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A NEW SPECIES OF MYXOZOAN (MYXOSPOREA) FROM THE BRAIN AND SPINAL CORD OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FROM IDAHO

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ABSTRACT: A new species of Myxosporea, *Myxobolus neurotropus* n. sp., is described from the brain and spinal cord of rainbow trout (*Oncorhynchus mykiss*) from Duncan Creek, Owyhee County, Idaho. Spores are oval, have 2 pyriform polar capsules, and possess a thick spore wall (sutural rim) with a short intracapsular offshoot. The mean spore dimensions are length 11.8 μm, width 10.8 μm, and thickness 8.8 μm. This myxozoan is compared to other described *Myxobolus* species found in cranial tissues of salmonids in terms of spore morphology and phylogenetic analysis. Because it is found in brain and spinal cord, it is encountered while performing screening tests for *Myxobolus cerebralis*, the causative agent of salmonid whirling disease. Where chronic inflammation and granulomatous lesions are associated with *M. cerebralis*, histological examination shows no host response to *M. neurotropus* n. sp. A diagnostic polymerase chain reaction (PCR) test is included as an aid in properly identifying the species.

Myxobolus Butschli, 1882 (Myxozoa, Myxosporea, Myxobolidae) includes over 700 described species, primarily from fish hosts (Eiras et al., 2005). Of these, several species have been detected in the cranial tissues of salmonids, including *Myxobolus cerebralis* (Hofer, 1903), *Myxobolus neurobius* (Schuberg & Schröder 1905), *Myxobolus kisutchi* (Yasutake & Wood 1957), *Myxobolus arcticus* (Pugachev & Khokhlov 1979), and *Myxobolus farionis* (Gonzalez-Lanza & Alvarez-Pellitero 1984). *Myxobolus cerebralis* is found in cartilage or bone, whereas *M. neurobius*, *M. kisutchi*, *M. arcticus*, and *M. farionis* have been described in nerve tissue. While screening salmonids for *M. cerebralis*, the causative agent of salmonid whirling disease, we observed another *Myxobolus* species. Herein, we describe this myxobolid as a new species from brain and spinal cord of rainbow trout (*Oncorhynchus mykiss*); also included are 18S SSU rDNA sequence information and results of a diagnostic polymerase chain reaction (PCR) test.

MATERIALS AND METHODS

Sample source

Rainbow trout were obtained from Duncan Creek, Owyhee County, Idaho in 2003, 2005, and 2006. For each necropsy, fish were given a lethal dose of tricaine methanesulfonate (Argent Laboratories, Redmond, Washington). In 2003, with the use of a dermal biopsy punch (Miltex Instrument Co., Bethpage, New York) and adult fish, we removed a 6-mm punch of tissue from the area posterior to the eye encompassing the brain and including the medulla oblongata, to be used for DNA extraction. In 2005, heads of juvenile fish were removed behind the operculum to include the brain and anteriormost portion of the spinal cord. The remaining body was divided into anterior, medial, and posterior sections. All tissues were fixed in 10% neutral buffered formalin for histological examination. In 2006, adult fish were obtained. Brain tissue and the anteriormost part of the spinal cord were removed and stored at 4 C to be processed later the same day for microscopic examination. The spinal cords were dissected from these fish, and cut into quarters for pepsin–trypsin digest (PTD) to be done on each quarter.

Microscopy

The brain and spinal-cord tissue were blended with sterile saline in a Waring blender, layered on 8 ml of 55% dextrose, centrifuged at 850 g for 30 min, and pelletized. The resulting spore pellet was washed with sterile saline and refrigerated for fresh, wet-mount preparations.

To determine spore morphology, slides were prepared from the fresh spore pellet alone, with the addition of India ink to visualize a mucous envelope, and with 2% Lugol's iodine to observe the presence of an iodinophilic vacuole. Spores in the fresh wet mount were photographed with the use of Nomarski differential interface contrast to aid in counting the polar filament coils within the polar capsules. Measurements, based on 31 spores, were obtained according to Lom and Arthur (1989) with a Nikon Optiphot microscope with the use of a Leica DC500 color digital camera and Image Pro Express v. 5.1 digitizing software. An aliquot was sent to the Utah Veternary Diagnostic Laboratory, Logan, Utah, where it was processed by standard techniques and photographed with the use of a scanning electron microscope.

