected in the Potomac and Cowpasture Rivers. Plasmodia intensity was scored as follows (y-axis): 0 = 0 plasmodia per gill filament, 1 = 1–10, 2 = 11–20, 3 = 21–50, and 4 = 51 or more. (**A**) Sample sizes were 121 in the spring, 152 in the summer, and 71 in the fall for the analysis including infected and uninfected bass. (**B**) Sample sizes were 80 in the spring, 12 in the summer, and 43 in the fall for the analysis of only infected bass. Columns with the same letter are not significantly different ($P > 0.05$).

Seasonal variation in intensity of *Myxobolus branchiarum* was observed in the gills of smallmouth bass from the South Branch Potomac River in 2004 and the Cowpasture River in 2006. Smallmouth bass gills were infected with the highest intensity of plasmodia, containing primarily mature spores, in the spring. The high intensity of plasmodia coincides with the spring mortality events that occurred in the Potomac River basin and its tributaries. The high plasmodia intensity in the spring could impair respiratory function and, if coupled with low dissolved oxygen, could cause asphyxia (Haaparanta et al., 1994). During the summer, smallmouth bass had the lowest intensity of infection, which was followed by an increase in plasmodia full of immature spores in the fall. Seasonality of infection is common

for other *Myxobolus* and *Henneguya* spp., which are known to exhibit increased plasmodia intensity in host tissues during the spring (Ching and Munday, 1984; Narasimhamurti and Kalavati, 1984; Haaparanta et al., 1994; Barse, 1998). The study by Haaparanta et al. (1994) associated low plasmodia intensity during the summer with increased water temperatures and immunity of the fish after the stress of spring spawning subsided. Temperature is regarded as a key factor for influencing myxozoan intensity, although the density of aquatic oligochaetes, alkalinity, dissolved oxygen, and turbidity are important as well (Narasimhamurti and Kalavati, 1984; Haaparanta et al., 1994; Barse, 1998; Marcogliese and Cone, 2001). Pollution and nutrient enrichment may increase the density of benthic invertebrate intermediate hosts, and an increase in temperature allows them to release more actinospores (Blazer et al., 2003; Lin and Yo, 2008). While these newly described *Myxobolus* spp. have not been identified as agents of disease in the spring mortality events, they may contribute additional stress.

Future studies to determine the aquatic invertebrate intermediate host of *M. branchiarum* and *M. micropterii* would be beneficial for understanding their life history. Knowledge of the life history and environmental influences on the intermediate host could be used to predict infection intensity and prevalence, determine whether *M. branchiarum* is associated with spring mortality events, and, perhaps, identify ways to manage water and sediment quality for healthier fish populations.

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