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Morphological and molecular confirmation of *Myxobolus cerebralis* myxospores infecting wild-caught and cultured trout in North Carolina (SE USA)

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ABSTRACT: We used microscopy and molecular biology to provide the first documentation of infections of *Myxobolus cerebralis* (Myxozoa: Myxobolidae), the etiological agent of whirling disease, in trout (Salmonidae) from North Carolina (USA) river basins. A total of 1085 rainbow trout *Oncorhynchus mykiss*, 696 brown trout *Salmo trutta*, and 319 brook trout *Salvelinus fontinalis* from 43 localities across 9 river basins were screened. Myxospores were observed microscopically in pepsin-trypsin digested heads of rainbow and brown trout from the Watauga River Basin. Those infections were confirmed using the prescribed nested polymerase chain reaction (PCR; 18S rDNA), which also detected infections in rainbow, brown, and brook trout from the French Broad River Basin and the Yadkin Pee-Dee River Basin. Myxospores were 9.0–10.0 µm (mean ± SD = 9.6 ± 0.4; N = 119) long, 8.0–10.0 µm (8.8 ± 0.6; 104) wide, and 6.0–7.5 µm (6.9 ± 0.5; 15) thick and had polar capsules 4.0–6.0 µm (5.0 ± 0.5; 104) long, 2.5–3.5 µm (3.1 ± 0.3; 104) wide, and with 5 or 6 polar filament coils. Myxospores from these hosts and rivers were morphologically indistinguishable and molecularly identical, indicating conspecificity, and the resulting 18S rDNA and ITS-1 sequences derived from these myxospores were 99.5–100% and 99.3–99.8% similar, respectively, to published GenBank sequences ascribed to *M. cerebralis*. This report comprises the first taxonomic circumscription and molecular confirmation of *M. cerebralis* in the southeastern USA south of Virginia.

KEY WORDS: Trout · Salmonid · Southeastern USA · Whirling disease · Morphology · Molecular diagnostics

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INTRODUCTION

Myxobolus cerebralis Hofer, 1903 (Bivalvulida: Myxobolidae), the causative agent of 'whirling disease' of salmonids (Sarker et al. 2015), infects rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Salmoniformes: Salmonidae), brown trout *Salmo trutta* Linnaeus, 1758, (Salmoniformes: Salmonidae), brook trout (char) *Salvelinus fontinalis* (Mitchill, 1814), (Salmoniformes: Salmonidae), and other salmonids in the USA and abroad (O'Grodnick 1979, Lorz et al. 1989, Hoffman 1990). It was first discovered infecting naive rain-

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bow trout imported (uninfected) from the USA (Hofer 1903). Those naive rainbow trout likely contracted infections in Germany that resulted in clinical signs of whirling disease (Sarker et al. 2015). The pathogen may have been introduced to the USA via infected rainbow trout (Hoffman 1990), and since then it has been introduced throughout the western and northeastern USA as a result of translocations of infected trout (Whipps et al. 2004, Sarker et al. 2015) and oligochaete hosts (*Tubifex tubifex* [Müller, 1774]) (Zendt & Bergersen 2000, Krueger et al. 2006). Because whirling disease can harm wild trout populations (Nehring & Walker 1996, Vincent 1996, Thompson et al. 1999), *M. cerebralis* is among the most well-studied of freshwater fish parasites and perhaps the most extensively studied myxozoan species. No previous record of the parasite exists from the southeastern USA south of Virginia (Hoffman 1970, 1999, Halliday 1976, Bartholomew & Reno 2002).

In summer 2015, anglers reported rainbow trout with clinical signs of whirling disease in North Carolina. Those reportedly abnormal rainbow trout were not recovered from the anglers, but several rainbow trout from that site (Watauga River, Aldridge Rd.; Table 1) were soon thereafter captured by the North Carolina Wildlife Resource Commission (NCWRC) and sent to the Southeastern Cooperative Fish Parasite and Disease Laboratory (SCFPDL; Auburn University) for screening. Two of those rainbow trout were infected by myxospores of a species of *Myxobolus*; myxospores were identified as *M. cerebralis* using morphological evidence and the nested PCR protocol of Andree et al. (1998) (see Tables 2–5). Thereafter and in collaboration with the SCFPDL, the NCWRC initiated a state-wide surveillance program for infections of *M. cerebralis* in trout. Rainbow, brown, and brook trout from selected trout waters in 9 major river basins (Broad, Catawba, French Broad, Hiwassee, Little Tennessee, New, Savannah, Watauga, and Yadkin Pee-Dee Rivers) were sampled, and the results of that surveillance program are presented herein.

MATERIALS AND METHODS

We followed the diagnostic protocol outlined in the US Fish and Wildlife Service and American Fisheries Society-Fish Health Section Blue Book (USFWS & AFS-FHS 2014), which prescribes the pepsin-trypsin digest (PTD) method of Markiw & Wolf (1974) and the species-specific nested PCR of Andree et al. (1998), to screen rainbow, brown, and brook trout for infection by *Myxobolus cerebralis*. Taxonomic authorities, dates, and scientific names for fishes follow Eschmeyer et al. (2017). Common names of fishes follow Page et al. (2013) and Froese & Pauly (2017).

Fish collection and necropsy

Rainbow trout (N = 1085), brown trout (696), and brook trout (319) were collected from 13 July 2015 through 19 July 2016 from 43 localities comprising 9 North Carolina river basins, 2 state fish hatcheries, and 3 private trout farms (Table 1, Fig. 1). Fish were collected using a backpack electroshocker, individually bagged, shipped overnight on ice to the SCFPDL, measured, weighed, and examined for the presence of parasites, lesions, and abnormalities. Fish were processed according to the diagnostic procedures outlined in Fish Health Section 1, Chapter 3.2.5, in the USFWS/AFS Blue Book (MacConnell & Bartholomew 2012). State hatchery samples were processed as per the animal health inspection guidelines in Fish Health Section 2, Chapter 5.2 of the Blue Book (USFWS & AFS-FHS 2014). The head of each fish was removed posterior to the insertion of the pectoral fin and bisected such that one half was frozen overnight before being subjected to PTD as per Markiw & Wolf (1974), and half was frozen at −20°C comprising an archived sample.

Pellet and myxospore examination

Each pellet resulting from a PTD, hereafter 'pellet,' contained no more than 5 fish head halves (no mixing of fish species). Each of the resulting 570 pellets (294 of rainbow trout, 188 of brown trout, and 88 of brook trout) was examined for 10 min using a Zeiss Axioskop with aid of a 40× objective, 15× wide-field ocular objectives, and differential interference contrast (DIC) optical components, and myxospore anatomy and dimensions were determined and illustrated with a 63× oil immersion objective or a 100× oil immersion objective and drawing tube affixed to that microscope. To mitigate excessive fluidity and capillary movement of wet-mounted myxospores, a drop of pellet mixture was placed in the center of a coverslip, inverted, and placed face down onto a glass slide with a thin (<1 mm) layer of 1.5% agar (Lom 1969). Photomicrographs were taken at 1600× total magnification with the aid of a Leica DMR compound microscope equipped with DIC, 1.6× magnifier, and a digital single lens reflex camera. Myxospore meas-

Table 1. Results of pepsin-trypsin digests (PTDs) conducted in the present study (presented chronologically). RBT: rainbow trout *Oncorhynchus mykiss*; BNT: brown trout *Salmo trutta*; BKT: brook trout *Salvelinus fontinalis*. Measurements reported as range (mean ± SD; sample size). For PTD results, N: number of resulting pellets after pooling ≤5 trout heads per PTD; M+: number of microscopy-positive pellets; PCR+: number of nested PCR positive pellets