Pepsin–trypsin digest (PTD)

PTD was performed on each quarter of the dissected spinal cords following American Fisheries Society Bluebook protocols (MacConnell, 2003) modified in that the spinal cord was not defleshed prior to adding pepsin.

Histology

With the use of the fixed tissues, heads were processed first by standard techniques, sectioned, and then stained with May-Grunwald Geimsa and H&E stains. Slides were examined under a compound microscope for the presence of parasite developmental stages and myxospores in brain and spinal cord. The fish determined to be positive for myxospores in the brain and spinal cord were used; the anterior, medial, and posterior sections were processed as above and all tissues were examined for the presence of the parasite.

PCR, sequencing, and phylogenetic analysis

Genomic DNA was isolated from 6-mm punches of brain tissue with the use of a QIAGEN DNeasy™ tissue kit (QIAGEN, Valencia, California) according to the manufacturer's protocol.

Following partial 18S SSU rDNA sequencing of isolates from the Duncan Cr. location (Hogge et al., 2004), additional primers were designed and used in conjunction with existing primers to amplify overlapping fragments of the 18S SSU rDNA. In the 5′ region, primers 18E (CTGGTTGATCCTGCCAGT) (Hillis and Dixon, 1991) and Mc3R (CCCGTAACCGAAAAACTTGA) (Hogge et al., 2004) produced a fragment ~1,200 base pairs (bp). Primers CH1 (GGCGTCAAAACTA TCAAGG) and CH1440 (TTGCCTCCAACGTCACAATA) (this study) produced a fragment ~1,400 bp. In the 3′ region Myxgen3F (GGAC TAACRAATGCGAAGGCA) (Kent et al., 2000) and 18R (CTACGG AAACCTTGTTACG) (Whipps et al., 2003) were used to amplify a fragment ~1,000 bp long.

The PCR was performed in 50-μl volumes consisting of 1× PCR buffer, 2.5 mM $MgCl_2$, 5 μM tetramethylammonium chloride, 400 μM deoxynucleotide triphosphates, 20 pmol of each primer, 2 U *Taq*, and 2 μl of template DNA. The DNA was denatured at 94 C for 5 min, followed by 35 cycles of 94 C for 1 min, 56–59 C for 1 min, and 72 C for 1–2 min. Amplification products were sequenced by Nevada Genomics Center (University of Nevada, Reno, Nevada) with the use of primers listed above and primer Mc5L683 (AACAACTGGAGGGCA AGTCT) (Hogge et al., 2004), then aligned and checked using Sequencher™ 4.6 (Gene Codes Corporation, Ann Arbor, Michigan).

For comparison, SSU rDNA sequences of other representative myxozoans were retrieved from GenBank, including *M. cerebralis* (Gen-

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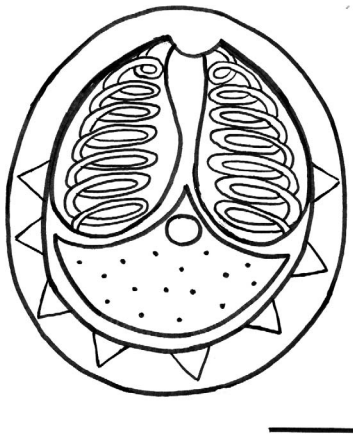

FIGURE 1. *Myxobolus neurotropus* n. sp. Line drawing of the myxospore. Scale bar = 5 μm.

