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TAXONOMY OF *MYXOBOLUS RIDOUTI* N. SP. AND *M. RIDGWAYI* N. SP. (MYXOZOA) FROM *PIMEPHALES NOTATUS* AND *SEMOTILUS ATROMACULATUS* (CYPRINIFORMES) IN ONTARIO

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ABSTRACT: *Myxobolus ridouti* n. sp. and *M. ridgwayi* n. sp. are described, respectively, from *Pimephales notatus* (Rafinesque) and *Semotilus atromaculatus* (Mitchill) in Algonquin Park, Ontario, Canada. Both are intracellular in striated muscles of the body flank. Spores of *M. ridouti* n. sp. are subcircular (9.5–10.5 μm long by 9.4–10.9 μm wide); those of *M. ridgwayi* n. sp. are oval (10.0–12.1 μm long by 9.5–10.5 μm). Both species have spores with a small, distinct swelling (1 μm) on the spore valve near the posterior sutural ridge, similar to that described for *Myxobolus insignis* Eiras, Malta, Varella, and Pavanelli, 2005. It looks to be non-nuclear in origin, apparently part of the internal face of a spore valve, and could be taxonomically useful. Partial sequence data of the 18S rDNA from both species are unique among those species in the genus that have been sequenced. The sequences have the greatest overall identity with various species of *Myxobolus* from cyprinids in North America, Europe, and Asia, with a constructed molecular phylogeny having the 2 new species forming a separate clade.

There are over 800 nominal species of *Myxobolus* Bütschli, 1882 (Myxozoa) described from host fishes worldwide (Eiras, Molnar et al., 2005; Lom and Dyková, 2006; Ferguson et al., 2008). These parasites are typically histozoic and tissue specific, developing, as a group, in all host organ systems (Lom and Dyková, 1992). Most species are host specific to freshwater bony fishes, with limited radiation having occurred within coastal marine fishes (Bahri et al., 2004). Available evidence suggests that species of *Myxobolus* are among the most diverse parasite lineages in freshwater fishes, with richness reaching as high as 5 species per individual host (Cone et al., 2004).

The taxonomy of *Myxobolus* is difficult because the spores of so many species resemble each other (see Chen and Ma, 1998). Contemporary species descriptions address this issue by providing as much detailed information as possible on spore and plasmodial structure (Eiras and D'Souza, 2004), ultrastructure (Ali et al., 2003; Tajdari et al., 2005), novel spore morphology (Eiras, Malta et al., 2005), pathology and nature of the infections (Longshaw et al., 2003; Levsen et al., 2004), sequence data of the 18S rDNA (Easy et al., 2005; Molnár et al., 2007, 2008, 2009; Ferguson et al., 2008), and ecological information on tissue and host specificity (Fomena et al., 2004; Molnár et al., 2007). Most authors now try to use as many of these other features with sequence data, forming an integrated taxonomic assessment (Lom and Dyková, 2006; Székely et al., 2009a, 2009b).

The present study describes a new species from striated muscle of *Pimephales notatus* (Rafinesque) (Cyprinidae) and a second from *Semotilus atromaculatus* (Mitchill) (Cyprinidae), both in Ontario. We use spore morphology and 18S rDNA sequence data as the basis for the descriptions.

MATERIALS AND METHODS

Bluntnose minnow (*P. notatus*) and creek chub (*S. atromaculatus*) were caught on 17 May 2004 in baited minnow traps set in Brewer Lake, Algonquin Park, Ontario, Canada. Live fish were anaesthetized, and organs were examined microscopically for myxozoans, by teasing of tissues with forceps, using incident lighting that helps create contrast between infected and non-infected cells. Plasmodia were examined first in wet mounts using coverslips and identified by spore morphology. These plasmodia were then scraped from the slide and placed in 5% formalin (for later morphological study, during which spores were photographed in bright field optics of a Zeiss AxioPlan microscope and studied with the Zeiss Image Analysis System [Zeiss, AxioVision 4.5 software, 2006, Carl Zeiss Canada Ltd., Toronto, Canada]) or in 95% ethanol for molecular taxonomy. Tissues with plasmodia were sectioned histologically as described by Cone et al. (2004). Measurements in the description are means in μm ± SD, followed by ranges in parentheses.

