

Discriminating between a Neurotropic *Myxobolus* sp. and *M. cerebralis*, the Causative Agent of Salmonid Whirling Disease

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Abstract.—While screening salmonids for *Myxobolus cerebralis*, the causative agent of whirling disease, we detected a neurotropic *Myxobolus* sp. that is morphologically similar to the *M. cerebralis* spore in brain and other nervous tissue. We developed a polymerase chain reaction (PCR)-based diagnostic technique to differentiate the two species of *Myxobolus*. Primers were designed for a 277-base-pair (bp) sequence within the 18S ribosomal DNA (rDNA) gene, and the restriction enzyme *Mse*-I was chosen to cut the amplified product into diagnostic fragments. When electrophoresed in an acrylamide gel, *M. cerebralis* yielded a two-band pattern while the neurotropic *Myxobolus* sp. yielded a three-band pattern. This diagnostic method has resolved cases in which the pepsin–trypsin digest screening test indicated *Myxobolus* spores but histological examination or PCR was negative for *M. cerebralis*. We were also able to eliminate *M. kisutchi* as the neurotropic *Myxobolus* sp. using spores from infected coho salmon *Oncorhynchus kisutch* obtained from Minter Creek, Washington (the type host and type location for *M. kisutchi*), by designing primers to amplify a larger 683-bp amplicon within the 18S rDNA gene. Sequencing of this 683-bp product from the neurotropic *Myxobolus* sp. and *M. kisutchi* was then performed. Sequence alignment and phylogenetic analysis on the neurotropic *Myxobolus* sp. and *M. kisutchi* as well as *M. cerebralis*, *M. arcticus*, *M. neurobius*, *M. insidiosus*, and *M. squamalis* suggest that the neurotropic *Myxobolus* sp. is a new species.

Myxobolus cerebralis, the causative agent of whirling disease, has been the object of considerable examination, investigation, and concern from fish health managers owing to the reported impact of the disease on salmonid fish populations in some river systems in the western United States. The parasite has been reported in 22 U.S. states (Bartholomew and Reno 2002). Accurate detection of *M. cerebralis* in wild and cultured salmonids is crucial for certifying fish (USFWS and AFS–FHS 2003) and monitoring and preventing the spread of the parasite.

Detection of *M. cerebralis* typically entails digesting cranial tissue with pepsin and trypsin, then performing microscopic screening for the spores (Markiw and Wolf 1974). Several other *Myxobolus* species with similar morphology have been detected in the cranial tissues of salmonids, including *M. neurobius* (as cited in Hoffman 1999), *M. kisutchi* (Yasutake and Wood 1957), and *M. arcticus* (as cited in Hoffman 1999). Using spore morphology and size alone makes identification difficult. Confirmation of *M. cerebralis* (MacConnell 2003) is obtained from histological examination of spores found in cartilage or bone or polymerase chain reaction (PCR; Andree et al. 1998). Histo-

logical examination is difficult and expensive and lacks sensitivity. Polymerase chain reaction methods are highly sensitive but expensive, and many laboratories are not equipped to perform the test. Laboratories having the equipment and expertise can accurately detect the presence of *M. cerebralis* with PCR. Identification is unresolved when *Myxobolus* spores are detected in pepsin–trypsin digest (PTD) but the *M. cerebralis* PCR is negative.

Since 1987, when *M. cerebralis* was first confirmed in Idaho waters, the Eagle Fish Health Laboratory of the Idaho Department of Fish and Game (with which we are affiliated) has sampled both cultured and wild salmonid populations from all river systems statewide using Blue Book protocols (MacConnell 2003). During these investigations we observed *Myxobolus* spores with morphology and dimensions overlapping *M. cerebralis* but that could not be confirmed as *M. cerebralis* by means of histology and PCR. Histology demonstrated *Myxobolus* spores in brain and other nerve tissues. This neurotropic *Myxobolus* sp. has been detected in fish from nearly all of the river systems in Idaho, including watersheds shown to be positive for *M. cerebralis*. After years of observing, measuring, and documenting these *Myxobolus* spores and performing tests to demonstrate that they are not *M. cerebralis*, we wanted to resolve the confusion surrounding this *Myxobolus* sp. Therefore, the objec-

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tives of this study were (1) to develop a PCR-based diagnostic technique to discriminate between the two species of *Myxobolus* and (2) to characterize the relatedness of this neurotropic species of *Myxobolus* to other known *Myxobolus* species.