Date	Locality	River basin	Coordinates	Host	Total length (mm)	PTD results		
						N	M+	PCR+
13 Jul 2015	Watauga River, Aldridge Rd.	Watauga	36°8′46″N, 81°47′29″W	RBT	134–209 (179 ± 23; 20)	5	3	5
28 Jul 2015	Armstrong State Fish Hatchery	Catawba	35°48′10″N, 82°5′48″W	RBT	73–364 (152 ± 70; 60)	15	0	0
29 Jul 2015	Bobby Setzer State Fish Hatchery	French Broad	35°17′4″N, 82°47′45″W	RBT	83–343 (166 ± 71; 19)	9	0	0
				BKT	106–200 (144 ± 30; 18)	6	0	0
04 Aug 2015	Watauga River, Grandfather Rd.	Watauga	36°7′57″N, 81°48′55″W	RBT	238 (1)	1	0	0
				BNT	61–182 (127 ± 40; 30)	6	0	3
04 Aug 2015	Watauga River, McLean Rd.	Watauga	36°8′29″N, 81°48′14″W	RBT	55–224 (124 ± 53; 32)	6	0	0
				BNT	66–192 (115 ± 43; 30)	6	0	0
04 Aug 2015	Watauga River, Calloway Rd.	Watauga	36°9′37″N, 81°45′48″W	RBT	131–297 (180 ± 37; 16)	4	1	2
				BNT	72–219 (153 ± 44; 28)	7	0	1
12 Aug 2015	Watauga River, Private trout farm 1	Watauga	36°8′41″N, 81°47′47″W	RBT	380–473 (410 ± 22; 31)	31	6	25
20 Aug 2015	Elk River	Watauga	36°10′17″N, 81°55′3″W	RBT	145–349 (190 ± 39; 31)	9	2	2
				BNT	77–332 (162 ± 55; 30)	9	3	5
21 Oct 2015	Private trout farm 2	Watauga	36°8′55″N, 81°59′15″W	RBT	111–307 (196 ± 53; 60)	23	0	0
26 Oct 2015	Private trout farm 3	French Broad	35°5′43″N, 82°46′34″W	RBT	128–198 (161 ± 15; 59)	15	0	0
01 Mar 2016	Armstrong Creek	Catawba	35°47′32″N, 82°7′45″W	RBT	84–170 (116 ± 28; 30)	6	0	0
02 Mar 2016	Jacobs Fork	Catawba	35°35′38″N, 81°38′22″W	RBT	63–126 (90 ± 19; 30)	6	0	0
03 Mar 2016	Fires Creek	Hiwassee	35°7′23″N, 83°47′24″W	RBT	88–194 (133 ± 31; 33)	7	0	0
08 Mar 2016	Linville River	Catawba	36°2′9″N, 81°53′32″W	BNT	114–266 (190 ± 30; 32)	8	0	0
09 Mar 2016	Big Snowbird Creek	Little Tennessee	35°15′18″N, 83°57′34″W	RBT	78–170 (103 ± 21; 21)	5	0	0
				BNT	100–214 (153 ± 50; 9)	2	0	0
				BKT	87–95 (91 ± 4; 4)	1	0	0
10 Mar 2016	Pounding Mill Branch	French Broad	35°20′31″N, 82°47′20″W	BKT	84–171 (124 ± 24; 24)	5	0	0
10 Mar 2016	Middle Prong	French Broad	35°20′31″N, 82°56′40″W	BKT	71–188 (116 ± 28; 31)	7	0	0
10 Mar 2016	Davidson River	French Broad	35°17′7″N, 82°49′49″W	RBT	75–182 (130 ± 27; 30)	6	0	0
				BNT	79–211 (155 ± 42; 30)	7	0	0
14 Mar 2016	North Fork Ivy Creek	French Broad	35°47′53″N, 82°22′13″W	RBT	74–165 (127 ± 31; 30)	6	0	0
				BKT	64–150 (102 ± 25; 30)	6	0	0
15 Mar 2016	Tucker Creek	French Broad	35°12′49″N, 82°52′11″W	BNT	76–197 (126 ± 30; 27)	6	0	0
15 Mar 2016	North Fork French Broad River	French Broad	35°14′50″N, 82°53′14″W	RBT	90–210 (137 ± 27; 30)	6	0	0
				BNT	108–164 (142 ± 30; 3)	1	0	0
16 Mar 2016	Chattooga River	Savannah	35°4′26″N, 83°6′25″W	BNT	109–259 (189 ± 49; 31)	7	0	0
21 Mar 2016	Caney Fork	Little Tennessee	35°19′30″N, 83°2′24″W	RBT	9–189 (121 ± 32; 24)	7	0	0
				BKT	75–172 (112 ± 25; 30)	6	0	0
23 Mar 2016	Little Rock Creek	French Broad	36°3′43″N, 82°6′58″W	RBT	123–225 (157 ± 25; 30)	6	0	0
				BNT	113–188 (140 ± 16; 30)	6	0	0
24 Mar 2016	Shawneehaw Creek	Watauga	36°9′39″N, 81°52′12″W	RBT	140–185 (166 ± 13; 30)	6	0	0
				BNT	98–204 (137 ± 31; 30)	6	0	0
30 Mar 2016	Anthony Creek	Catawba	36°4′15″N, 81°44′19″W	RBT	95–147 (120 ± 11; 30)	6	0	0
30 Mar 2016	Wilson Creek	Catawba	36°4′28″N, 81°47′44″W	BNT	96–190 (138 ± 23; 30)	6	0	0

(continued on next page)

Table 1. (continued)

Date	Locality	River basin	Coordinates	Host	Total length (mm)	PTD results N	M+	PCR+
31 Mar 2016	Curtis Creek	Catawba	35°40′51″N, 82°12′5″W	RBT	90–200 (126 ± 28; 30)	6	0	0
31 Mar 2016	Newberry Creek	Catawba	35°40′45″N, 82°12′49″W	BKT	83–145 (104 ± 13; 30)	6	0	0
04 Apr 2016	South Toe River	French Broad	35°45′0″N, 82°12′52″W	RBT	78–156 (111 ± 23; 30)	6	0	0
				BNT	85–192 (128 ± 36; 30)	6	0	1
06 Apr 2016	Cove Creek	Broad	35°15′50″N, 82°17′2″W	RBT	99–203 (136 ± 23; 30)	6	0	0
07 Apr 2016	Bradley Creek	French Broad	35°23′0″N, 82°41′12″W	RBT	75–156 (117 ± 24; 20)	4	0	0
				BNT	94–200 (157 ± 40; 15)	3	0	0
07 Apr 2016	South Fork Mills River	French Broad	35°21′59″N, 81°31′58″W	RBT	75–205 (130 ± 51; 11)	3	0	0
				BNT	37–205 (145 ± 48; 15)	4	0	0
11 Apr 2016	Crab Orchard Creek	Watauga	36°12′14″N, 81°48′18″W	RBT	126–195 (156 ± 32; 4)	1	0	0
				BNT	98–182 (137 ± 23; 25)	5	0	0
12 Apr 2016	Big Horse Creek	New	36°34′33″N, 81°34′20″W	RBT	112–205 (157 ± 27; 31)	7	0	0
12 Apr 2016	Buffalo Creek	New	36°25′33″N, 81°31′40″W	RBT	95–210 (142 ± 34; 32)	7	0	0
				BNT	135–196 (163 ± 31; 3)	1	0	0
14 Apr 2016	Laurel Creek	Yadkin Pee-Dee	36°10′19″N, 81°32′47″W	BNT	135–229 (168 ± 34; 29)	6	0	1
				BKT	98–180 (120 ± 19; 30)	6	0	1
19 Apr 2016	Boone Fork	Watauga	36°7′21″N, 81°46′24″W	RBT	84–183 (129 ± 25; 30)	6	0	0
				BNT	103–203 (137 ± 27; 26)	6	0	1
25 Apr 2016	Basin Creek, site 1	Yadkin Pee-Dee	35°43′54″N, 82°1′36″W	RBT	148–223 (188 ± 20; 14)	3	0	0
27 Apr 2016	Beech Creek	Watauga	36°14′22″N, 81°53′33″W	RBT	94–186 (139 ± 22; 31)	7	0	0
				BNT	114–203 (139 ± 22; 30)	6	0	0
27 Apr 2016	Meat Camp Creek	New	36°16′18″N, 81°39′27″W	RBT	132–204 (178 ± 16; 30)	6	0	0
				BNT	111–210 (166 ± 29; 30)	6	0	0
28 Apr 2016	Roaring Creek	French Broad	36°3′48″N, 82°0′54″W	RBT	80–227 (149 ± 45; 30)	6	0	1
				BNT	90–212 (144 ± 41; 30)	6	0	0
3 May 2016	Basin Creek, site 2	Yadkin Pee-Dee	36°23′5″N, 81°9′26″W	RBT	153–205 (184 ± 18; 15)	3	0	0
13 Jun 2016	Armstrong State Fish Hatchery	Catawba	35°48′10″N, 82°5′48″W	BKT	75–442 (168 ± 92; 64)	20	0	0
14 Jun 2016	Bobby Setzer State Fish Hatchery	French Broad	35°17′4″N, 82°47′45″W	BKT	66–417 (211 ± 100; 66)	25	0	0
18 Jul 2016	Bobby Setzer State Fish Hatchery	French Broad	35°17′3.62′N, 82°47′45″W	RBT	45–442 (182 ± 113; 60)	23	0	0
				BNT	69–507 (173 ± 123; 64)	26	0	0
19 Jul 2016	Armstrong State Fish Hatchery	Catawba	35°48′10″N, 82°5′48″W	RBT	53–381 (185 ± 104; 40)	15	0	0
				BNT	203–544 (312 ± 58; 58)	36	0	0