Bank accession number U96492), *M. neurobius* (AF085180), *M. arcticus* (AF085176), *Myxobolus insidiosus* (U96494), *Myxobolus squamalis* (U96495), *M. kisutchi* (EF431919), and *Ceratomyxa shasta* (AF001579). These sequences were truncated to match the sequence of the new species and aligned with the use of ClustalW (Thompson et al., 1994) in the San Diego Supercomputer Center (SDSC) Biology Workbench (http://workbench.sdsc.edu). The software program PAUP version 4.0b10 (Swofford, 1998) was used to estimate uncorrected pairwise genetic distances between aligned sequences. A maximum-likelihood estimation (MLE) phylogram was constructed, also with PAUP, under the settings for the General Time Reversible plus Gamma model as identified with the use of the software program FINDMODEL (http://www.hiv.lanl.gov/content/hiv-db/findmodel/findmodel.html). Support for nodes were assessed with the use of 100 bootstrap replications, with *C. shasta* included as an outgroup.

Diagnostic PCR

In the SDSC Biology Workbench, the *M. neurotropus* n. sp. sequence and GenBank sequences from the various *Myxobolus* species listed above were aligned with the use of ClustalW. Primer 3 (SDSC Biology Workbench) was used to identify primer pairs in regions that would only amplify *M. neurotropus* n. sp. PCR was performed as above with the use of the resulting primers CH260L (TTACCGTGAACGTGACT CAGC) and CH260R (GATGTGAACTACACCCACAGCTA) amplifying at 94 C for 5 min, followed by 35 cycles of 94 C for 1 min, 59 C for 45 sec, and 72 C for 1 min to produce a 260-bp amplicon. This diagnostic PCR was then tested for specificity with 9 isolates of the new species from separate locations throughout Idaho representing all fish host species known to date, cutthroat trout (*Oncorhynchus clarki*, both Yellowstone and westslope forms), steelhead and rainbow trout

FIGURE 2. *Myxobolus neurotropus* n. sp. spore in wet mount, Nomarski differential interface contrast. Scale bar = 5 μm.

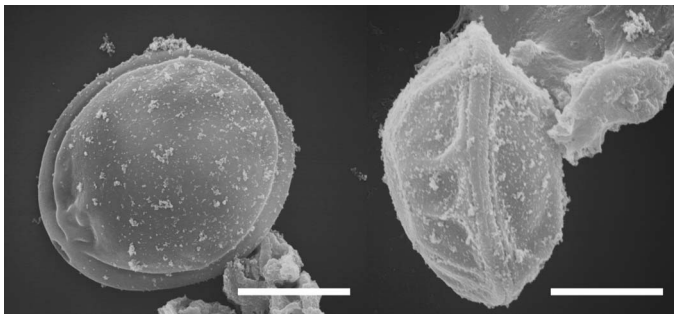

FIGURE 3. Scanning electron microscope (SEM) image of *Myxobolus neurotropus* n. sp. Scale bar = 4.29 μm.

(*O. mykiss*), kokanee and sockeye salmon (*Oncorhynchus nerka*), Chinook salmon (*Oncorhynchus tshawytscha*), and bull trout (*Salvelinus confluentus*). Eight *M. cerebralis* isolates (PCR confirmed; Andree et al., 1998) from varying regions in Idaho were tested, as well as *M. kisutchi* (Washington), 2 *M. squamalis* isolates (California and Oregon), *M. insidiosus* (Oregon), *Henneguya salmincola* (Idaho), *Henneguya* sp. (Idaho), and *Myxobolus* sp. free isolates of rainbow trout, cutthroat trout (*O. clarki*), brook trout (*Salvelinus fontinalis*), bull trout (*S. confluentus*), Chinook salmon (*O. tshawytscha*), and sockeye salmon (*O. nerka*). Amplification products were electrophoresed through a 2% agarose gel, stained with 1% ethidium bromide, and visualized under ultraviolet light.

DESCRIPTION

Myxobolus neurotropus n. sp.

(Figs. 1–4)

Plasmodia: Cysts in brain and spinal cord not present. No trophozoites observed.