DNA was extracted from myxozoans using the methods from Easy et al. (2005). Briefly, tissues containing parasitic spores were lysed in 10 mM Tris-Cl pH 8.0, 1 mM EDTA (TE), 1% SDS with proteinase K (200 μg/ml) for 2 hr in a 37 C water bath. Lysates were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), and the DNA was precipitated with cold 100% ethanol and 3 M sodium acetate (pH 7.0), followed by centrifugation at 9,300 *g* for 10 min. The pellet was washed once with 70% ethanol and air-dried at room temperature. Genomic DNA was re-suspended in 50 μl TE and stored at 4 C. Quantification of DNA was completed using a Beckman Spectrophotometer (Beckman Coulter Canada, Mississauga, Ontario, Canada). The 18S rDNA was amplified using primers 18r (5′-CTACGGAAACCTTGTTACG-3′) (Whipps et al., 2003) and 18e (5′-TGGTTGATCCTGCCAGT-3′) (Hillis and Dixon, 1991). Primers were synthesised by IDT (Stender Way, Santa Clara, California) and re-suspended in ddH_2O to a stock concentration of 10 μM. PCR was performed in 50-μl reaction volumes containing 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.25 units Taq polymerase, 1 mM of each primer, and 300 ng DNA. Amplifications were performed on a Perkin-Elmer Gene Amp 9700 Thermocycler (Perkin Elmer, Life and Analytical Services, Waltham, Massachusetts). Cycling parameters were: initial denaturation at 95 C for 5 min, followed by 35 cycles of 94 C for 1 min, 55 C for 1 min, 72 C for 1.5 min, and a final extension at 72 C for 10 min. The PCR products were excised from an agarose gel and purified using UltraClean from ABgene (ABgene, Mississauga, Ontario). Sequencing reactions were performed using ET terminator chemistry (Amersham Biosciences, Piscataway, New Jersey) and sequenced on a MegaBACE 1000 capillary sequencer (Amersham Biosciences). The data were edited using Sequencher (Gene Codes, Ann Arbor, Michigan) and submitted for database searching using BLASTX (Altschul et al., 1997). Sequences were aligned using ClustalX software (Thompson et al., 1997). Phylogenetic analyses were conducted using PAUP version 4.0b10 (PAUP, Sinauer Associates Inc., Sunderland, Massachusetts) and included character-based analysis (maximum parsimony) and distance methods (UPGMA, minimum evolution and neighbor joining), with bootstrap analysis of 1,000 replicates using *Ceratomyxa shasta* as the outgroup.

Sequences used in the phylogeny were: *Ceratomyxa shasta* Noble, 1950 (AF001579), *M. pseudodispar* Gorbunova, 1936 (EF466088.1), *Myxobolus* sp. CMW-2004 (AY591531.1), *M. stanlii nomen nudum* (DQ779996.2), *M. pendula* Kent, Andree, and Bartholomew, 2001 (AF378340), *M. procerus* Easy, Johnson, and Cone, 2005 (AY665926), *M. intramusculi* Easy, Johnson, and Cone, 2005 (AY665297), *M. musculi* Keysselitz, 1908 (AF380141), *M. cyprini* Doflein, 1898 (AF380140), *M. bartai* Salim and Desser, 2000 (AF186835), *M. terengganuensis* Székely, Shaharom-Harrison, Cech, Ostoros, and Molnár, 2009 (EU643629.1), *M. intimus* Zaika, 1965 (AY325285), *M. bilobus* Cone, Jang, Sun, and Easy, 2005 (DQ008579.1),