urements are herein reported in micrometers and presented as a range followed by the mean, standard deviation (SD), and number of specimens measured (N) in parentheses. Morphological terms and nomenclature for myxospores follow Shul'man (1966). Herein, we consider Myxozoa as a parasitic clade of Cnidaria (Siddall et al. 1995, Kent et al. 2001).

Comparative morphology of myxospores

We primarily used Hofer (1903) and Lom & Hoffman (1971) to morphologically diagnose *M. cerebralis*; we especially appreciated the latter work because of the level of descriptive detail included therein. Considering all nominal species of *Myxobolus* and the morphological criteria of Eiras et al. (2005) (i.e. myxospore length, width, and thickness; polar capsule length, width, and symmetry; number of polar filament coils; intercapsular process presence/absence), we compared all congeners that were morphologically indistinguishable from *M. cerebralis.* We also compared any species of *Myxobolus* that infects a salmonid, regardless of myxospore morphology. This yielded a total of 39 *Myxobolus* spp., comprising 23 that were indistinguishable from *M.*

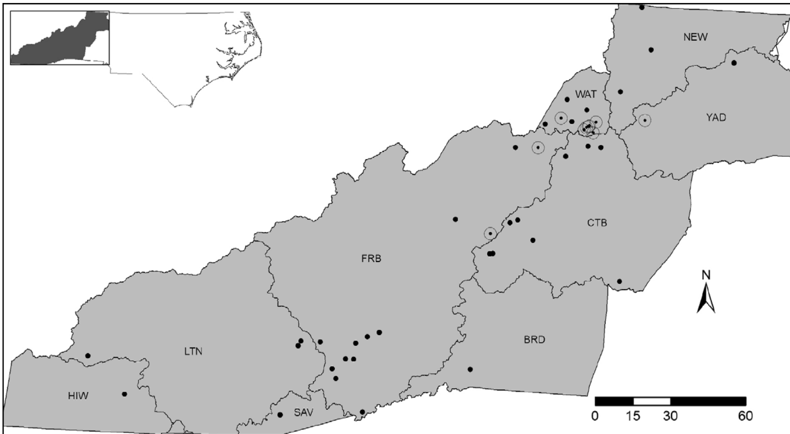


Fig. 1. North Carolina (USA) collection localities (N = 43) comprising 9 river basins screened for infections of *Myxobolus cerebralis* (Myxozoa: Bivalvulida) in rainbow trout *Oncorhynchus mykiss*, brown trout *Salmo trutta*, and brook trout *Salvelinus fontinalis*. Encircled dots = infection localities. HIW: Hiwassee; LTN: Little Tennessee, SAV: Savannah, FRB: French Broad; BRD: Broad; CTB: Catawba; YAD: Yadkin Pee-Dee; NEW: New; WAT: Watauga

cerebralis (Cone & Overstreet 1998, Lom & Cone 1996, Easy & Cone 2009, Yokoyama et al. 2010, Buchmann et al. 2012, Molnár et al. 2012, Iwanowicz et al. 2013, Borkhanuddin et al. 2014, Cech et al. 2015, Karlsbakk et al. 2017), including *M. squamalis*, which infects a salmonid (Polley et al. 2013); plus the 16 additional species that infect salmonids (Eiras et al. 2005, Ferguson et al. 2008) (see Table 3). We tested for the presence of a mucous envelope using India ink (Lom & Vávra 1963) in a subsample of myxospores taken from formalin-fixed and thawed pellets but failed to visualize the mucous membrane. Lom & Hoffman (1971) used scanning electron microscopy (SEM) in addition to India ink to show this feature and stated that refrigeration of PTD pellets with myxospores could cause the mucous membrane to diminish over months. Other than artifactitious shrinking of formalin-fixed myxospores (Kudo 1921), Lom & Hoffman (1971) did not report any effect of freezing on the mucous envelope.

DNA extraction, nested PCR, and sequencing of the 18S rDNA

All resulting pellets, regardless of microscopy results, were subjected to nested PCR. DNA from 200 µl of pellet material was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's directions. Total DNA concentrations were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies), diluted to 50 ng µl^{-1}, and stored at −20°C. Samples were amplified using outer primers Tr5-16 (5′-GCA TTG GTT TAC GCT GAT GTA GCG A-3′) and Tr3-16 (5′-GAA TCG CCG AAA CAA TCA TCG AGC TA-3′), and as inner primers Tr5-17 (5′-GCC CTA TTA ACT AGT TGG TAG TAT AGA AGC-3′) and Tr3-17 (5′-GGC ACA CTA CTC CAA CAC TGA ATT TG-3′) (Andree et al. 1998). PCR reagents were purchased from Promega. Morphologically confirmed positive samples from the Watauga River basin (Watauga River, Aldridge Rd.) were sent to Lucigen Corporation (Middleton, WI) for automated bidirectional sequencing with inner primers Tr3-17 and Tr5-17. Sequences were trimmed and contigs made using BioNumerics v. 7.0 (Applied Maths). Sequences of our isolates (KY522969, KY522970, KY522971) were compared with published 18S sequences of *Myxobolus* spp. that are morphologically indistinguishable from *M. cerebralis* (see Table 3) using BLAST (Altschul et al. 1990). Further, we compared our isolates to existing sequences of *M. cerebralis* present in GenBank (AF115253.1, AF115254.1, AF115255.1, EF370481.1; Andree et al. 1999, Arsan et al. 2007) and percent similarity values were determined using BioNumerics v. 7.0 (see Table 4).

Amplification, cloning, and sequencing of ITS-1

DNA was extracted from a microscopy-confirmed and nested PCR-positive pellet from the Watauga River basin (Watauga River, Aldridge Rd.) and amplified with forward primer Mc18S1F (5′-AAT ACG CTG GGA TCG ATG-3′) and reverse primer Mc5S1R (5′-ATG ACT CAC TAG GCT TGC-3′) (Whipps et al. 2004). PCR bands were gel extracted and purified using a QIAquick™ Gel Extraction Kit (Qiagen) according to the manufacturer's instructions (this step was necessary to isolate the targeted 600 base pair amplicon). Selected isolates were cloned following standard protocols (Sambrook et al. 1989). Bidirectional sequencing of 3 clones was performed using the universal primers T7 forward (5′-TAA TAC GAC TCA CTA TAG GG-3′) and T3 reverse (5′-AAT TAA CCC TCA CTA AAG GG-3′). Resulting sequences were trimmed and contigs made as above. Resulting ITS-1 sequences (MF374491, MF374492, MF374493)

were compared to those in GenBank, and percent similarity values were determined using BioNumerics v. 7.0 (see Table 5).

