Molecular characterization of the myxosporean associated with parasitic encephalitis of farmed Atlantic salmon *Salmo salar* in Ireland

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ABSTRACT: During seasonal epizootics of neurologic disease and mass mortality in the summers of 1992, 1993 and 1994 on a sea-farm in Ireland, Atlantic salmon *Salmo salar* smolts suffered from encephalitis associated with infection by a neurotropic parasite. Based on ultrastructural studies, this neurotropic parasite was identified as an intercellular presporogonic multicellular developmental stage of a histozoic myxosporean, possibly a *Myxobolus* species. In order to generate sequence data for phylogenetic comparisons to substantiate the present morphological identification of this myxosporean in the absence of detectable sporogony, polymerase chain reaction (PCR), Southern blot hybridization, dideoxynucleotide chain-termination DNA sequencing, and *in situ* hybridization (ISH) were used in concert to characterize segments of the small subunit ribosomal RNA (SSU rRNA) gene. Oligonucleotide primers were created from sequences of the SSU rRNA gene of *M. cerebralis* and were employed in PCR experiments using DNA extracted from formalin-fixed paraffin-embedded tissue sections of brains from Atlantic salmon smolts in which the myxosporean had been detected by light microscopy. Five segments of the SSU rRNA gene of the myxosporean, ranging in length from 187 to 287 base pairs, were amplified, detected by hybridization with sequence-specific probes, and sequenced. Consensus sequences from these segments were aligned to create a partial sequence of the SSU rRNA gene of the myxosporean. Assessments of sequence identity were made between this partial sequence and sequences of SSU rRNA genes from 7 myxosporeans, including *Ceratomyxa shasta*, *Henneguya doori*, *M. arcticus*, *M. cerebralis*, *M. insidiosus*, *M. neurobius*, and *M. squamalis*. The partial SSU rRNA gene sequence from the myxosporean had more sequence identity with SSU rRNA gene sequences from neurotropic and myotrophic species of *Myxobolus* than to those from epitheliotropic species of *Myxobolus* or *Henneguya*, or the enterotrophic species of *Ceratomyxa*, and was identical to regions of the SSU rRNA gene of *M. cerebralis*. Digoxigenin-labeled oligonucleotide DNA probes complementary to multiple segments of the SSU rRNA gene of *M. cerebralis* hybridized with DNA of the parasite in histologic sections of brain in ISH experiments, demonstrating definitively that the segments of genome amplified were from the organisms identified by histology and ultrastructural analysis. Based on sequence data derived entirely from genetic material of extrasporogonic stages, the SSU rDNA sequence identity discovered in this study supports the hypothesis that the myxosporean associated with encephalitis of farmed Atlantic salmon smolts is a neurotropic species of the genus *Myxobolus*, with sequences identical to those of *M. cerebralis*.

KEY WORDS: Atlantic salmon · Myxosporean · *Myxobolus cerebralis* · Small subunit ribosomal DNA · PCR · *In situ* hybridization

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INTRODUCTION

In the summers of 1992, 1993 and 1994, epizootics of parasitic encephalitis and mass mortality occurred in netpen-reared Atlantic salmon *Salmo salar* smolts on a sea-farm in Ireland, with mortalities that exceeded 90% of total smolt stocks in 1993 alone (Rodger et al. 1995, Scullion et al. 1996). Prior to death, affected smolts displayed neurologic clinical signs which included gyrating and circling swimming patterns, inappropriate postures in the water column, and periods of apparent unconsciousness (Rodger et al. 1995). Epizootologic studies conducted in the winter of 1995 revealed that parasitic infection was detectable with and without host inflammatory cell infiltrates and that netpen populations of infected smolts neither developed neurologic clinical signs nor underwent significant increases in cumulative mortalities (Frasca et al. 1998).

The ultrastructural morphology of the parasite associated with encephalitis of Atlantic salmon smolts was consistent with that of an intercellular presporogonic multicellular developmental stage of a histozoic myxosporean, having morphologic features shared by proliferative stages of members of Family Myxobolidae (Frasca et al. 1998). No spores of this myxosporean were detected in histologic sections of brain or coelomic viscera (Rodger et al. 1995, Frasca et al. 1998). Because taxonomic classification of myxosporeans is currently based on spore morphology (Lom & Dyková 1992), the absence of detectable spores in tissues of infected smolts precluded definitive taxonomic classification of this myxosporean in much the same way as the absence of detectable spores from sites of inflammation hampered taxonomic classifications of the myxosporeans responsible for proliferative kidney disease (Kent & Hedrick 1985) and proliferative gill disease (Hedrick et al. 1990).