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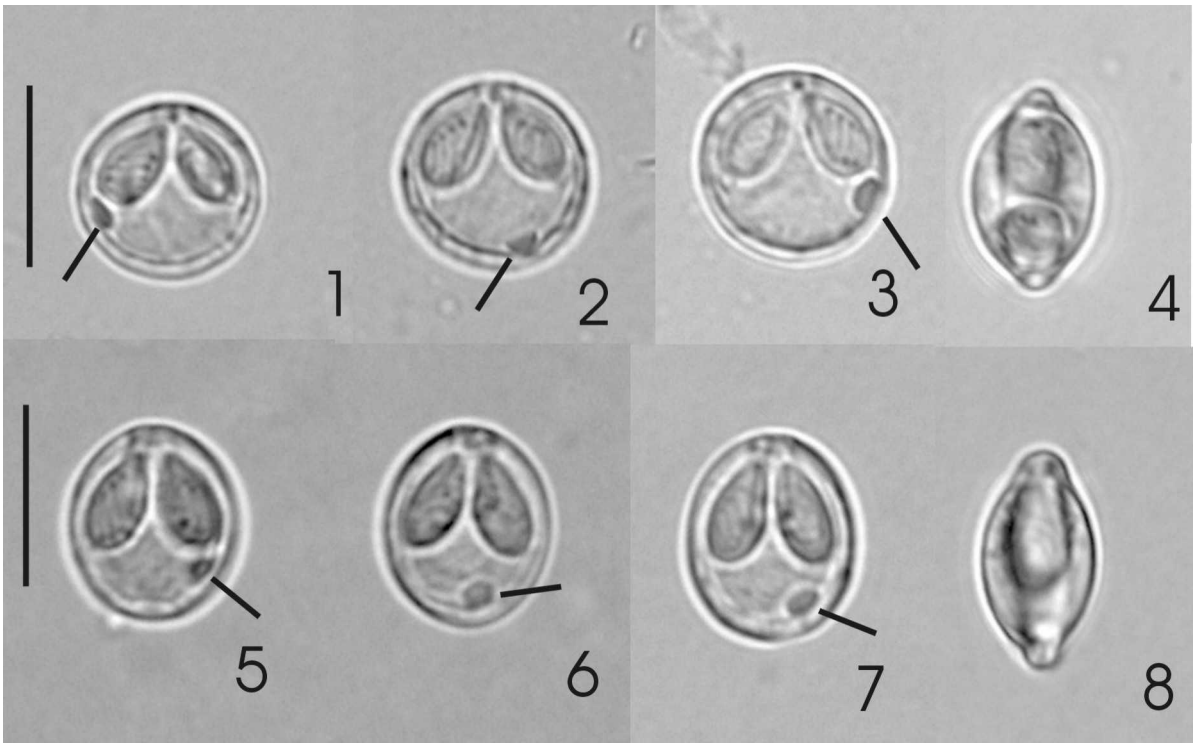


FIGURES 1–8. **(1–4)** Fixed spores of *Myxobolus ridouti* n. sp. **(5–8)** Fixed spores of *Myxobolus ridgwayi* n. sp. Bar = 10 μm.

M. dujardini Thélohan, 1892 (DQ439804.1), *M. hungaricus* Jaczó, 1940 (AF448444.1), *Myxobolus* sp. Hungary-EE-2003 (AY325283.1), and *M. obesus* Gurley, 1893 (AY325286).

DESCRIPTIONS

Myxobolus ridouti n. sp.

(Figs. 1–4, 9)

Diagnosis: Plasmodia intracellular in striated muscle cells, fusiform and up to 300 long; polysporous. Formalin-fixed spores mostly subcircular in frontal view, 9.9 ± 0.3 (9.5–10.5) (n = 15) long, 10.1 ± 0.4 (9.4–10.9) wide, and 6.7 ± 0.01 (6.7–6.8) (n = 2) thick. Five or 6 often indistinct, sutural ridge folds. Prominent swelling (1 μm) in posterior of shell of the majority of spores. Spore length–width 0.98 ± 0.04 (0.92–0.05). Polar capsules pyriform, converging anteriorly, 5.2 ± 0.3 (4.6–5.6) (n = 30) long by 3.0 ± 0.27 (2.6–3.6) (n = 30) wide; 3–4 filament coils arranged loosely within capsule. No intercapsular appendix, iodinophilous vacuole, or mucous coat.

Taxonomic summary

Type host: Pimephales notatus (Rafinesque) (bluntnose minnow, Cyprinidae).

Site of infection: Intracellular in striated muscle of body flank.

Type locality: Brewer Lake, Algonquin Park, Ontario (45°35′N, 78°19′W).

Prevalence and intensity: Six of 15 fish were infected with an undetermined number of plasmodia.

Etymology: This species is named for Gary Ridout of the Ontario Ministry of Natural Resources, who has helped parasitologists collect fish hosts for many years.

Phototypes: A black and white photograph of a portion of the contents (photosyntypes) from a single cyst is deposited in the United States National Parasite Collection (USNPC) (Accession no. 102126.00), Beltsville, Maryland.

Molecular sequence data: A 765-base pair segment of the 18S rDNA is deposited in GenBank (GQ292745).