Methods

Samples and sample processing.—The Eagle Fish Health Laboratory routinely samples cultured and wild salmonids for the presence of *M. cerebralis*. Whole heads or head wedges are typically split in half, one half-head being used for PTD screening and the other being stored at -20°C for *M. cerebralis* confirmation. Half-heads are combined in pools of five fish for the PTD. The findings for each pool of fish are reported as the number of spores per 30 microscope fields at $400\times$ magnification, and spore measurements are noted. For *M. cerebralis* confirmation, either histology or PCR is performed.

For this study, whirling disease analysis data from 1995 to 2003 were reviewed. Ninety-four individual archived half-head samples were chosen to represent three categories: (1) cases in which the PTD report indicated that *Myxobolus* spores were detected but *M. cerebralis* was not confirmed; (2) cases in which histology reports indicated that *Myxobolus* spores were present in brain or other nerve tissue; and (3) cases confirmed positive for *M. cerebralis*. The samples encompassed 22 locations from eight river drainages in Idaho. Host species included rainbow trout *Oncorhynchus mykiss*, cutthroat trout *O. clarki*, and Chinook salmon *O. tshawytscha*. An *M. cerebralis*, PCR-positive control (plasmid p18Tr29; Andree et al. 2002) and a *Myxobolus*-negative rainbow trout were included.

Using a dermal biopsy punch (Miltex Instrument Co., Bethpage, New York), we took a 6-mm punch of tissue from the area immediately posterior to the eye and encompassing the brain and ventral cranium. Genomic DNA was isolated using a Qia-gen DNeasy tissue kit (Qiagen, Valencia, California) following the manufacturer's protocol.

Oligonucleotide design and PCR.—GenBank sequences of approximately 1,600 base pairs (bp) of the 18S ribosomal DNA (rDNA) gene from 12 different species in the genus *Myxobolus* (*M. arcticus*, *bramae*, *cerebralis*, *cyprini*, *djragini*, *elipsoides*, *insidiosus*, *musculi*, *neurobius*, *portucalensis*, *sandrae*, and *squamalis*) were aligned using the CLUSTALW (Thompson et al. 1994) multiple-sequence alignment program available on the San Diego Supercomputer Center (SDSC) Biology Workbench (<http://workbench.sdsc.edu>). The primer

construction program PRIMER 3 from the SDSC workbench was used to identify primer pairs in highly conserved regions capable of amplifying sequences of varying length in all 12 of the *Myxobolus* species listed above. The following primer pair was selected to amplify 277 bp within the 18S rDNA gene: Mc5L (5'-TCCGTATTGGGGTGATGATT-3') and Mc3R (5'-CCCCTAACCGAAAAACTTGA-3').

Using the DNA extracted from a subset of the 94 samples, the PCR was optimized in 50- μL reactions consisting of $1\times$ PCR buffer, 2.5 mM MgCl_2 , 5 μM tetramethylammonium chloride, 400 μM deoxynucleotide triphosphates, 20 pmol Mc5L primer, 20 pmol Mc3R primer, 2 U *Taq*, and 2 μL template DNA. The DNA was denatured at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 56°C for 45 s, and 72°C for 1 min. Amplification was performed and the amplification products sequenced. These sequences were aligned using CLUSTALW and analyzed with the online DNA restriction enzyme mapping program NEB-cutter V1.0 (New England Biolabs, Beverly, Massachusetts; <http://tools.neb.com/NEBcutter2/index.php>) to identify restriction enzymes capable of identifying differences in the sequences with diagnostic fragments suitably sized for viewing on a gel. We determined that *Mse*-I (New England BioLabs) would yield optimal results.