Mature spores: Oval, occasionally almost circular in valvular view. Thick spore wall (sutural rim) with short intracapsular offshoot (Fig. 1). Two polar capsules pyriform, 1 slightly longer than the other, extending past the midlength of spore, 6–8 polar filament coils (Fig. 2). Mean spore measurements in micrometers ± SD (n = 31); length, 11.8 ± 0.48; width, 10.8 ± 0.49; thickness (n = 5) 8.8 ± 0.31. Polar capsule

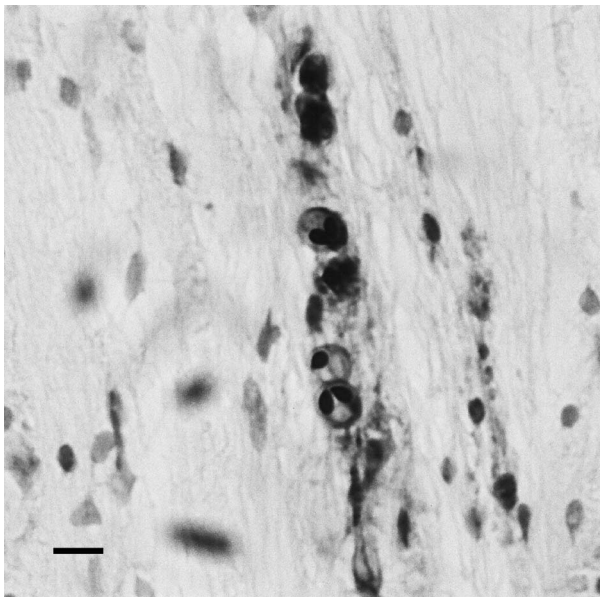

FIGURE 4. Histological section of anterior spinal cord. *Myxobolus neurotropus* n. sp. spores lying along white matter tracks. Giemsa. Scale bar = 10 μm.

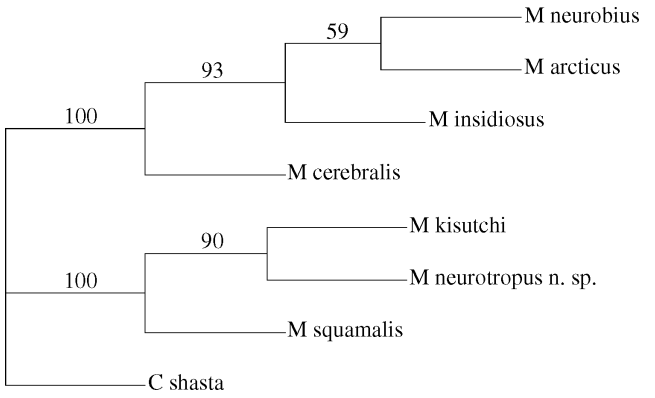

FIGURE 5. Unrooted phylogram showing relationships among 18s rDNA sequences of *Myxobolus* spp. and *Ceratomyxa shasta* under the General Time Reversible plus Gamma model. Comparisons were based on 1,661 bp of homologous sequence. Bootstrap confidence levels are based on 100 resamplings. See text for GenBank accession numbers of the specimens examined.

measurements (n = 31); 5.6 ± 0.55 × 3.7 ± 0.54, and 5.9 ± 0.57 × 3.7 ± 0.43. Spore lenticular in sutural view (Fig. 3), valve surface smooth. Suture line ridged with prominent groove on valve edge; 5–10 sutural markings along the sutural margin. Polar filament pores elongate, situated on sutural rim at anterior end (Fig. 3). Mucous envelope on posterior sometimes observed; iodinophilic vacuole present.

Taxonomic summary

Host: Rainbow trout, *Oncorhynchus mykiss* Walbaum, 1792.
Site of infection: Brain and spinal cord.
Locality: Duncan Creek, Owyhee County, Idaho (580334E 4711749N, mapping datum WGS84).
Prevalence: Seven of 10 fish positive by PTD and PCR.
Material deposited: At the U.S. National Parasite Collection, Beltsville, Maryland: spores in 10% NBF as syntypes USNPC no. 99657 (Aliquot in 95% ethanol retained at Eagle Fish Health Lab until USNPC can accept alcohol fixed tissues.), histological slides with spores in spinal cord as syntypes USNPC no. 99658, and histological slides with spores in brain as syntypes USNPC no. 99659.
Etymology: The specific name refers to the organism's affinity for nerve tissue.