Comparative analyses of eukaryotic small subunit ribosomal RNA (SSU rRNA) gene sequences have been employed to evaluate phylogenetic distances between and within taxonomic groups of organisms (Sogin et al. 1986, Gunderson et al. 1987, Sogin & Gunderson 1987); this is possible because the SSU rRNA gene is universally distributed, functionally equivalent, and occurs in sizes large enough that interspersed patterns of conserved and nonconserved regions allow for statistically valid interpretations of genetic relatedness (Sogin & Gunderson 1987). SSU rRNA gene sequences have been used to develop sensitive and specific molecular diagnostic tests and to elucidate taxonomic relationships between members of both the Microspora and the Myxozoa (Kent et al. 1996). Such molecular techniques can overcome the shortcomings of other diagnostic techniques, such as

light and electron microscopy, when pathogens are present in small numbers and lack easily detectable morphologic criteria for their speciation (Fedorko & Hijazi 1996).

The application of molecular techniques to the study of myxosporeans has provided further evidence elucidating their phylogenetic relationships and life cycles. SSU rDNA sequence analyses have supported the morphologically derived hypothesis that myxozoans are more closely related to metazoans than to protozoans (Smothers et al. 1994, Siddall et al. 1995, Schlegel et al. 1996), and SSU rDNA sequences have been used to unite alternate actinosporean and myxosporean stages of *Myxobolus cerebralis* (Andree et al. 1997) in support of the actinosporean-myxosporean alternation theory of Wolf & Markiw (1984). Of the 1200 species of myxosporeans, SSU rDNA sequences have been determined for a small number of species from several genera, including *Ceratomyxa* (Bartholomew et al. 1997), *Henneguya* (Kent et al. 1996), *Kudoa* (Kent et al. 1996), *Myxobolus* (Smothers et al. 1994, Andree et al. 1999), and *Myxidium* (Schlegel et al. 1996), allowing for sequence comparisons to determine the genetic relatedness of isolates.

In this study, we characterized segments of the SSU rRNA gene from proliferative stages of a myxosporean associated with encephalitis of farmed Atlantic salmon. The resultant SSU rDNA sequence data was used to elucidate the phylogenetic relationship of this myxosporean to others and confirmed that it was most likely a neurotropic species of the genus *Myxobolus*.

METHODS

Sample acquisition. The brains of 12 clinically normal Atlantic salmon *Salmo salar* smolts from a freshwater hatchery with no history of parasitic encephalitis or recent disease were collected at necropsy in 1997 and fixed in 1.5% paraformaldehyde, 1.5% glutaraldehyde, and 3 mM MgCl₂ in 0.1 M sodium cacodylate buffer (pH 7.3) for 3 d prior to paraffin embedding. These brains served as the source of genomic Atlantic salmon DNA (i.e. negative control DNA).

Brains of 20 Atlantic salmon smolts that died or suffered from neurologic disease during an epizootic of mass mortality at Site A (Frasca et al. 1998) in 1994 served as the source of parasite DNA (i.e. test DNA). These brains were collected at necropsy in 1994 and stored in 10% neutral buffered formalin at 4°C for 3 yr.

Three rainbow trout *Oncorhynchus mykiss* fingerlings, experimentally infected with the triactinomyxon stage of *Myxobolus cerebralis*, were obtained from the Fish Health Laboratory, University of California, Davis, California, USA, fixed whole in 10% neutral buffered

formalin for 3 d, and embedded in paraffin. These samples served as the source of myxobolus DNA (i.e. positive control DNA). *M. cerebralis* was selected because it is a neurotropic myxobolus, it shares ultrastructural features with the Atlantic salmon parasite and members of Family Myxobolidae (El-Matbouli et al. 1995, Frasca et al. 1998), and its SSU rRNA gene has been sequenced (Andree et al. 1997).