All DNA samples were first analyzed for the presence of *M. cerebralis* using either nested or single-round PCR following the protocol of Andree et al. (1998). Each sample was then amplified with the discriminatory primers (Mc5L and Mc3R) and digested with *Mse*-I following the manufacturer's protocol. Products were electrophoresed in a 3% acrylamide gel and visualized under ultraviolet light.

Sequencing and phylogenetic analyses.—Our second objective was to look at the relatedness of this species to other known *Myxobolus* species. Of the three *Myxobolus* species described in cranial tissue of salmonids, *M. kisutchi* was the only one without a sequence in GenBank. To sequence *M. kisutchi*, coho salmon *O. kisutch* from Minter Creek, Washington, were obtained (i.e., the type host and type location for *M. kisutchi*; Yasutake and Wood 1957) and DNA isolated as above. DNA from 4 neurotropic *Myxobolus* samples from the 94 samples in the study was also chosen for sequencing. Monetary constraints determined the number of samples we could sequence. Since we wanted these samples to fully represent our Idaho neurotropic *Myxobolus*, we chose samples from



FIGURE 1.—Map of Idaho showing the locations at which samples of a neurotropic *Myxobolus* species were obtained for sequencing.

three locations that would encompass the state and have biological significance. The first location, White Bird Creek in north-central Idaho, flows into the Salmon River and thus is accessible by anadromous fishes (Figure 1). The second location, Duncan Creek in southwestern Idaho, is located in an isolated, sparsely populated part of the state; it lies within the Snake River basin, but is intermittent (i.e., connected briefly during some high-water years) and is inhabited by indigenous redband trout (a subspecies of rainbow trout). Two samples from Duncan Creek were selected, representing two sampling years. The third sample location, the Blackfoot River in southeastern Idaho, is inaccessible to anadromous fish owing to a natural barrier, Shoshone Falls.

To obtain a longer sequence for comparison, we developed a second forward primer, Mc5L683 (5'-AACAAGTGGAGGGCAAGTCT-3'; see the section on oligonucleotide design above) to use with the 277-bp reverse primer, Mc3R, and create a 683-bp amplification product. The myxosporean 18S

rDNA of the four neurotropic *Myxobolus* samples and two *M. kisutchi* samples was amplified with primers Mc5L683 and Mc3R (see PCR amplification above) and sequenced.

The sequences retrieved from GenBank for *M. cerebralis* (GenBank accession number U96492), *M. neurobius* (AF085180), *M. arcticus* (AF085176), *M. insidiosus* (U96494), *M. squamalis* (U96495), *Myxobolus* sp. (AF378342), *Ceratomyxa shasta* (AF001579), and *Kudoa thyrsites* (AF031412) were truncated to compare homologous sequences with the 683-bp *M. kisutchi* and neurotropic *Myxobolus* sp. sequences. Sequences were aligned using CLUSTALW in the SDSC Biology Workbench as described above. To estimate phylogenetic relationships among sequences, phylograms were generated using maximum likelihood as well as maximum parsimony methods in PAUP version 4.0b10 (Swofford 1998). For the maximum likelihood analyses we used the branch-and-bound algorithm with the HKY85 model of nucleotide substitution (Hasegawa et al. 1985) and the following options: addition sequence = as is, initial upperbound computed heuristically, and the Multrees option in effect. We employed an exhaustive search option algorithm for the parsimony analyses with zero length branches collapsed, no weighting, gaps ignored, addition sequence = furthest, and the Multrees option in effect. Support for the nodes in the parsimony-constructed phylogram was sought via 1,000 bootstrap replicates. *Ceratomyxa shasta* and *K. thyrsites* were included as outgroup comparisons.