RESULTS

PTD and histology

Myxobolus neurotropus n. sp. spores were found in the brain and in all 4 quarters of the spinal cord. Histological sections that exposed the brain continuous with the spinal cord revealed spores in the diencephalon, myelencephalon, and spinal cord. Within the brain, spores lay freely in small pockets. In the spinal cord, spores tended to lie along white-matter tracts (Fig. 4). No inflammatory or reactive responses were observed in the brain or spinal cord. No spores or prespore stages were found in other tissues.

DNA sequencing and phylogenetic analysis

A 2,027-bp sequence of *M. neurotropus* n. sp.18S SSU rDNA was obtained (GenBank DQ846661). Of this, 1,661 bp were compared to homologous sequences of 6 reference *Myxobolus* species (Fig. 5). Pairwise genetic distances among the *Myxobolus* species sequences ranged from 0.008 (*M. neurobius* versus *M. arcticus*), to 0.238 (*M. cerebralis* versus *M. squamalis*) (Table I). The *M. neurotropus* n. sp. sequence exhibited the least genetic distance in comparisons against *M. kisutchi* (0.035), and subsequently clustered with *M. kisutchi* in a well-supported clade (100% bootstrap support) that included *M. squamalis*. The other clade contained the remaining *Myxobolus* species (*M. neurobius*, *M. arcticus*, *M. insidiosus*, and *M. cerebralis*) and also exhibited 100% bootstrap support.

Diagnostic PCR

The expected 260-bp product was visualized in all 9 *M. neurotropus* n. sp. samples. No product was visualized with *M. cerebralis*, *M. kisutchi*, *M. squamalis*, *M. insidiosus*, *Henneguya salmonicola*, and *Henneguya* sp., or with *Myxobolus* sp.—free rainbow trout, cutthroat trout, brook trout, bull trout, Chinook salmon, and sockeye salmon (Fig. 6).

Remarks

Myxobolus species exhibit a high degree of host specificity, especially with regard to salmonid fishes. Therefore, we are limiting our comparison to *Myxobolus* species that have been described from the cranial tissues of Salmonidae.

Myxobolus neurotropus n. sp. differs from other described species in spore morphology and tissue tropism (Table II). Although the spore of *M. neurotropus* n. sp. resembles that of *M. cerebralis*, the overall size is larger on average and it is not found in cartilage. It is smaller than *M. arcticus* and does not have the pyriform shape. It is larger than *M. farionis*, which is described to be oviform, in some occasions pyriform or ellipsoidal. Unfortunately, species descriptions were not made from fresh wet mounts in the case of *M. kisutchi* (formalin-fixed tissue) and *M. neurobius* (originally described from alcohol-fixed tissue). Shrinkage of spores caused by preservatives typically

TABLE I. Uncorrected pairwise genetic distances among homologous 18S rDNA sequences from *Myxobolus* spp. and the outgroup *Ceratomyxa shasta*.

	M. kisutchi	*M. neurotropus*	*M. squamalis*	*M. neurobius*	*M. arcticus*	*M. insidiosus*	*M. cerebralis*	*C. shasta*
M. kisutchi	—							
M. neurotropus	0.035	—						
M. squamalis	0.091	0.087	—					
M. neurobius	0.230	0.227	0.232	—				
M. arcticus	0.231	0.228	0.236	0.008	–			
M. insidiosus	0.234	0.229	0.237	0.019	0.025	—		
M. cerebralis	0.220	0.226	0.238	0.068	0.072	0.080	—	
C. shasta	0.288	0.280	0.298	0.282	0.280	0.291	0.290	—