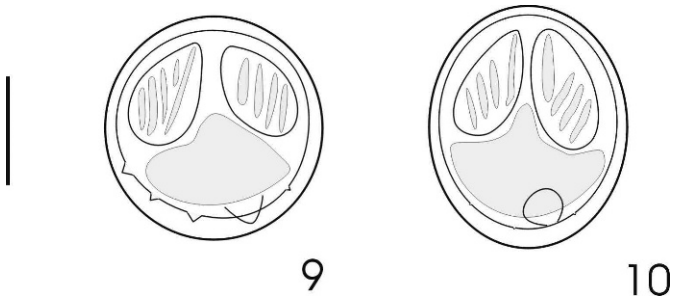
Remarks

Spores of *M. ridouti* n. sp. resemble most closely those of *M. endovasus* (Davis, 1947) from gill filaments of *Ictiobus bubalis* (Catostomidae), *M. bartai* Salim and Desser, 2000, *M. nodosus* Kudo, 1934 from integument of *P. notatus*, and *M. transversalis* Fantham, Porter, and Richardson, 1939 from muscle of *Notropis cornutus* (Cyprinidae) in overall size and shape of the spores. However, spores of *M. endovaus* and *M. bartai* have polar capsules that fill much more of the spore interior and miniscule sutural ridge folds. Spores of *M. nodosus* have a markedly irregular posterior edge as opposed to being smooth in *M. ridouti*. Spores of *M. transversalis* have a small intercapsular appendix.

Myxobolus ridgwayi n. sp.

(Figs. 5–8, 10)

Diagnosis: Plasmodium intracellular in striated muscle cells, fusiform, and up to 300 long; polysporous. Formalin-fixed spores oval in frontal view, 11.3 ± 0.5 (10.0–12.1) (n = 15) long, 10.4 ± 0.3 (9.5–10.5) wide, 6.5 ± 0.01 (6.6, 6.7) (n = 2). Spore length–width 1.13 ± 0.06 (1.03–1.2). Five or 6 indistinct sutural ridge folds. Prominent swelling (1 μm) in posterior



FIGURES 9–10. Diagrammatic drawings of fixed spores in frontal view. **(9)** *Myxobolus ridouti* n. sp. **(10)** *Myxobolus ridgwayi* n. sp. Bar = 5 μm.