Results

Fish Database Review

Microscopic examination of the PTDs of the samples in this study revealed *Myxobolus* spores with a wide range of sizes. The dimensions of the spores from nervous tissue were frequently larger than those of *M. cerebralis*, although there were instances when the size approximated the expected spore size for *M. cerebralis* (Figure 2). The spore morphology of the two species was similar (Figure 3). Often the neurotropic *Myxobolus* spores were sparse and concentrated within small pockets in the brain (Figure 4). It was sometimes necessary to repeat the entire histology preparation, recutting and restaining the tissues, before spores were observed, either in brain tissue (neurotropic *Myxobolus*) or in cartilage (*M. cerebralis*).

Discriminatory PCR

Digestion of the 277-bp product with *Mse*-I resulted in two banding patterns: pattern "A," which

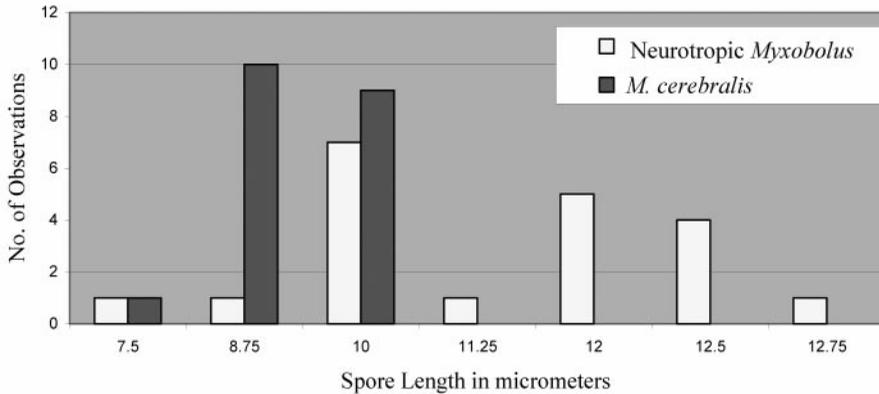


FIGURE 2.—Distributions of spore lengths of the neurotropic *Myxobolus* sp. and *M. cerebralis*, showing the degree of overlap. The samples of *M. cerebralis* were obtained from the Pahsimeroi River, Idaho; those of the neurotropic *Myxobolus* sp. were obtained from the locations indicated in Figure 1.

has two fragments (168 and 109 bp) corresponding to *M. cerebralis* PCR-positive samples; and pattern “B,” which has three fragments (109, 95, and 73 bp) corresponding to *M. cerebralis* PCR-negative, histologically-confirmed neurotropic samples (Figure 5). In some cases, the two banding patterns were demonstrated in separate fish within a single pool from one location. In other cases, both banding patterns were demonstrated in individual fish (Table 1). Of the 94 samples analyzed, the expected banding pattern based on *M. cerebralis* nested PCR was demonstrated in all but 5.

Phylogenetic analyses

Pairwise alignment of the sequences from the White Bird Creek, Duncan Creek, and Blackfoot River isolates showed 99.7% similarity, with differences at only two sites in the 683-bp region. Samples representing two sampling years at Duncan Creek showed 100% similarity. The sequence

alignment of the four neurotropic samples with the *M. kisutchi* samples showed differences at 22 of the 683 sites (96.8% similarity). Alignment comparison of sequences from the neurotropic samples with *M. cerebralis* showed differences at 118 sites (81.6% similarity).

The phylogenetic trees obtained from maximum parsimony and maximum likelihood analyses showed concordant topologies. They were not sensitive to the outgroup used, *C. shasta* or *K. thyrsites*. The species of *Myxobolus* analyzed separated out into two major clades, with bootstrap support of 100% (Figure 6). The first clade contains *M. neurobius*, *M. arcticus*, *M. insidiosus*, and *M. cerebralis*. The second clade contains *M. kisutchi* and *M. squamalis* as well as the Idaho neurotropic *Myxobolus* sp. and a California neurotropic *Myxobolus* sp. from GenBank (AF378342) located by a BLAST (Basic Local Alignment Search Tool; National Center for Biotechnology Information) search. The three geographically separated Idaho samples of *Myxobolus* sp. are very closely related to the neurotropic *Myxobolus* sp. from California. *Myxobolus kisutchi* is more distantly related and is basal to this group, and *M. squamalis* is basal to *M. kisutchi*.