Sample evaluation and target selection. To ascertain that myxosporeans were present in tissue sections for DNA extraction, Atlantic salmon brains and rainbow trout fingerlings were sliced sagittally along the median longitudinal axis, routinely processed for paraffin embedding, sectioned at 3 µm, stained with hematoxylin and eosin (H&E), and examined by light microscopy. Whenever myxosporean parasites were detected, 2 sequential 5 µm sections were cut for DNA extraction. Histologic sections of control Atlantic salmon brains were examined to ensure that no myxosporeans were detectable by light microscopy, and 2 successive 5 µm sections were submitted for extraction.

DNA extraction and quantification. DNA was extracted using the QIAamp DNA extraction system (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer's protocol for paraffin-embedded tissue, except that samples were lysed in tissue lysis buffer with 400 µg proteinase K at 55°C for 3 to 5 d. After a 3-step elution, DNA was quantitated by UV spectrophotometry. Eluates from samples that did not register absorbance readings for DNA were combined, subjected to an additional round of membrane absorption and washes, eluted with preheated elution buffer to a final volume of 100 µl, and quantitated again.

Synthesis of primers. Oligonucleotide primers for amplification of myxosporean DNA in polymerase chain reaction (PCR) experiments were designed from sequences of the SSU rRNA gene of *Myxobolus cerebralis* (Andree et al. 1997), except for SM5-3, which was designed from a conserved region of the SSU rRNA genes of *M. cerebralis*, *M. arcticus*, and *M. neurobius* (Andree et al. 1999). Primers were tested for self-complementarity or homodimer and heterodimer formation using the computer software programs Amplify (University of Wisconsin Genetics, Madison, WI, USA) and Oligo 4.05 (National Biosciences, Inc., Plymouth, MN, USA). Primers for PCR and DNA sequencing reactions were prepared either by Gibco BRL (Gaithersburg, MD, USA) or the University of Connecticut Biotechnology

Center (Storrs, CT, USA). The sequences of the primers and their positions relative to the *M. cerebralis* SSU rRNA gene are listed in Table 1.

Polymerase chain reaction amplification of DNA. In order to determine whether extractions from paraffin-embedded tissues had yielded amplifiable DNA, samples were subjected to PCR amplification using oligonucleotide primers designed from a segment of the repetitive Bg/I element of the Atlantic salmon genome (Goodier & Davidson 1993). The sequence of the forward primer (5'-CCCTCTTGCTGTATCTCTCATTG-3') corresponded to positions 11 to 36 of the Bg/I element (GenBank L01505), and the sequence of the reverse primer (5'-CCACTGTGGTCCCAAGTCAGT-3') corresponded to positions 707 to 727 (Moran et al. 1997). DNA was amplified in 50 µl reactions containing 100 to 500 ng of sample DNA, 5 µl 10× Buffer I (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin), 200 µM dNTPs, 40 ng µl⁻¹ forward and reverse primers, and 2 U AmpliTaq Gold polymerase (Perkin-Elmer, Foster City, CA, USA). PCR was performed in a Perkin-Elmer Model #2400 thermal cycler. Reactions were initiated by a 9 min incubation at 94°C, followed by 40 amplification cycles, each consisting of a 1 min denaturation step at 94°C, a 1 min primer annealing step at 55°C, and a 1 min extension step at 72°C. A prolonged extension step of 10 min at 72°C completed each reaction. PCR products were separated by electrophoresis in 8% polyacrylamide gels, visualized using ethidium bromide staining and UV trans-illumination, and recorded using a Stratagene Eagle Eye II-Still Video System (Stratagene Inc., LaJolla, CA, USA).

Multiple segments of the SSU rRNA gene from the myxosporean parasite were amplified using 5 different primer pairs (Table 1). DNA was amplified in separate

Table 1. Oligonucleotide primers used to amplify myxosporean DNA in polymerase chain reactions. Location refers to the position of the primer relative to the nucleotide sequence of the *Myxobolus cerebralis* small subunit ribosomal RNA (SSU rRNA) gene

Primer	5'→3' sequence	Location	Reverse primer	Product size
SM5-3	CGCAGTGTAAGTTGGTAAGCTGA	639–661	SM3-5	287 bp
SM3-5	CGTTATTGGTGGTTGGTGCTG	948–968		
SM5-4	AGGACTAACGAATGCGAAGG	1059–1078	SM3-4	187 bp
SM3-4	CGTCAATTCCTTTAAGTTTCAGC	1265–1287		
SM5-6	GAGAAACGGCTACCACATCCAT	394–416	SM3-6	247 bp
SM3-6	GAGCAGTTAGTAGCACCATTCTG	663–684		
SM5-7	GTTTTTCGGTTACGGGGAGAG	1232–1252	SM3-7	244 bp
SM3-7	ATCATCGAGCTACACTATCTTCTTC	1496–1520		
SM5-8	TATTCTCCATTTGATGAGCGGAAG	1476–1499	SM3-8	258 bp
SM3-8	GGACATCTTAGGGCATCACAGACC	1757–1780		