Infection data

The total number of pellets per collection event per salmonid species was recorded and parsed into the number of microscopy-positive pellets and the number of nested PCR-positive pellets (Table 1). Prevalence and intensity (sensu Bush et al. 1997) were not obtained because samples were pooled as per AFS Blue Book protocols, and samples were not purified to further concentrate myxospores (Landolt 1973, Contos & Rothenbacher 1974, Markiw & Wolf 1974). Further, we did not resuspend PTD pellets in any known volume for enumeration using a counting tool such as a hemacytometer (Prasher et al. 1971, Tidd et al. 1973, O'Grodnick 1975, Lorz et al. 1989, Hedrick et al. 1999a,b, Kelley et al. 2004).

Treatment and deposition of museum voucher material

Aliquots of myxospores from 2 microscopy- and nested PCR-positive pellets were fixed in 5% neutral buffered formalin, rinsed with deionized water, and slowly dehydrated with a graded ethanol series to a final solution of 70% EtOH. Those samples were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (Smithsonian Institution, US National Museum Collection Nos. 1456975, 1456990).

RESULTS

Infections of *Myxobolus cerebralis* were confirmed using morphology and molecular biology in rainbow, brown, and brook trout from the French Broad River, Watauga River, and Yadkin-Pee-Dee River basins. Below, we provide a morphological description of the observed myxospores and present associated molecular sequence data sourced from myxospores.

Microscopy results

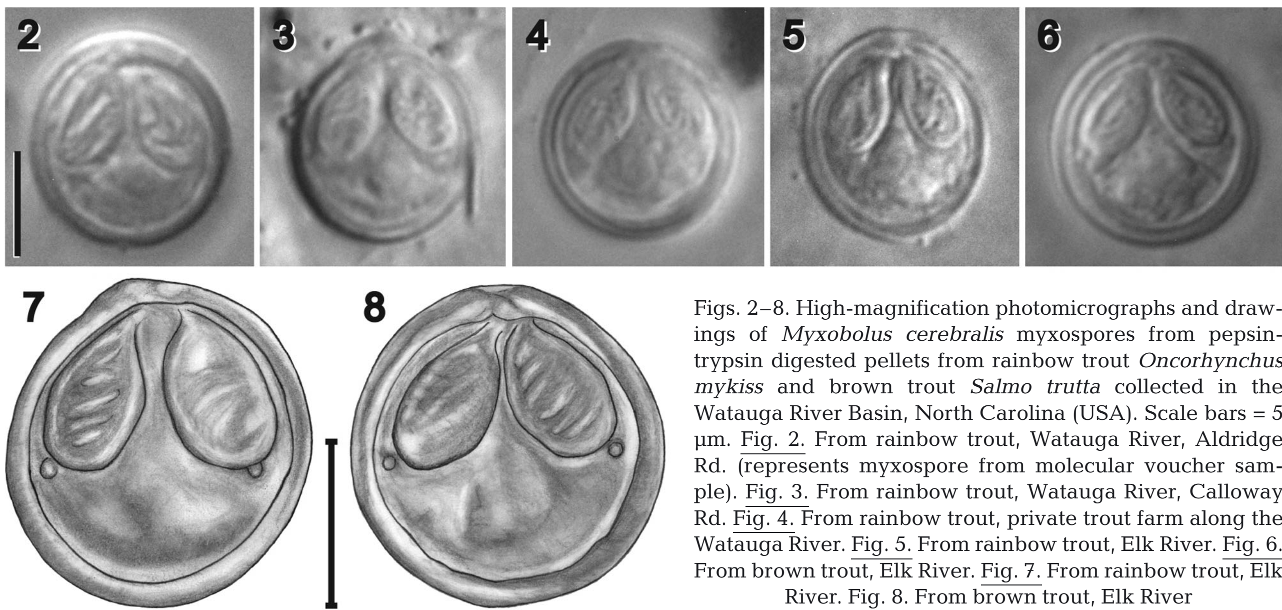
A total of 15 out of 570 PTD pellets (3%) were positive for *M. cerebralis* using light microscopy (Table 1): 12 of 294 (4%) from rainbow trout collected from the Watauga River Basin (Watauga River [Aldridge Rd., Calloway Rd., private trout farm]; Elk River) and 3 of 188 (2%) from brown trout in the Watauga River Basin (Elk River). No myxospore was detected microscopically in brook trout.

Taxonomic description of observed myxospores of *M. cerebralis* Hofer, 1903

The following description is based on 119 myxospores examined with light microscopy. Myxospores comprising 2 valves juxtaposed at seam of suture wall, 2 polar capsules, and sporoplasm (Shul'man 1966), ovoid (87 of 104 myxospores [84%]), subcircular (12 of 104 [12%]), or broader than long (5 of 104 [5%]) in frontal profile, with subtle or conspicuous bilateral asymmetry in frontal view (Figs. 2–8), lenticular in sutural view, 9.0–10.0 (9.6 ± 0.4; 119) long in frontal view, 8.0–10.0 (8.8 ± 0.6; 104) wide in frontal view, 6.0–7.5 (6.9 ± 0.5; 15) thick in sutural view (Table 2); suture wall of uneven thickness, <1 thick along lateral and posterior margins, typically smooth along inner posterior margin (11 of 104 [10.6%] with irregular inner margin; Figs. 7 & 8), ≥1 thick at anterior margin and forming crescent-shaped ledge (Figs. 5, 6, & 8) or slight protrusion (Figs. 3 & 7) in frontal view, lacking intercapsular process, without flanking lateral ridges in sutural view. Polar capsules ovoid, typically equal in length (2 of 104 [2%] were subequal), with non-overlapping anterior extensions, 4.0–6.0 (5.0 ± 0.5; 104) long, 2.5–3.5 (3.1 ± 0.3; 104) wide; polar filaments inconspicuous, loosely coiled with inconsistent intracapsular orientation, coiling 5 (45 of 48 [94%]; Figs. 4, 5, & 7) or 6 times (3 of 48 [6%]; Figs. 6 & 8); coils indistinct in most (71 of 119 [60%]; Figs. 2 & 3) specimens. Sporoplasm lacking vacuole or other organelle visible with light microscopy, with 2 nuclei near or touching posterior margins of polar capsules (Figs. 6–8).

Comparison with conspecific myxospores

Our myxospores resembled those of *M. cerebralis* described by Hofer (1903) and Lom & Hoffman (1971) (as *Myxosoma cerebralis*). We based our species-level identification on (1) myxospore shape (with same level of intraspecific variability reported by Lom & Hoffman 1971), (2) myxospore dimensions (our myxospores were 9.0–10.0 [9.6 ± 0.4; 119] long, 8.0–10.0 [8.8 ± 0.6; 104] wide, and 6.0–7.5 [6.9 ± 0.5; 15] thick compared to 7.4–9.7 [mean = 8.7] long, 7.0–



Figs. 2–8. High-magnification photomicrographs and drawings of *Myxobolus cerebralis* myxospores from pepsin-trypsin digested pellets from rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* collected in the Watauga River Basin, North Carolina (USA). Scale bars = 5 µm. Fig. 2. From rainbow trout, Watauga River, Aldridge Rd. (represents myxospore from molecular voucher sample). Fig. 3. From rainbow trout, Watauga River, Calloway Rd. Fig. 4. From rainbow trout, private trout farm along the Watauga River. Fig. 5. From rainbow trout, Elk River. Fig. 6. From brown trout, Elk River. Fig. 7. From rainbow trout, Elk River. Fig. 8. From brown trout, Elk River

Table 2. Myxospores (MX) of *Myxobolus cerebralis* from pepsin-trypsin digested rainbow trout *Oncorhynchus mykiss* (RBT) and brown trout *Salmo trutta* (BNT) from the Watauga River Basin, North Carolina. PC: polar capsule; PF: polar filament. Measurements are in µm; data are presented as range (mean ± SD; sample size)