Discussion

This study began amid concern that while screening for *M. cerebralis* another species of *Myxobolus* was routinely being encountered. We recognized the potential for misdiagnosis of *M. cerebralis* in fish infected with spores of similar size and morphology and wanted an additional PCR-

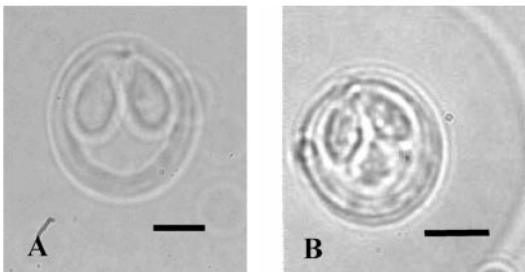


FIGURE 3.—Pepsin-trypsin digests of the spores of (A) neurotropic *Myxobolus* sp. and (B) *M. cerebralis* showing no obvious morphological differences; bars = 4 μ m.

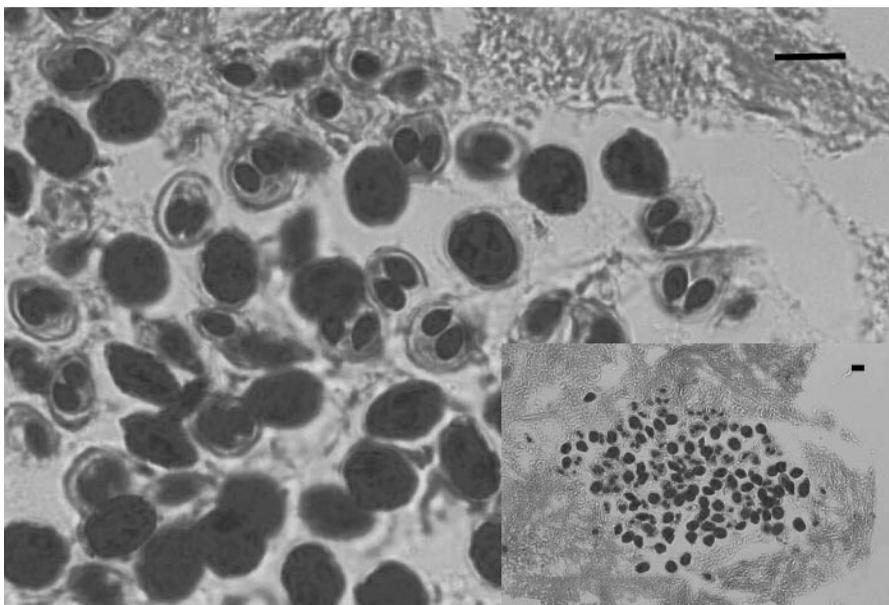


FIGURE 4.—Photomicrographs of an unusually large cluster of neurotropic *Myxobolus* sp. in brain tissue (large photomicrograph = 1,000 \times magnification; insert = 400 \times). Giemsa staining was used; bar = 9 μ m.

based diagnostic test to discriminate between this neurotropic *Myxobolus* sp. and *M. cerebralis*. This test, in combination with PTD and *M. cerebralis* PCR, gives us a clear identification of the *Myxobolus* spores encountered in fish from Idaho waters.