50 µl reaction mixtures, as described above. Reactions were initiated by a 9 min incubation at 94°C, followed by 40 amplification cycles, each consisting of a 30 s denaturation step at 94°C, a 45 s primer annealing step at 50°C, and a 30 s extension step at 72°C. Reactions were completed with a prolonged extension step of 10 min at 72°C. Amplification products were separated by electrophoresis in either 2% agarose or 8% polyacrylamide gels.

Southern hybridization. Digoxigenin-labeled DNA probes used in Southern hybridizations were created by random oligonucleotide primed synthesis according to the manufacturer's directions for the High Prime labeling kit (Boehringer Mannheim Corp., Indianapolis, IN, USA) and using Klenow fragment polymerase, a mixture of deoxyribonucleoside-triphosphates containing digoxigenin-labeled deoxyuridine-triphosphate (DIG DNA Labeling Mix, 10× conc., Boehringer Mannheim Corp.), and PCR product amplified from positive control DNA.

Amplification products were separated by electrophoresis in 2% agarose gels, depurinated in 0.25 M HCl for 5 min, rinsed in distilled H₂O, and denatured in 0.1 M NaOH for 15 to 20 min. DNA was transferred to nylon membranes (Boehringer Mannheim Corp.) by capillary action using 10× sodium chloride-sodium citrate (SSC) buffer in standard Southern transfer pyramids with transfer times ranging from 3 h to overnight and was fixed by incubation at 80 to 90°C for 30 min. Blots were incubated with 20 ml of prehybridization solution (equal parts of DIG Easy Hyb solution [Boehringer Mannheim Corp.] and 5× SSC, 1% glycine, 1% SDS [sodium dodecyl sulfate], 45% deionized formamide, and 10% dextran sulfate) per 100 cm² of membrane for 1 to 2 h, followed by overnight incubation with 13 to 15 ml of hybridization solution (5× SSC, 1% glycine, 1% SDS, 45% deionized formamide, 10% dextran sulfate, and probe at 8 to 10 ng ml⁻¹). After hybridization, blots were washed, blocked with a solution of 1% (w/v) blocking reagent in 0.1 M maleic acid buffer for 30 min, and incubated with 1:10 000 dilution of sheep polyclonal anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim Corp.) for 30 min at room temperature. Blots were dipped in CSPD (Boehringer Mannheim Corp.), and incubated in the dark for 10 min at room temperature, followed by 5 min at 37°C. Radiographic films were exposed to processed blots for 1 to 10 min and developed routinely.

Sequencing of PCR products and sequence alignments. For each of the 5 different primer pairs, 5 µl samples from at least 5 separate PCR mixtures that yielded detectable product were subjected to a second round of PCR to generate amplicons in sufficient concentration to serve as templates in sequencing reac-

tions. Second-round PCR products were purified (QIAquick PCR Purification Kit, Qiagen Inc.), visualized in 8% polyacrylamide gels, compared to molecular weight markers to estimate their concentrations, and submitted for sequencing.

Nucleotide sequences were determined by oligonucleotide-directed dideoxynucleotide chain-termination cycling sequencing reactions performed by the HHMI Biopolymer/W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University, New Haven, Connecticut, USA, using an automated Applied Biosystems 377 DNA sequencer (Perkin-Elmer), 10 to 20 ng of template DNA per 200 bases of product, 27 ng µl⁻¹ forward or reverse PCR primers, and AmpliTaq FS DNA polymerase. MacDNAsis version 3.5 (Hitachi Software Engineering America, Ltd, San Bruno, CA, USA) was used to generate consensus sequences of the products from each primer pair through the alignment of a minimum of 5 single-stranded and 10 double-stranded sequences. Discrepancies at base positions were resolved by simple majority rule. The resultant consensus sequences were aligned to create a partial SSU rDNA sequence of the parasite, which was compared to the SSU rRNA gene sequences of 3 neurotropic myxobolids, e.g. *Myxobolus arcticus*, *M. cerebralis* and *M. neurobii*.