Locality	Host	MX length	MX width	MX thickness	PC length	PC width	PF coils
Watauga River, Aldridge Rd.	RBT	9.0–10.0 (9.7 ± 0.4; 23)	8.0–10.0 (9.0 ± 0.5; 20)	6.5–7.5 (7.0 ± 0.5; 3)	4.0–6.0 (5.1 ± 0.6; 20)	2.5–3.5 (3.1 ± 0.3; 20)	5 (11)–6 (1)
Watauga River, Calloway Rd.	RBT	9.0–10.0 (9.6 ± 0.4; 5)	8.0–9.5 (8.9 ± 0.6; 4)	7.0 (N=1)	5.0–5.5 (5.1 ± 0.3; 4)	3.0–3.5 (3.1 ± 0.3; 4)	5 (3)
Watauga River, Private trout farm 1	RBT	9.0–10.0 (9.6 ± 0.4; 45)	8.0–10.0 (8.7 ± 0.6; 40)	6.0–7.5 (6.9 ± 0.7; 5)	4.0–6.0 (5.0 ± 0.6; 40)	2.5–3.5 (3.1 ± 0.3; 40)	5 (10)
Elk River	RBT	9.0–10.0 (9.7 ± 0.4; 23)	8.0–9.5 (8.8 ± 0.5; 20)	6.0–7.0 (6.7 ± 0.6; 3)	4.0–6.0 (5.0 ± 0.4; 20)	2.5–3.5 (3.2 ± 0.3; 20)	5 (9)–6 (1)
	BNT	9.0–10.0 (9.6 ± 0.4; 23)	8.0–10.0 (8.8 ± 0.6; 20)	6.5–7.0 (6.8 ± 0.3; 3)	4.0–6.0 (5.2 ± 0.3; 20)	2.5–3.5 (3.2 ± 0.2; 20)	5 (12)–6 (1)
Combined		9.0–10.0 (9.6 ± 0.4; 119)	8.0–10.0 (8.8 ± 0.6; 104)	6.0–7.5 (6.9 ± 0.5; 15)	4.0–6.0 (5.0 ± 0.5; 104)	2.5–3.5 (3.1 ± 0.3; 104)	5 (45)–6 (3)

10.0 [8.2] wide, and 6.2–7.4 [6.3] thick as documented by Lom & Hoffman 1971), and (3) polar capsule features (our specimens were 4.0–6.0 [5.0 ± 0.5; 104] long, 2.5–3.5 [3.1 ± 0.3; 104] wide, and contained 5–6 polar filament coils compared to 5.0–6.0 [5.1] long, 3.0–3.5 wide [3.2], with 5–6 polar filament coils as documented by Lom & Hoffman 1971) (Table 2).

Comparison with congeneric myxospores

The myxospores of several *Myxobolus* spp. infecting salmonids morphologically intergrade with those of *M. cerebralis* (Table 3): *M. farionis* Gonzalez-Lanza and Alvarez-Pellitero, 1984; *M. kisutchi* Yasutake and Wood, 1957; *M. krokhini* Konovalov and Shulman, 1966; *M. neurobius* Schuberg and Schröder, 1905; *M. salmonis* Donec, Vartanyan, and Mkrtchyan, 1973; *M. squamalis* (Iversen, 1954) Lom and Noble, 1984; *M. thymalli* Konovalov, 1966; and *M. vartanyanae* (Donec, Vartanyan, and Mkrtchyan, 1973) Landsberg and Lom, 1991 (Table 3). However, despite some overlapping measurements and similar morphological features, these species can be morphologically differentiated from *M. cerebralis*: *M. farionis* (ex. brain, nerve cord; brown trout; Spain) has 8–9 polar filament coils (Eiras et al. 2005); *M. kisutchi* (ex. brain, nerve cord; coho salmon *Onco-*

Table 3. *Myxobolus* spp. infecting salmonids and/or that are morphologically and/or molecularly similar to *M. cerebralis* (asterisks indicate that measurements overlap with those of *M. cerebralis*). MX: myxospore; PC: polar capsule; PCE: PCs symmetrical (equal in size; Y: yes; N: no); PFC: number of polar filament coils; IP intercapsular process (P: present; A: absent). Hosts are G: Gadidae; S: Salmonidae; C: Cyprinidae; E: Eleotridae; Ca: Carangidae; Pl: Pleuronectidae; Go: Gobiidae; Ce: Centrarchidae; Cat: Catastomidae; Ga: Galaxiidae; Ci: Cichlidae; Ma: Macrouridae. Sites of infection include scale (1); head cartilage (2); gill lamellae (3); gill filament (or gill) (4); muscle (5); brain (6); lateral line nerve (7); nerve cord (8); bile duct (9); liver (10); buccal cavity (11); heart (12); kidney (13); spleen (14); ureter (15); serosa (16); eye (17); urinary bladder (18); gall bladder (19); cartilage (20); skin (21). AL: Alabama; AMB: Amur Basin; AUS: Australia; CAN: Canada; CHI: China; GRE: Greenland; HUN: Hungary; ICE: Iceland; ID: Idaho; IL: Illinois; ISR: Israel; JAP: Japan; LE: Lake Erie; MAL: Malaysia; NOR: Norway; OR: Oregon; PNW: Pacific Northwest; RUS: Russia; SPA: Spain; SWI: Switzerland; UKN: Ukraine; WA: Washington; WV: West Virginia. Measurements are in µm; data are presented as mean; range