This discriminatory PCR results in two banding

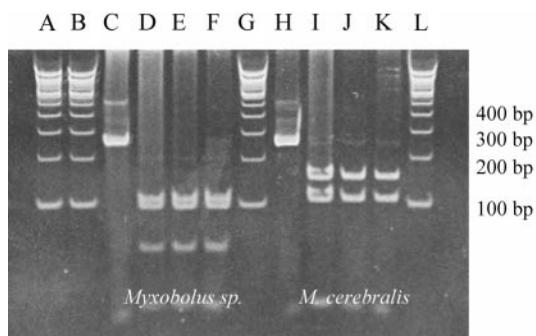


FIGURE 5.—Acrylamide gel of the rDNA of the neurotropic *Myxobolus* sp. and *M. cerebralis* showing the different banding patterns obtained from cutting a 277-bp product with *Mse*-I. Lanes A, B, G, and L show the 100-bp ladder; lanes C and H show uncut product; lanes I–K show pattern “A” (two fragments [168 and 109 bp] corresponding to *M. cerebralis* PCR-positive samples); and lanes D–F show pattern “B” (three fragments [109, 95, and 73 bp] corresponding to the *M. cerebralis* PCR-negative and histologically confirmed neurotropic *Myxobolus* sp.

patterns: pattern A having two bands (168 and 109 bp) corresponding to *M. cerebralis* PCR-positive samples, and pattern B having three bands (109, 95, and 73 bp) corresponding to *M. cerebralis* PCR-negative, histologically-confirmed neurotropic *Myxobolus* sp. Of 94 samples tested with the discriminatory PCR technique, the expected banding pattern based on *M. cerebralis* nested PCR was demonstrated in all but 5. Three of these five samples were fish from one five-fish pool that contained *Henneguya* sp. along with *Myxobolus* spores. Banding pattern A was demonstrated when we expected the neurotropic pattern B. In reviewing the *Henneguya* sequence, we realized that *Mse*-I cuts it into fragments similar to banding pattern A in size. The inconsistent banding pattern was resolved by cutting the 277-bp product with another restriction enzyme, *Dde*-I. In the other two samples in which we did not observe the expected banding pattern, the expected pattern would have been both pattern A and pattern B based on histology and *M. cerebralis* PCR. In both samples we only demonstrated pattern B. In these two samples, the *M. cerebralis* PCR was negative in the round I amplification and positive in the round II or nested amplification. From this we determined that both *M. cerebralis* and the neurotropic *Myxobolus* were probably present but that the *M. cerebralis* was not visualized owing to its low numbers. The

TABLE 1.—Characteristics of a new neurotropic *Myxobolus* species from Idaho, including banding patterns as determined by polymerase chain reaction (PCR) and acrylamide gel electrophoresis, spore size as determined by pepsin-trypsin digest, and tissue specificity as determined by histopathological analysis.

Sample	PCR ^a			Spore size ^b	Tissue specificity
	WD	D-277	<i>Mse</i> -I		
Pool 1				7.5–8.8 × 10	Cartilage and brain
1	+	+	A		
2	+	+	A		
3	0	+	B		
4	0	+	B		
5	0	+	B		
Pool 2				7.5 × 7.5–10	Cartilage and nerve
6	0	+	B		
7	0	+	B		
8	0	+	B		
9	0	+	B		
10	+	+	A, B		

^a A plus indicates amplification, a zero no amplification. The designation WD refers to *M. cerebralis* primers, the designation D-277 to a discriminatory primer for a 277-base-pair (bp) sequence of 18S rDNA. The designation *Mse*-I refers to the banding patterns resulting from digestion of the D-277 product with the endonuclease *Mse*-I. Pattern A consists of two fragments (168 and 109 bp) corresponding to *M. cerebralis* samples; pattern B consists of three fragments (109, 95, and 73 bp) corresponding to samples of the new neurotropic *Myxobolus* species from Idaho.

^b Width × length (μm).

application of this discriminatory technique is made in combination with PTD and *M. cerebralis* PCR. If multiple parasite species are present in the PTD (as was true in our case, both *Henneguya* sp. and neurotropic *Myxobolus* sp. occurring in one group of fish), a Percoll gradient could be used to separate them before DNA is extracted (Andree et al. 1999).