Analysis of percent sequence identity. Assessments of percent sequence identity were obtained by comparison of the consensus SSU rDNA sequence of the Atlantic salmon parasite with the SSU rDNA sequences of 7 myxosporeans, including several neurotropic myxobolids of salmon (*Myxobolus arcticus* [Andree et al. 1999], *M. cerebralis* [Genbank accession no. U96493], and *M. neurobii* [Andree et al. 1999]), a myotrophic myxobolid of salmon (*M. insidiosus* [U96494]), 2 epitheliotropic myxosporeans (one of salmonid hosts, *M. squamalis* [U96495], and one of non-salmonid hosts, *Henneguya doori* [H DU37549]), and a non-myxobolid enterotropic myxosporean of salmon from Family Ceratomyxidae (*Ceratomyxa shasta* [AF001579]). Selection of species for sequence comparisons was based on availability of complete SSU rRNA gene sequence data, tissue tropism, host range, and phylogenetic classification. Percent sequence identities were calculated using MacDNAsis version 3.5 (Hitachi Software Engineering America, Ltd).

Preparation of tissue sections and probes for *in situ* hybridization. The paraffin-embedded tissue blocks, which had been prepared for target selection for PCR, were used in the *in situ* hybridization (ISH) experiments. Tissue sections were cut at 4 µm, mounted on glass slides, stained with H&E, and examined by light microscopy. Whenever foci of parasites were identified in these sections, successive serial tissue sections were cut at 4 µm, mounted un-

stained on silane-coated glass slides, and submitted for ISH experiments.

Digoxigenin-labeled single-stranded oligonucleotide DNA probes complementary to sequences specific to the SSU rRNA gene of *Myxobolus cerebralis* (i.e. Tr5-16, Tr3-16, Tr3-17) were synthesized by Genmed Synthesis Inc., San Francisco, California, USA, and were labeled at their 3'-OH ends with digoxigenin-11-deoxyuridine triphosphate in standard tailing reactions. The nucleotide sequences of probes and their positions relative to the *M. cerebralis* rRNA gene are listed in Table 2.

In situ hybridization procedure. The protocol for *in situ* hybridization (ISH) was based on procedures described by Crabbe et al. (1992), Gan et al. (1994), and Antonio et al. (1998). The ISH was performed using an automated GenII *In Situ* Hybridization Processor (Ventana Medical Systems Inc., Tucson, AZ, USA) in the Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA. Unstained tissue sections mounted on silane-coated glass slides were deparaffinized, rehydrated, incubated for 3 min in Tris-CaCl₂ buffer (pH 7.2), and digested for 24 min in a solution of proteinase K (200 µg ml⁻¹) and 10 mM Tris buffer (pH 7.5). After digestion, sections were covered with 100 µl of hybridization solution (5.0 ml deionized formamide, 2.0 ml 20× SSC, 0.5 ml of dextran sulfate, 0.2 ml of 50× Denhardt's solution) containing a mixture of 3 digoxigenin-labeled DNA probes each at 5 ng µl⁻¹, heated to 95°C for 5 min, and hybridized for 10 h at 37°C. Following three 4 min washes with 2× SSC at 42°C, slides were incubated for 32 min at 42°C in an anti-digoxigenin antibody solution consisting of 2% normal sheep serum, 0.3% Triton X-100, and 1:100 dilution of sheep polyclonal anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim Corp.). Slides were washed multiple times at 42°C in alkaline phosphatase K (APK) washing solution (Ventana Medical System Inc.), immersed in color substrate solution consisting of 4-nitro blue tetrazolium chloride (NBT 75 mg ml⁻¹ in 70% dimethylformamide) and 5-bromo-4-chloro-3-indolylphosphate (X-phosphate 50 mg ml⁻¹ in 100% dimethylformamide) in color substrate buffer (100 mM Tris-HCl, 100 mM NaCl, and 50 mM MgCl₂ at