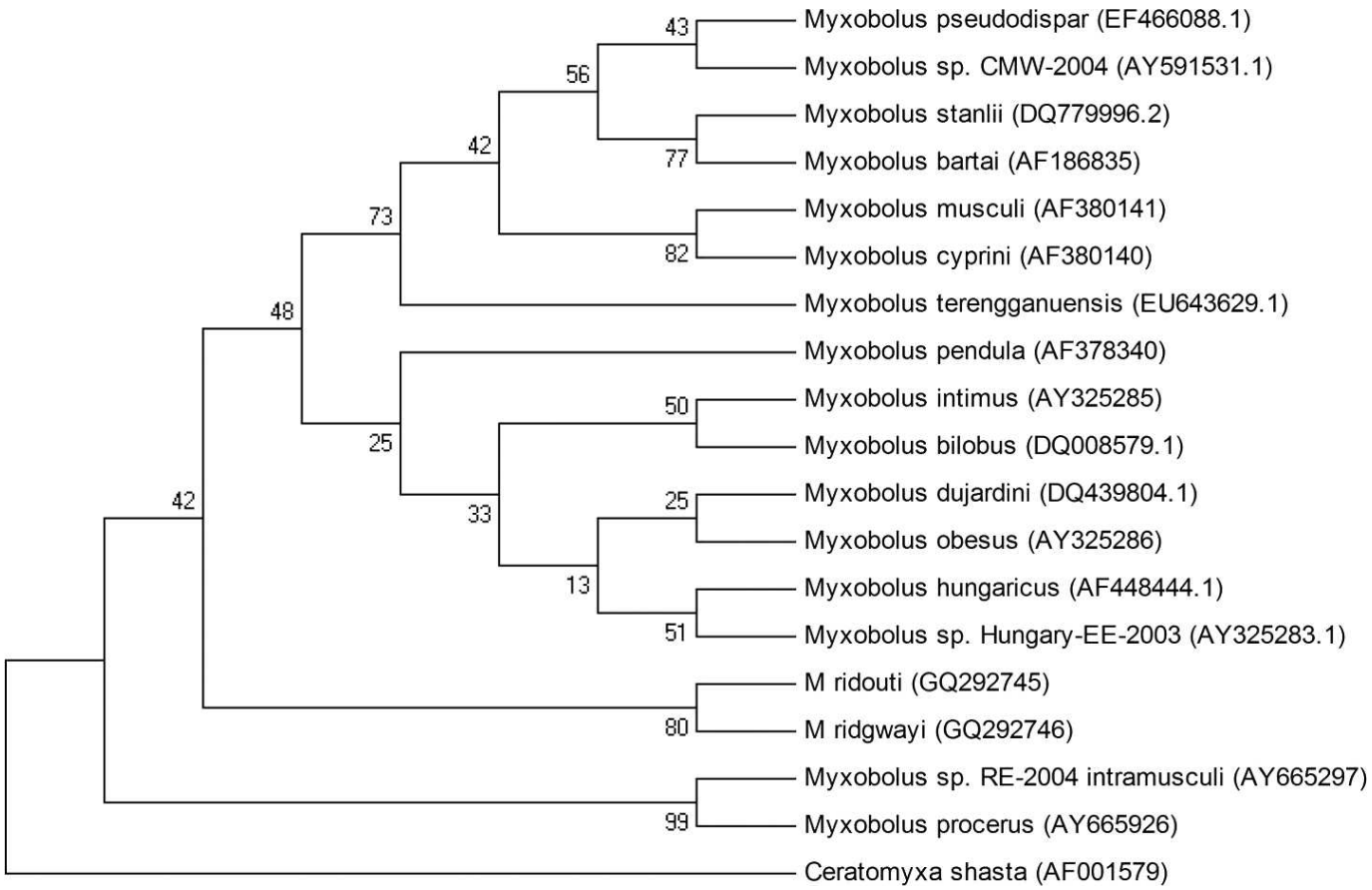


FIGURE 11. Maximum parsimony tree of 18S rDNA sequences of *Myxobolus* species described in this paper with other myxozoan species. GenBank accession numbers are shown in parentheses. Bootstrap values represent 1,000 replicates.

of shell of most spores. Polar capsules pyriform, converging anteriorly, 6.5 ± 0.3 (5.8–7.0) (n = 13) long, 3.2 ± 0.3 (2.0–3.8) wide; 4–5 polar filament coils, arranged loosely in capsule and oblique to capsule length. No intercapsular appendix, iodinophilous vacuole, or mucous coat.

Taxonomic summary

Type host: Semotilus atromaculatus (Mitchill) (Creek Chub, Cyprinidae).
Site of infection: Intracellular in striated muscle of body flank.
Type locality: Brewer Lake, Algonquin Park, Ontario (45°35′N, 78°19′W).
Prevalence and intensity: Two of 11 fish infected with undetermined number of intracellular plasmodia.
Etymology: This species is named for Mark Ridgway, the director of the Harkness Fisheries Research Laboratory, Algonquin Park, Ontario, for his long-time support of fish parasitology research.
Phototypes: A black and white photograph of spores (photosyntypes) from a single cyst is deposited in the United States National Parasite Collection (USNPC) (Accession no. 102127.00), Beltsville, Maryland.
Molecular sequence data: An 839-base pair segment of the 18S rDNA is deposited in GenBank (GQ292746).

Remarks

Overall spore size and shape of *M. ridgwayi* n. sp. resembles most closely *M. enoblei* Lom and Cone, 1996 from gills of *Ictiobus bubalis* (Catostomidae). However, spores of this latter species have polar capsules that are more globose in shape and larger relative to the size of the spore. *Myxobolus ridgwayi* also resembles *M. wellerae* Li et Desser, 1985 from muscle of *Notropis cornutus* (Cyprinidae) and *M. heterolepis* Li and Desser, 1985 from nervous tissue and eye of *N. heterolepis* (Cyprinidae), but both these species have spores with a well-developed intercapsular appendix. Also, *M. wellerae* has spores with a prominent posterior mucous coat. The new species differs significantly from *M. pendula* Gurley, 1964 (syn. *M. pellicides* Li and Desser, 1985) in base pair makeup of the 18S rDNA.