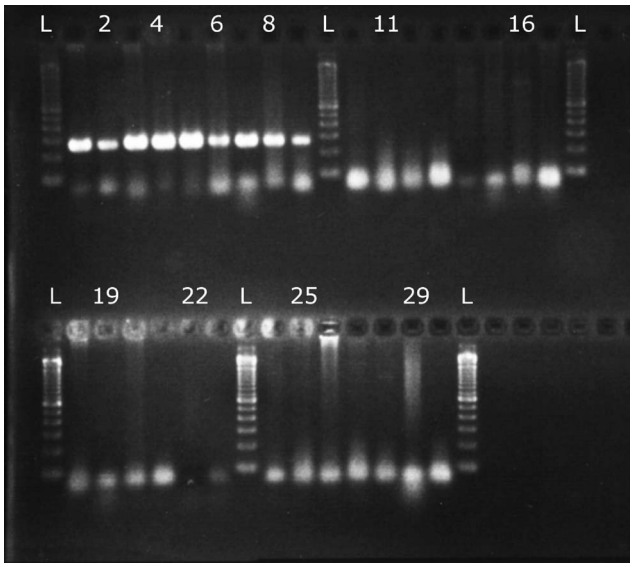

FIGURE 6. Two percent agarose gel showing the 260-bp rDNA amplicon from the *Myxobolus neurotropus* n. sp. diagnostic PCR. In the top row lanes 1–9 are *M. neurotropus* n. sp. positive samples from host species (see text) *Oncorhynchus mykiss* (lanes 1, 2, 8, and 9), *Salvelinus confluentus* (lane 3), *Oncorhynchus clarki* (lanes 4 and 5), *Oncorhynchus tshawytscha* (lane 6), and *Oncorhynchus nerka* (lane 7). Lanes 10–17 are *Myxobolus cerebralis* positive samples (PCR confirmed; Andree et al. 1998). In the bottom row lanes 18–23 are isolates of *Myxobolus kisutchi* (WA), *Myxobolus squamalis* (CA), *M. squamalis* (OR), *Myxobolus insidiosus* (OR), *Henneguya* sp. (ID), and *Henneguya salminicola* (ID) respectively; and lanes 24–29 are pepsin–trypsin digest confirmed *Myxobolus* sp. negative *O. mykiss*, *O. tshawytscha*, *O. nerka*, *O. clarki*, *Salvelinus* fontinalis, and *S. confluentus*. Lane 30 is a master mix negative control.

does not exceed 15% (Hedrick et al., 1991). Therefore, even with shrinkage considered, *M. kisutchi* is smaller than *M. neurotropus* n. sp. The last species to consider is *M. neurobius*.

An effort was made by the authors to obtain samples of *M. neurobius* from trout in the Gutach, Germany (type host and type location) for current morphological and molecular comparison. Not only was reference material for *M. neurobius* unavailable, German colleagues report that they no longer see infected fish despite considerable routine diagnostic screening (M. El-Matbouli, pers. comm.). Thus, comparisons are based on the original description of *M. neurobius* by Schuberg and Schröder (1905) from alcohol-fixed nerve tissue and a later description by Pugachev and Khokhlov (1979), presumably from fresh material. Although the latter authors indicate a spore size for *M. neurobius* that is larger than *M. neurotropus* n. sp., clearly the difference, reported in both descriptions of *M. neurobius*, is that the spore width and breadth is much less than those of *M. neurotropus* n. sp. (see Table II). Line drawings and text describing the *M. neurobius* spore report a rounded posterior end, but a tapered anterior. The *M. neurotropus* n. sp. spore is oval or occasionally almost circular, but not pyriform. Schuberg and Schröder (1905) indicate 8–10 polar filament coils in their original description of *M. neurobius*, whereas drawings given by Shulman (1966) indicate 13 coils both figures higher than the 6-8 coils we report for *M. neurotropus* n. sp.

The inability to consistently distinguish the morphologically similar spores of *M. cerebralis* and *Myxobolus neurotropus* n. sp. was the impetus for this research. At times, the *M. neurotropus* n. sp. spores observed in PTD appeared larger than the range for *M. cerebralis*. Other times, the spore size was similar to those of *M. cerebralis*. Despite this morphological similarity, phylogenetic analysis indicates that *Myxobolus neurotropus* n.

TABLE II. Comparison of mean measurements (in micrometers) and ranges (in parentheses) of *Myxobolus neurotropus* n. sp. spores with those of other *Myxobolus* species found in cranial tissues of salmonids.