Species	GenBank 18S (% sim)	MX length	MX width	MX thickness	PC length	PC width	PCE	PFC	IP	Host	Site	Locality	Reference
*M. aeglefini**	KX886271.1 (78)	10.2; 9.0–11.3	9.8; 9.0–10.5	6.9; 5.8–7.7	4.8; 4.0–5.6	3.1; 2.6–3.6	Y	5–6	A	G	2	ICE	Karlsbakk et al. (2017)
M. arcticus	AB353129.1 (89)	13.2; 11.9–14.4	8.4; 7.8–9.4	6.6; 5.5–7.0	8.3; 7.0–9.5	3.1; 2.3–3.7	–	9–13	–	S	6	JAP	Urawa et al. (2009)
M. branchialis	JQ388887.1 (92)	9.7; 9.0–10.4	7.6; 7.0–8.6	6.6; 6.3–7.1	5.3; 4.9–6.0	3.0; 2.2–4.1	Y	6	A	C	3	HUN	Molnár et al. (2012)
*M. branchilateralis**	JQ388888.1 (92)	9.7; 9.4–10.4	8.2; 7.5–9.1	6.4; 5.2–7.2	5.6; 5.3–6.8	3.0; 2.5–3.3	Y	6	A	C	4	HUN	Molnár et al. (2012)
*M. cartilaginis**	–	10.2; 9.5–10.5	8.9; 8.4–9.5	6.4; 6.3–7.3	5.3; 5.2–5.6	3.3; 3.0–3.5	Y	5–7	A	Ce	20	WV, USA	Eiras et al. (2005)
*M. conei**	–	8.5; 6.7–10.0	9.0; 7.6–10.4	7.3; 7.0–7.8	4.3; 3.2–5.2	3.0; 2.5–3.4	Y	4–5	A	Ca	10	AUS	Eiras et al. (2005)
M. evdokimovae	–	10.0–11.0	8.7–9.2	–	5.0–5.2	3.1–3.3	Y	–	P	S	11	RUS	Eiras et al. (2005)
*M. farionis**	–	9.1; 8.5–10.0	6.6; 6.0–7.5	4.7; 4.5–5.0	4.8; 4.5–5.5	2.3; 2.0–2.8	Y	8–9	P	S	6, 8	SPA	Eiras et al. (2005)
M. fryeri	EU346372.1 (90)	12.9; 11.1–14.8	8.6; 7.2–10.1	7.2; 6.4–7.7	6.9; 5.9–8.1	2.8; 2.0–3.3	N	8–10	–	S	5	OR, USA	Ferguson et al. (2008)
*M. groenlandicus**	JF694785.1 (77)	10.3; 8.5–11.0	10.1; 9.1–11.2	6.2; 4.9–7.1	4.4; 4.0–5.1	2.5; 2.1–4.1	Y	5–6	A	Pl	5	GRE	Buchmann et al. (2012)
*M. heteromorpha**	–	10.9; 9.1–11.8	9.5; 8.8–10.3	5.7; 5.0–7.0	5.2; 4.4–5.9	3.6; 2.9–4.4	Y	5	A	C	12, 13	CHI	Eiras et al. (2005)
M. ibericus	–	10.0; 9.0–11.0	8.6; 8.0–9.5	6.5; 6.0–7.0	4.9; 4.0–6.0	2.6; 2.2–3.5	N	7–8	P	S	10, 13, 14, 15	SPA	Eiras et al. (2005)
M. insidiosus	EU346373.1 (90)	14.7; 13.3–15.9	9.4; 7.9–10.5	7.4; 6.8–8.3	7.7; 5.3–9.3	3.2; 1.9–3.9	–	–	–	S	5	OR, USA	Ferguson et al. (2008)
M. insidiosus clarki[a]	–	12.5; 11.5–13.5	8.4; 7.5–9.0	7.4; 7.3–8.4	8.3; 7.5–9.5	2.9; 2.5–3.5	–	–	A	S	5	OR, USA	Eiras et al. (2005)
*M. kisutchi**	AB469988.1 (89)	9.4; 8.6–10.1	8.0; 7.2–9.0	5.6; 5.2–6.2	5.2; 4.5–6.0	2.8; 2.3–3.1	–	6–8	–	S	8	WA, USA	Urawa et al. (2009)
*M. krokhini**	–	9.6–12.0	7.5–10.5	6.6–6.9	5.0–6.6	2.4–4.0	Y	–	P	S	16	RUS	Eiras et al. (2005)
*M. lairdi**	–	9.9; 9.0–11.0	9.9; 9.0–11.0	6.1; 5.0–7.0	5.0; 4.5–6.0	1.8; 1.5–2.5	Y	6–8	A	Ma	6, 17	NOR	Eiras et al. (2005)
*M. luciogobii**	–	9.0; 7.7–10.7	7.7; 6.9–9.2	6.4; 5.0–7.9	3.7; 3.0–4.6	2.6; 2.3–2.9	Y	6	A	Go	18	JAP	Eiras et al. (2005)
*M. manueli**	–	10.8; 10.0–11.0	9.1; 8.0–10.0	7.0; 6.5–7.0	5.3; 4.5–6.0	2.9; 2.5–3.0	Y	6–7	A	Ce	12	LE, USA	Cone & Overstreet (1998)
*M. morrisonae**	–	10.0; 9.6–10.5	9.5; 9.1–10.3	5.0	5.5; 5.3–5.8	3.7; 3.4–4.0	Y	6	A	Cat	4	IL, USA	Lom & Cone (1996)
*M. neurobius**	AB469986.1 (90)	9.2; 8.6–10.5	7.6; 7.0–8.2	6.1; 5.4–7.0	5.0; 3.9–5.5	2.4; 2.1–2.7	–	6–8	–	S	7	NOR	Urawa et al. (2009)
M. neurotropus	DQ846661.1 (89)	11.8; 11.2–13.0	10.8; 10.4–12.3	8.8; 8.4–9.1	5.9; 5.0–6.9*	3.7*	N	6–8	P	S	6, 8	ID, USA	Hogge et al. (2008a)
*M. olidus**	–	9.0–10.0	7.0–8.0	4.0–5.5	4.2–5.0	2.2–2.5	Y	6	A	Ga	8	AUS	Eiras et al. (2005)
*M. ophiocarae**	KF211436.1 (89)	10.3; 9.3–11.3	8.8 (7.9–9.5	4.5; 4.1–5.2	4.7; 3.9–5.5	2.9; 2.2–3.3	Y	5–6	A	E	3	MAL	Borkhanuddin et al. (2014)
*M. ridgwayi**	–	11.3; 10.0–12.1	10.4; 9.5–10.5	6.6–6.7	6.5; 5.8–7.0	3.2; 2.0–3.8	Y	4–5	A	C	5	CAN	Easy & Cone (2009)
*M. salmonis**	–	8.2–10.4	7.4–9.5	5.5–8.3	3.6–5.8	2.1–3.4	Y	5–6	P	S	1	RUS	Eiras et al. (2005)
*M. sarigi**	–	11.3; 9.9–13.1	8.4; 7.9–9.6	5.2; 4.8–5.9	4.5; 4.1–5.2	3.2; 2.9–4.0	Y	4–5	A	Ci	13, 14	ISR	Eiras et al. (2005)
*M. sichuanensis**	–	10.2; 9.6–10.4	7.5; 7.2–8.0	6.0; 5.6–6.1	4.0; 4.0–4.8	2.5; 2.4–3.2	Y	5	A	C	4	CHI	Eiras et al. (2005)
M. soldatovi	–	8.0–9.5	NA	NA	4.0–4.2	2.2–2.2	Y	–	–	S	21	AMB	Eiras et al. (2005)
M. sphaeralis	–	9.0	9.0	–	–	–	–	–	–	S	4	SWI	Eiras et al. (2005)
*M. spinibarbus**	–	9.0; 8.8–9.5	6.6; 6.4–7.2	6.2; 6.0–6.4	4.7; 4.6–5.0	2.3; 2.1–2.5	Y	5	A	C	4	CHI	Eiras et al. (2005)
*M. spirosulcatus**	AB530263.1 (87)	8.9; 7.5–10.0	7.8; 7.5–8.5	6.7; 6.0–7.5	4.1; 3.5–5.0	2.6; 2.0–3.0	Y	4–5	A	Ca	9	JAP	Eiras et al. (2005), Yokoyama et al. (2010)
*M. squamalis**	JX910362.1 (96)	7.3–10.2	6.1–9.6	5.2–7.2	3.3–5.1	2.1–3.9	Y	4–6	A	S	1	PNW, USA	Polley et al. (2013)
*M. stanlii**	DQ779995.2 (89)	7.5–11.0	(8.8 6.3–11.3	6.3; 6.2–8.6	4.6; 4.5–6.9	2.5; 2.1–4.3	Y	5–7	A	C	5	AL, USA	Iwanowics et al. (2013)
*M. szentendrensis**	KP025686.1 (90)	9.2; 8.8–9.6	7.9; 7.6–8.0	6.7; 6.4–7.1	5.3; 4.8–5.6	3.0; 2.8–3.2	Y	6	A	C	3	HUN	Cech et al. (2015)
*M. thymalli**	–	9.0–11.0	8.0–10.5	5.9–7.2	5.2–6.5	2.7–3.6	Y	–	P	S	19	RUS	Eiras et al. (2005)
*M. uvuliferus**	–	9.0; 7.0–12.0	11.5; 10.0–13.0	6.5; 6.0–7.0	4.5; 3.0–5.0	2.5; 2.0–3.0	Y	5–7	A	Ce	–	CAN	Eiras et al. (2005)
*M. vartanyanae**	–	9.5–12.3	8.5–10.0	7.3–8.0	4.5–6.0	2.5–3.3	N	4–5	P	S	5, 10, 13, 14	UKN	Eiras et al. (2005)
*M. xinanensis**	–	10.5; 9.6–11.8	9.2; 8.8–9.3	5.0–5.6	5.2; 4.8–5.6	3.2	Y	5–6	A	C	4, 13	CHI	Eiras et al. (2005)

[a]Ferguson et al. (2008) suggested that this sub-species was probably *M. fryeri*

rhynchus kisutch [Walbaum, 1792], and Chinook salmon *O. tshawytscha* [Walbaum, 1792]; Washington and Oregon) (Hoffman 1999, Hogge et al. 2004, Urawa et al. 2009) has a thick sutural wall (Hogge et al. 2008b) with pointed ends in sutural view (spindle-shaped) and polar capsules with 6−8 polar filament coils (Urawa et al. 2009); *M. krokhini* (ex. 'abdominal serosa'; Arctic char *Salvelinus alpinus* [Linnaeus, 1758]; Russia) and *M. salmonis* (ex. scales; chum salmon *O. keta* [Walbaum, 1792]; Russia) have a large intercapsular process (Eiras et al. 2005); *M. neurobius* (ex. brain, nerve cord; brown trout; Norway) (Hoffman 1999) has 6−8 polar filament coils (Urawa et al. 2009); *M. squamalis* (ex. scales; chum salmon, coho salmon, and rainbow trout; Oregon) has 2 prominent flanking sutural ridges (Iversen 1954, Polley et al. 2013); and *M. thymalli* (ex. gall bladder; Arctic grayling, *Thymallus arcticus* [Pallas, 1776]; Russia) and *M. vartanyanae* (ex. kidney, muscle, liver, spleen; brown trout and rainbow trout; Ukraine) have a small intercapsular process (Eiras et al. 2005).