The sequencing results and corresponding phylogenetic analyses reveal a large genetic divergence between the Idaho neurotropic *Myxobolus* sp. and *M. cerebralis*. This divergence probably explains why the development of a diagnostic marker between the two was relatively straightforward. These results also demonstrate that while the Idaho neurotropic *Myxobolus* groups with *M. kisutchi*, it is genetically distinct and deserves its own taxonomic status. The percent sequence similarity between the neurotropic *Myxobolus* sp. and *M. kisutchi* is 96.8%. Previous sequencing of the 18S rDNA gene demonstrated 95.2–96.9% sequence similarity between six other taxonomically distinct *Myxobolus* sp. (*M. arcticus*, *bramae*, *djragini*, *elipsoides*, *insidiosus*, and *neurobius*; Andree et al. 1999).

Although we have not correlated the presence of the neurotropic *Myxobolus* with any disease state, findings of the species are important. Additional sampling suggests that the neurotropic species is widespread. In Oregon, while screening

for *M. cerebralis*, Lorz et al. (1989) reported two different-sized spores with similar morphology. The spores were differentiated by means of histology, and those found in brain tissue were deemed an unidentified *Myxobolus* sp. In California, three myxosporeans were found in the cranial tissues of a single population of rainbow trout (Hedrick et al. 1991); these spores measured 12.7 × 10.5 μm. In Utah, PTD revealed *Myxobolus* spores from Bear Lake cutthroat trout. Although PCR was negative for *M. cerebralis*, our discriminatory PCR confirmed the presence of the neurotropic *Myxobolus* sp. A BLAST search produced a nearly identical match between the Idaho neurotropic *Myxobolus* and a neurotropic *Myxobolus* sp. from California, further indicating a widespread distribution.

Management Implications

The discriminatory PCR that we developed is an important tool in identifying a neurotropic species present in spore digests. Separation of neurotropic spores from those of *M. cerebralis* in free-ranging fish samples is important for fish management. When spores are detected in PTD and the nested PCR fails to confirm the presence of *M. cerebralis*, the interpretation is not clear. Recording such findings as “*Myxobolus* sp.” in databases has been problematic, especially when the databases are accessed by outside researchers (Bar-

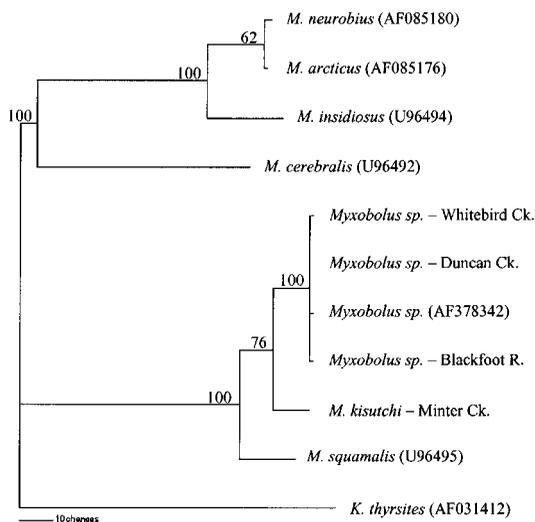


FIGURE 6.—Phylogram generated by parsimony analysis showing the relationships among the three neurotropic *Myxobolus* samples from Idaho, *M. kisutchi*, and other *Myxobolus* species for which sequences were obtained from GenBank (accession numbers in parentheses) based on 683 bp of 18S rDNA. The outgroup is *Kudoa thyrsites*; bootstrap confidence values are shown at the nodes.

tholomew and Reno 2002). The new technique allows us to positively identify the neurotropic species and enter these cases in our database as such.

Myxobolus spores have occasionally been encountered in Chinook salmon smolts and adults at the Idaho Fish and Game's Rapid River Hatchery. The *M. cerebralis* nested PCR examination of these samples has routinely proved negative. When the number of adult Chinook salmon exceeds the hatchery's production needs, the surplus fish are used to supplement natural spawning in the nearby lower Salmon River tributaries and the Clearwater River. In addition, carcasses from adult salmon are in demand for nutrient enrichment programs. Fish health managers are concerned about introducing *M. cerebralis* into new locations. In recent sampling at the hatchery, discriminatory PCR allowed us to quickly determine that the spores seen in PTD were of the neurotropic species.