A posterior swelling, similar to that observed near the sutural ridge in spores of *M. ridouti* n. sp. and *M. ridgwayi* n. sp., has been reported (Eiras, Malta et al., 2005) in *Myxobolus insignis* Eiras, Malta, Varella, and Pavanelli, 2005. We initially thought it was a degenerating nucleus. However, in Giemsa-stained histological sections valvular, capsular, and sporoplasmic nuclei stained brilliantly blue–purple while the swelling remained unstained and hyaline. This suggests it is not nuclear in origin. The fact that the structure was not seen when spores were viewed from the side suggests that it is positioned on the internal face of a spore valve (this also was the impression one had from histological sections of spores) and may be a character of taxonomic significance.

Molecular phylogeny

Analysis of a 765-base pair region of the 18S rDNA from *M. ridouti* n. sp. and *M. ridgwayi* n. sp. showed >10% sequence divergence between the 2 species. However, both species formed a distinct separate clade in a phylogeny (Fig. 11) prepared of those myxobolids, with the greatest sequence similarity from the BLAST search. Distance methods revealed similar topology to character-based analyses (maximum parsimony). Nodal support for trees constructed by the 4 methods revealed groupings of lower confidence; however, the separate clade of *M. ridouti* and *M. ridgwayi* showed ≥80 bootstrap support in all analyses. All of these represent species reported from cyprinids in North America, Europe, and Asia.

DISCUSSION

With the 2 new additions, there are now 126 nominal species of *Myxobolus* described or reported from freshwater fishes in North

America. Of these, 12 are suspected to represent misidentifications, i.e., *M. artus* Akhmerov, 1960, *M. cyprini* Doflein, 1898, *M. cyprinicola* Reuss, 1906, *M. dujardini* (Thélohan, 1892), *M. microlatus* Li and Nie, 1973, *M. muelleri* Butschli, 1882, *M. multiplicatus* (Reuss, 1906), *M. musculi* Keysselitz, 1908, *M. nemachili* Weiser, 1949, *M. neurobius* Schuberg et Schröder, 1905, *M. scardini* Reuss, 1906, and *M. squamae* Keysselitz, 1908. We suspect these taxa are misidentified because these parasites infect fishes on other continents with no zoogeographical connection to the hosts examined herein (Grinham and Cone, 1990).

The remaining 114 species are distributed throughout 13 host families (Cyprinidae 42 species, Catostomatidae 23, Centrarchidae 21, Salmonidae 8, Cyprinodontidae 6, Esocidae 3, Percidae 4, Percopsidae 2, Clupeidae 1, Cottidae 1, Ictaluridae 1, Gasterosteidae 1, and Poeciliidae 1) and have been reported mostly from hosts in North America. One of these, *M. cerebralis* Hofer, 1903, is a well-documented introduction from Europe. Interestingly, *Myxobolus catostomi*, Farnham, Porter and Richardson, 1939 is known to occur in *Catostomus catostomus* in eastern Asia (Pugachev, 1980).

The molecular phylogeny of freshwater species of *Myxobolus*, based on GenBank depositions of about 120 species, most of which are from North America, Europe, and Asia, shows paraphyly–polyphyly and an overall poor correlation between strict spore morphology, tissue specificity, features of the actinospores, and the geographical distribution in freshwater host families (Fiala, 2006). Embedded in this complex phylogeny, however, are terminal clades showing some distinction with these factors. For example, Molnár et al. (2008) describe a lineage involving 4 myxobolids with ellipsoidal shaped spores, all with similar 18S rDNA sequences and all parasitizing gills of the roach (*Rutilus rutilus*) and bleak (*Alburnus alburnus*) in Europe, that forms a terminal clade embedded among 24 other species of *Myxobolus* from cyprinid fishes. Cone et al. (2005) found that *Myxobolus bilobus* Cone, Yang, Sun, and Easy, 2005 occurred, with high bootstrap support, within a clade that included 10 other species of the genus from gills or muscle tissue of various cyprinid fishes from North America and Eurasia. Similarly, Ferguson et al. (2008) examined species of *Myxobolus* from salmonids in western North America and Japan, most of which had pyriform-shaped spores and most of which developed in the brain, spinal cord, or peripheral nerves. These species formed another terminal clade positioned among a complex phylogeny of different spore types infecting a variety of fish families. The present results indicate that the greatest percent similarity of the 18S rDNA of the new species lies with those species in the genus described from other cyprinid fishes in the Northern Hemisphere. The molecular phylogeny further suggests that amongst these species, *M. ridouti* n. sp. and *M. ridgwayi* n. sp. are members of a separate clade.

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