The newly collected myxospores of *M. cerebralis* had features and measurements that, to various degrees, overlapped with or matched an additional 22 species of *Myxobolus*. In fact, these species are indistinguishable using the characters in Eiras et al. (2005) alone. Yet, a combination of myxospore shape, length, and width; polar filament coil number; posterior ridges; and sutural wall thickness and surface features differentiate these species. Presented below, these exhaustive comparisons strongly support the diagnosis of our myxospores as *M. cerebralis* (Table 3).

Regarding myxospore length and width, the following species reportedly have myxospores that exceed the maximum length and/or width for those of *M. cerebralis*: *M. groenlandicus* Buchmann, Skovgaard, and Kania, 2012 (ex. cartilage; Greenland halibut *Reinhardtius hippoglossoides* [Walbaum, 1792]; Greenland) (11.0 long; 10.1 wide) (Buchmann et al. 2012); *M. heteromorpha* Ma, 1993 (ex. heart, kidney; common carp *Cyprinus carpio* Linnaeus, 1758; China) (11.8 long) (Eiras et al. 2005); *M. luciogobii* (Ishizaki, 1957) Landsberg and Lom, 1991 (ex. urinary bladder; flat-headed goby *Luciogobius guttatus* Gill, 1859; Japan) (10.7 long) (Eiras et al. 2005); *M. ophiocarae* Borkhanuddin, Cech, Mazelan, Shaharom-Harrison, Molnár, and Székely, 2014 (ex. gill lamellae; mud gudgeon *Ophiocara porocephala* [Valenciennes, 1837]; Malaysia) (11.3 long) (Borkhanuddin et al. 2014); *M. ridgwayi* Easy and Cone, 2009 (ex. somatic muscle; creek chub *Semotilus atromaculatus* Mitchell, 1818; Canada) (12.1 long) (Easy & Cone 2009); *M. sarigi* (Landsberg, 1985) Landsberg and Lom, 1991 (ex. kidney, spleen; hybridized tilapia *Oreochromis aureus* × *O. niloticus*; Israel) (13.1 long) (Eiras et al. 2005); *M. stanlii* Iwanowicz, Iwanowicz, Howerth, Schill, Blazer, and Johnson, 2013 (ex. somatic muscle; largescale stoneroller *Campostoma oligolepis* Hubbs and Greene, 1935; Alabama) (11.0 long) (Iwanowicz et al. 2013); *M. uvuliferus* Cone and Anderson, 1977 (ex. pumpkinseed sunfish *Lepomis gibbosus* [Linnaeus, 1758]; Canada) (12.0 long; 13.0 wide) (Eiras et al. 2005); and *M. xinanensis* Ma and Chen, 1998 (ex. gill, kidney; a cyprinid, *Schizothorax meridionalis* Tsao, 1964; China) (11.8 long) (Eiras et al. 2005).

Regarding myxospore shape, *M. cerebralis* is ovoid to sub-circular, whereas *M. branchialis* (Markevitsch, 1932) Landsberg and Lom, 1991 (ex. gill lamellae; common barbel *Barbus barbus* [Linnaeus, 1758], and Iberian barbel *Luciobarbus bocagei* [Steindachner, 1864]; Hungary) is pyriform (Molnár et al. 2012); *M. branchilateralis* Molnár, Eszterbauer, Marton, Székely, and Eiras, 2012 (ex. gill filament; common barbel and Iberian barbel; Hungary) is elongate-ovoid (Molnár et al. 2012); and *M. szentendrensis* Cech, Borzák, Molnár, and Székely, 2015 (ex. gill lamellae; common nase *Chondrostoma nasus* [Linnaeus, 1758]; Hungary) is pyriform in frontal view (Cech et al. 2015).

Regarding polar filament coils, *M. cerebralis* has 5 or 6 and no more than 6, whereas *M. lairdi* Moser and Noble, 1977 (ex. brain, eye; roundnose grenadier *Coryphaenoides rupestris* Gunnerus, 1765; Norway) has 6−8 (Eiras et al. 2005); *M. manueli* Cone and Overstreet, 1998 (ex. heart; black crappie *Pomoxis nigromaculatus* [Lesueur, 1829]; Lake Erie) has 6−7 (Cone & Overstreet 1998); *M. morrisonae* Lom and Cone, 1996 (ex. gill filament; smallmouth buffalo *Ictiobus bubalus* [Rafinesque, 1818]; Illinois) has a minimum of 6 (Lom & Cone 1996); *M. olidus* (Langdon, 1990) Kalavati, Brickle, and Mackenzie, 2000 (ex. nerve cord; mountain galaxias *Galaxias olidus* Günther, 1866; Australia) has a minimum of 6 (Kalavati et al. 2000); *M. stanlii* has 5−7 (Iwanowicz et al. 2013); and *M. uvuliferus* has 5−7 (Eiras et al. 2005).

Regarding posterior ridges, *M. cerebralis* has none, but *M. aeglefini* Auerbach, 1906 (ex. cranial cartilage; Atlantic cod *Gadus morhua* Linnaeus, 1758; Iceland) has 4−7 in frontal view (Karlsbakk et al. 2017). Regarding sutural wall thickness and surface features (markings, knobs, furrows, ridges), *M. cerebralis* has a relatively thin sutural wall (1 µm thick) with furrows observable with SEM only (Lom & Hoffman 1971), whereas *M. branchialis* and *M. branchilateralis* have a thick sutural wall (Molnár et al. 2012); *M. cartilaginis* (Hoffman, Putz, and Dunbar,

1965) Landsberg and Lom, 1991 (ex. cartilage; bluegill *Lepomis macrochirus* Rafinesque, 1819; West Virginia) has 6–9 sutural ridges on the posterior margin (Lom & Hoffman 1971); *M. conei* Lom and Dyková, 1994 (ex. liver; white trevally *Pseudocaranx dentex* [Bloch and Schneider, 1801]; Australia) has 2 sublateral knobs (Cone & Overstreet 1998); *M. manueli* has 2 sublateral knobs (Cone & Overstreet 1998); *M. morrisonae* has several markings along the sutural wall (Lom & Cone 1996); *M. ridgwayi* has 5–6 posterior ridges (Easy & Cone 2009); *M. spirosulcatus* Maeno, Sorimachi, Ogawa, and Kearn, 1995 (ex. bile duct; cultured yellowtail *Seriola quinqueradiata* Temminck and Schlegel, 1845; Japan) has spiral-like furrows (Maeno et al. 1995); and *M. szentendrensis* has an anteriorly-thickened sutural wall (Cech et al. 2015).

Because their original descriptions were incomplete, *M. sichuanensis* (Ma and Zhao, 1992) Eiras, Molnár, and Lu, 2005 (ex. gill; *Schizothorax davidi* [Sauvage, 1880], China) and *M. spinibarbus* (Ma, 1998) Eiras, Molnár, and Lu, 2005 (ex. gill; *Spinibarbus denticulatus* [Oshima, 1926], China) cannot not be differentiated with morphology from published descriptions of *M. cerebralis* nor our newly collected specimens.

Molecular biology results

All microscopy positive pellets were confirmed positive by nested PCR (Andree et al. 1998), and no microscopy-positive pellet was PCR-negative. A total of 48 of 570 (8%) pellets were PCR-positive: 35 of 294 (12%) pellets from rainbow trout in the Watauga River Basin (Watauga River [Aldridge Rd., Calloway Rd., private trout farm]; Elk River) plus the French Broad River Basin (Roaring Creek); 12 of 188 (6%) pellets comprising brown trout from the Watauga River Basin (Watauga River [Grandfather Rd., Calloway Rd.]; Elk River; Boone Fork), Yadkin Pee-Dee River Basin (Laurel Creek), and the French Broad River Basin (South Toe River); and 1 of 88 (1%) pellets comprising brook trout from Yadkin Pee-Dee River Basin (Laurel Creek) (Table 1). Salmonids collected from 34 localities within the Catawba River, Little Tennessee River, New River, Savannah River, Hiwassee River, and Broad River basins were negative for infection by *M. cerebralis* by microscopy and nested PCR. No infection was detected in trout from the Armstrong and Bobby Setzer state fish hatcheries (Table 1).