Fig. 1. Polyacrylamide gel electrophoresis (8%) of PCR products from paraffin-embedded tissues using primers to the repetitive Bg/I element of *Salmo salar*. Lane 1. PhiX174 DNA/Hae III molecular weight marker (Promega Corp., Madison, WI, USA). Lane 2: reagent control. Lanes 3 and 4: *Oncorhynchus keta* genomic DNA (Sigma Chemical Co., St. Louis, MO, USA). Lanes 5 and 6: repeated extractions of *S. salar* genomic DNA from uninfected smolts fixed in 1997. Lanes 7 to 11: *S. salar* genomic DNA from uninfected smolts fixed in 1997 (i.e. negative control DNA). Lanes 12 to 16: DNA from myxosporean-infected *S. salar* fixed in 1994 (i.e. test DNA). Lanes 17 and 18: DNA from *Myxobolus cerebralis*-infected *Oncorhynchus mykiss* fixed in 1997 (i.e. positive control DNA). Lane 19: human genomic DNA. Uninfected *S. salar* DNA from 1997 yielded multiple products at approximately 700, 560, 290, 250, and 225 base pairs, whereas 1 sample of myxosporean-infected *S. salar* DNA from 1994 yielded products at 700, 560, and 250 base pairs (Lane 14). Faint bands are detectable in Lanes 3, 4, and 18 from DNA of *Oncorhynchus* spp. There was no amplification of human genomic DNA (Lane 19).

pH 9.5), rinsed in distilled water, counterstained with nuclear fast red, dehydrated, and coverslipped with Permunt (Fischer Scientific, Fairlawn, NJ, USA).

RESULTS

PCR and Southern hybridizations

Based on UV spectrophotometry, yields of extracted DNA were similar for pooled test and control paraffin-embedded tissues and ranged from 70 to 140 ng µl⁻¹. In PCR experiments performed at an annealing temperature of 55°C, using DNA extracted from paraffin-embedded test and control tissues and primers to the Atlantic salmon Bg/I element, there was amplification of DNA from all negative control samples as well as from representative samples of test and positive control tissues (Fig. 1), indicating that the extraction procedure was adequate for paraffin-embedded tissues.

The Bg/I repeat element is tandemly arrayed and located in the vicinity of the rRNA gene of the *Salmo salar* genome (Goodier & Davidson 1993). According to

Table 2. Oligonucleotide DNA probes used in the *in situ* hybridization experiments. Location refers to the position of the probe relative to the nucleotide sequence of the SSU rRNA gene of *Myxobolus cerebralis*

Probe	5'→3' sequence	Location
Tr5-16	GCATTGGTTTACGCTGATGTAGCGA	212-237
Tr3-16	GAATCGCCGAAACAATCATCGAGCTA	1509-1535
Tr3-17	GGCACACTACTCCAACACTGAATTTG	698-723

Moran et al. (1997), at an annealing temperature of 62°C, amplification of a portion of the Bg/I element using the forward and reverse primers corresponding

to positions 11 to 36 and 707 to 727, respectively, generates fragments of 700 and 280 nucleotides in Atlantic salmon *S. salar* and 650 and 250 nucleotides in brown trout *S. trutta*. In this study, at an annealing temperature of 55°C, uninfected control Atlantic salmon genomic DNA from paraffin-embedded brains fixed in 1997 yielded multiple amplicons, which included fragments of approximately 700, 560, 290, 250, and 225 base pairs. DNA of myxosporean-infected Atlantic salmon from 1994 also yielded amplification products, although with less consistency than did the negative control DNA. DNA extractions from tissue sections from the same paraffin blocks of uninfected control Atlantic salmon brains from 1997 were repeated later in the study using the same protocol and yielded amplification products identical to those of the original negative control samples (Fig. 1). *Oncorhynchus mykiss* DNA from *Myxobolus cerebralis*-infected rainbow trout inconsistently yielded faint amplification products using the *S. salar* Bg/I primers.

With few exceptions, the amplification products from samples of the Atlantic salmon myxosporean using each of the 5 primer pairs were not detectable in agarose gels, although the amplification products from *Myxobolus cerebralis*-infected rainbow trout tissue were visible (Fig. 2A). However, Southern hybridization with sequence-specific digoxigenin-labeled DNA