Conclusion

Phylogenetic analysis suggests that the Idaho neurotropic *Myxobolus* sp. is a new, previously undescribed species. The possibility that its distribution is widespread is important, as the fish health community is committed to screening large

numbers of salmonids for *M. cerebralis*. Knowledge of the existence of the neurotropic *Myxobolus* species and our discriminatory PCR test contribute to the battery of information from which we can draw to assess fish health.

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References

- Andree, K. B., R. P. Hedrick and E. MacConnell. 2002. A review of the approaches to detect *Myxobolus cerebralis*, the cause of salmonid whirling disease. Pages 197–211 in J. L. Bartholomew and J. C. Wilson, editors. Whirling Disease: Reviews and Current Topics. American Fisheries Society, Symposium 29, Bethesda, Maryland.
- Andree, K. B., T. S. McDowell, S. J. Gresoviak, and R. P. Hedrick. 1998. A polymerase chain reaction test for the detection of *Myxobolus cerebralis*, the causative agent of salmonid whirling disease in fish, and a comparison to existing detection techniques. *Diseases of Aquatic Organisms* 34:145–154.
- Andree, K. B., C. Szekely, K. Molmar, S. J. Gresoviak, and R. P. Hedrick. 1999. Relationships among members of the genus *Myxobolus* (Myxozoa: Bivalvidae) based on small subunit ribosomal DNA sequences. *Journal of Parasitology* 85:68–74.
- Bartholomew, J. L., and P. W. Reno. 2002. The history and dissemination of whirling disease. Pages 3–24 in J. L. Bartholomew and J. C. Wilson, editors. Whirling Disease: Reviews and Current Topics. American Fisheries Society Symposium 29, Bethesda, Maryland.
- Hasegawa, M., H. Kishino, and T. Yano. 1985. Dating the human–ape split by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 22:160–174.
- Hedrick, R. P., A. Wishkovsky, J. C. Modin, and R. J. Toth. 1991. Three Myxosporeans found in the cranial and branchial tissues of rainbow trout in California. *Journal of Aquatic Animal Health* 3:55–62.

- Hoffman, G. L. 1999. Parasites of North American Freshwater Fishes, 2nd edition. Comstock Publishing Associates, Ithaca, New York.
- Lorz, H. V., A. Amandi, C. R. Banner, and J. S. Rohovec. 1989. Detection of *Myxobolus (Myxosoma) cerebralis* in salmonid fishes in Oregon. *Journal of Aquatic Animal Health* 1:217–221.
- MacConnell, E. 2003. Whirling Disease of Salmonids. *in* Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 5th edition. American Fisheries Society, Fish Health Section, Bethesda, Maryland.
- Markiw, E. M., and K. Wolf. 1974. *Myxosoma cerebralis*: isolation and concentration from fish skeletal elements—sequential enzymatic digestions and purification by differential centrifugation. *Journal of the Fisheries Research Board of Canada* 31:245–251.
- Swofford, D. L. 1998. PAUP: Phylogenetic analysis using parsimony (and other methods), version 4. Sinauer Associates, Sunderland, Massachusetts.
- Thompson, J. D., K. G. Higgins, and T. J. Gibson. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. *Nucleic Acids Research* 22: 4673–4680.
- USFWS (U.S. Fish and Wildlife Service) and AFS–FHS (American Fisheries Society, Fish Health Section). 2003. Standard procedures for aquatic animal health inspections. *In* Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 5th edition. American Fisheries Society, Fish Health Section, Bethesda, Maryland.
- Yasutake, W. T., and E. M. Wood. 1957. Some Myxosporidia found in Pacific Northwest salmonids. *Journal of Parasitology* 43:633–642.