Three nested PCR positive and microscopy positive pellets from the Watauga River (Aldridge Rd.) were used to obtain molecular sequence data for the 18S rDNA. These sequences (KY522969, KY522970, KY522971) were identical to each other as well as to isolates from California (AF115253.1) and Alaska (EF370481.1). Collectively, these isolates were 99.5% similar to isolates from West Virginia (AF115254.1) and Munich, Germany (AF115255.1) (Table 4). No published sequence used in the 18S BLAST comparison herein yielded a percent identity >96% when compared to the isolates from North Carolina. A comparison of our isolates to published 18S GenBank sequences representing congeners revealed a range of similarities: *M. groenlandicus* (77%; Buchmann et al. 2012), *M. kisutchi* (89%; Urawa et al. 2009), *M. neurobius* (90%; Urawa et al. 2009), *M. ophiocarae* (90%; Borkhanuddin et al. 2014), *M. squamalis* (96%; Polley et al. 2013), and *M. stanlii* (89%; Iwanowicz et al. 2013) (Table 3). The ITS-1 sequences from 3 clones (MF374491, MF374492, MF374493) were identical to each other and 99.3–99.8% similar to published sequences (DNA sourced from triactinomyxons; TAMs) of *M. cerebralis* (Table 5).

DISCUSSION

Determining species boundaries within *Myxobolus* is not simple. Like many groups of metazoan parasites, no 'yardstick' or universal criteria exist for strictly defining a species of *Myxobolus* with 18S rDNA or ITS-1 sequence data (Lom & Dyková 2006). Yet, when coupled with morphology, the 18S rDNA can facilitate species identification (Hogge et al. 2004, Urawa et al. 2009, Polley et al. 2013), and no published work refutes the notion that identical 18S sequences indicate conspecificity. Our results confirm that the sequences of Andree et al. (1999) and Arsan et al. (2007) are conspecific with those linked to our morphologically confirmed North Carolina isolates of *M. cerebralis*. Likewise, our ITS-1 se-

Table 4. Percent similarities of 18S rDNA sequences for *Myxobolus cerebralis* from rainbow trout *Oncorhynchus mykiss*

GenBank sequence (origin)		(1)	(2)	(3)	(4)	(5)
AF115255.1 (Munich, Germany)	(1)	100	100	99.5	99.5	99.5
AF115254.1 (West Virginia, USA)	(2)	–	100	99.5	99.5	99.5
KY522969 (North Carolina, USA; present study)	(3)	–	–	100	100	100
EF370481.1 (Alaska, USA)	(4)	–	–	–	100	100
AF115253.1 (California, USA)	(5)	–	–	–	–	100

Table 5. ITS-1 sequence comparisons for *Myxobolus cerebralis* from rainbow trout *Oncorhynchus mykiss* in the USA. NC: North Carolina; CA: California; MT: Montana

GenBank sequence (origin)		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
AY479922.1 (CA)	(1)	100	99.8	99.5	99.3	99.3	99.3	99.5	99.0	99.3	98.8	98.5
AY479924.1 (CA)	(2)	–	100	99.3	99.5	99.5	99.5	99.3	99.3	99.3	99.0	98.8
JN134180.1 (MT)	(3)	–	–	100	99.3	99.3	99.3	99.5	99.0	99.0	98.8	99.0
MF374491 (NC; present study)	(4)	–	–	–	100	100	100	99.8	99.8	99.3	99.5	99.3
MF374492 (NC; present study)	(5)	–	–	–	–	100	100	99.8	99.8	99.3	99.5	99.3
MF374493 (NC; present study)	(6)	–	–	–	–	–	100	99.8	99.8	99.3	99.5	99.3
AY479923.1 (CA)	(7)	–	–	–	–	–	–	100	99.5	99.3	99.3	99.0
JN134181.1 (MT)	(8)	–	–	–	–	–	–	–	100	99.0	99.3	99.0
AY479925.1 (CA)	(9)	–	–	–	–	–	–	–	–	100	98.8	98.5
JN134170.1 (MT)	(10)	–	–	–	–	–	–	–	–	–	100	99.8
JN134182.1 (MT)	(11)	–	–	–	–	–	–	–	–	–	–	100

quences (Table 5) confirm the taxonomic identity of the myxospores we collected in North Carolina salmonids as *M. cerebralis*.

Previous authors have reported intraspecific variation in the ITS-1 of *M. cerebralis*. In contrast, our cloned ITS-1 sequences were identical, despite having come from a pellet (with numerous myxospores) rather than a single myxospore or TAM. Whipps et al. (2004) reported intraspecific variation (1.7%) in cloned ITS-1 sequences sourced from a single TAM, and Lodh et al. (2012) reported intraspecific variation (16 haplotypes in 20 sequences) in clones sourced from TAMs in Montana. Future comparisons with larger sample sizes from geographically distinct isolates in North Carolina will help explore the level of intraspecific and geographic variation among infections of *M. cerebralis* in North Carolina salmonids.

The GenBank sequences generated herein for *M. cerebralis* are unique in that they are accompanied by a published morphological description of the myxospore. As the present work highlights, several *Myxobolus* spp. morphologically intergrade (see above; Table 3). Because of this, complementary morphological and molecular sequence data, which document intraspecific variation, are needed for identifying infections by *M. cerebralis* and in diagnosing/describing myxozoans (Yokoyama et al. 2004, Whipps & Diggles 2006, Carriero et al. 2013). Bahri et al. (2003) and Ferguson et al. (2008) as well as Whipps & Diggles (2006), Camus & Griffin (2010), Yokoyama et al. (2010), Molnár et al. (2011), Urawa et al. (2011), Zhao et al. (2013), Székely et al. (2015), and Rosser et al. (2017) comprise good examples of the use of complementary morphological and molecular data in myxozoan taxonomy regarding variation between and among species, respectively.

Amplification and cloning of the ITS-1 from a PTD sample has not been reported previously; Whipps et al. (2004) obtained clones from EtOH-preserved TAMs. Also noteworthy is that the present study confirmed that the PCR primers of Whipps et al. (2004) did not amplify trout DNA from a PTD pellet, which is relevant to diagnosticians performing hatchery checks if ITS-1 emerges as an additional confirmatory molecular diagnostic tool for infections by *M. cerebralis*.

Our report extends the known geographic range of *M. cerebralis* into the southeastern USA south of Virginia. The detection of myxospores in trout coupled with unpublished data (S. A. Bullard and C. R. Arias) of infections of TAMs of *M. cerebralis* in oligochaetes in North Carolina rivers indicates that the life cycle of *M. cerebralis* is being completed there, beyond the confines of fish hatcheries and within wild trout populations. We find it highly unlikely that stocked, infected trout are the only source of infections. Yet, clearly, much remains to be studied regarding the biology of this parasite and its ability to induce whirling disease in the southeastern USA. When, how, and from where it was introduced is unknown; population-level effects on trout there are indeterminate; and fine details of the life cycle remain unstudied in this region of North America. Seasonal monitoring of salmonid populations (Granath & Vincent 2010), field studies (Thompson et al. 1999, Baldwin et al. 2000), and laboratory challenges (Hoffman & Putz 1969, Markiw 1991, Hedrick et al. 1999b, 2001) using trout and oligochaetes from the region may reveal additional information about the plasticity of the life cycle (Zendt & Bergersen 2000, Gilbert & Granath 2001) of *M. cerebralis*, perhaps resulting from local adaptations, or additional details about the presence/incidence of whirling disease (which we did not

confirm in this study). In addition, subsequent molecular analyses using faster-evolving molecular markers (Whipps et al. 2004) could reveal details about how the parasite was introduced into, and became established in, this region. Moreover, baseline infection data and routine pathogen and disease monitoring are needed for scientific epidemiological investigations that lead to natural resource modeling and management decisions (Bartholomew et al. 2005). This approach is urgently needed in the southeastern USA, where no regional aquatic animal health plan exists currently.

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