Species	Spore morphology					Site	Reference
	Length	Width	Thickness	Shape	PC,* length		
M. neurotropus	11.8† (11.2–13)	10.8 (10.4–12.3)	8.8 (8.4–9.1)	Oval to circular	5.6 and 5.9 (5.0–6.9)	Fresh brain and spinal cord. No cyst formation.	This study
M. kisutchi	(7–8.5)	(6.5–7)	(3.5–3.8)	Oval	(3.8–5.5)	Fixed spinal cord. No cyst formation.	Yasutake and Wood (1957)
M. cerebralis	(7.4–9.7)	(7–10)	(6.2–7.4)	Oval to circular	(4.2–6)	Fresh cartilage	Lom and Dykova (1992)
M. neurobius	(10–12)	8	6	Oval, tapered anterior	(6–7)	Fixed nerve tissue. Cyst formation.	Schuberg and Schröder (1905)
M. neurobius	(13.4–14)	(8.5–9.2)	(6.8–7.4)	Oval, tapered anterior	(7.8–8.5)	Fresh nerve tissue	Pugachev and Khokhlov (1979)
M. arcticus	(14.3–16.5)	(7.6–7.7)	(6.8–7.4)	Pyriform	(6.6–9)	Brain and spinal cord	Pugachev and Khokhlov (1979)
M. farionis	9.15 (8.5–10)	6.67 (6–7.5)	4.7 (4.5–5)	Oviform-Pyriform	4.85 (4.5–5.5)	Fresh brain and spinal cord	Gonzalez–Lanza and Alvarez–Pellitero (1984)

* Polar capsule.
† Mean (micrometers), (range).

sp. is very distinct from *M. cerebralis*, but rather emphasizes the relatedness to *M. kisutchi*. Of the 1,661 nucleotide sites compared, *M. neurotropus* n. sp. and *M. kisutchi* have differences at 60, including 4 insertion/deletions. This is greater than the pairwise differences observed when comparing *M. neurobius*, *M. arcticus*, and *M. insidiosus* over the same region.

In light of the distinctive spore morphology and phylogenetic analysis, this species should be considered a new one, which we designate *M. neurotropus* n. sp. in reference to its affinity for nerve tissue.

DISCUSSION

The *M. neurotropus* n. sp. spore has a similar morphology to that of *M. cerebralis*, except that the *M. neurotropus* n. sp. spore is larger overall. The 2 species differ in their tissue tropism and corresponding host response. *Myxobolus neurotropus* n. sp. does not induce an inflammatory response or nerve tissue destruction, whereas *M. cerebralis* is associated with granulomatous lesions and chronic inflammation of host cartilage. Indeed, *M. cerebralis* is the causative agent of salmonid whirling disease and has been reported to be responsible for host population declines in certain western rivers (Nehring and Walker, 1996). Because we have not detected any lesions associated with *M. neurotropus* n. sp., it does not appear to be pathogenic to its salmonid host.

Due to the morphological similarity of the spores, it is important to clearly distinguish between *M. neurotropus* n. sp. and *M. cerebralis*. We have demonstrated both parasite species in a single fish and in a group of fish collected from the same location. The diagnostic PCR can be used in conjunction with the *M. cerebralis* diagnostic PCR (Andree et al., 1998) to clarify species identification and to identify mixed infections.

In addition to the type host, rainbow trout, *M. neurotropus* n. sp. infects cutthroat trout (both Yellowstone and westslope forms), bull trout, Chinook salmon, and kokanee and sockeye salmon in Idaho. The geographic range within the state encompasses waters of all 7 fisheries management regions (Hogge et al., 2004).

The distribution of *M. neurotropus* n. sp. appears to be widespread. With the use of PCR, we have confirmed *M. neurotropus* n. sp. in rainbow trout (*O. mykiss*) from Washington and in cutthroat trout (*O. clarki*) from Utah and it may also be present in Oregon (Lorz et al., 1989) and California (Hedrick et al., 1991).

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