Fig. 2. Electrophoretic and Southern hybridization patterns of product 5636 (247 base pairs) amplified from formalin-fixed paraffin-embedded brain of *Salmo salar* using primers SM5-6 and SM3-6. (A) 2% agarose gel electrophoresis of PCR products and (B) Southern blot of PCR products hybridized with digoxigenin-labeled double-stranded DNA probes created from the 5636 positive control amplification product. (A,B) Lane 1: pGEM molecular weight marker (Promega Corp.). Lane 2: digoxigenin-labeled DNA molecular weight marker VIII (Boehringer Mannheim Corp., Indianapolis, IN, USA). Lanes 3 and 4: genomic DNA from uninfected *S. salar* brain. Lanes 5 and 15: *Oncorhynchus mykiss* experimentally infected with *Myxobolus cerebralis*. Lanes 6 to 14: DNA from pooled extracts of *S. salar* brain infected with myxosporean developmental stages. Products in Lanes 6 to 14 are not detectable in the 2% agarose gel by ethidium bromide staining and UV trans-illumination, whereas those in Lanes 5 and 15 are visible. Hybridization with digoxigenin-labeled DNA probe reveals amplicons in Lanes 8, 10, and 14 with estimated lengths consistent with the *M. cerebralis* product at <300 and >242 base pairs. (C) Polyacrylamide gel electrophoresis (8%) of purified products of second-round PCRs. (C) Lane 1: blank. Lane 2: reagent control. Lane 3: genomic DNA of uninfected *S. salar*. Lane 4: *M. cerebralis*-infected *O. mykiss* DNA. Lane 5: PhiX174 DNA/Hae III molecular weight marker (Promega Corp.). Lanes 6 to 18: DNA of myxosporean-infected *S. salar* brains that yielded amplification products detectable by Southern hybridization. All second-round PCR products are the same size as that from *M. cerebralis*.

probes followed by chemiluminescent detection allowed for identification of PCR products that were not detectable by ethidium bromide staining and UV trans-illumination alone (Fig. 2B). Whenever extractions of DNA from Atlantic salmon infected with the myxosporean produced amplicons, those products were of the same molecular weight as amplicons from DNA of *M. cerebralis*-infected rainbow trout. The sizes of amplification products ranged from 187 to 287 base pairs for the different primer pairs (Table 1). DNA from *M. cerebralis*-infected rainbow trout consistently produced robust amplifications with each primer pair, whereas DNA extracted from Atlantic salmon smolts, which were determined by light microscopy to be free of myxosporeans, consistently failed to produce amplicons with any of the primer pairs.

Products of each primer pair were detectable by Southern hybridization using sequence-specific probes. The 5 µl samples from first-round PCR mixtures subjected to a second round of PCR yielded amplification products of sufficient concentration (i.e. 25 to 100 ng µl⁻¹) to be utilized in subsequent sequencing reactions. Purified second-round PCR products were detectable after electrophoresis in 8% polyacrylamide gels (Fig. 2C).

Consensus sequences and sequence alignments

A consensus sequence for the amplification product of each of the 5 different primer pairs was obtained from alignments of the forward and reverse sequencing reactions of a minimum of 5 separate PCR products. Overall, there was minimal variation in the sequences of the products of each primer pair with discrepancy between bases at less than 2% of the positions.

The consensus sequences of the 5 separate amplification products from the myxosporean were aligned with the SSU rDNA sequence of *Myxobolus cerebralis* (Fig. 3). Consensus sequences of products were consolidated at positions of overlap, resulting in a partial SSU rDNA sequence of the parasite consisting of a 5' fragment derived from products 5636 and 5335, followed by a gap of undetermined bases, followed by a 3' fragment derived from products 5434, 5737 and 5838. This partial sequence was aligned with the SSU rDNA sequences of 3 neurotropic myxobolid parasites of salmonids, e.g. *M. arcticus*, *M. cere-*

Fig. 3. Alignments of the SSU rDNA sequence of *Myxobolus cerebralis* (Row 1) with the consensus sequences of the 5 PCR products amplified from DNA of *Salmo salar* brains infected with myxosporean developmental stages (Rows 2 to 6). Note that products are identified by the numerical designations of the primers responsible for their amplification

bralis, and *M. neurobius* (Fig. 4). The reported bases of the Atlantic salmon myxosporean were identical to those of *M. cerebralis* at 78 of 81 locations of unmatched bases between the 4 sequences.

Analysis of percent sequence identity

Percent sequence identities between the SSU rDNA sequence of the parasite and regions of the SSU rRNA gene sequences of the myxosporeans of the study group were calculated separately for the 529-base pair and 661-base pair fragments and did not include the intervening gap of undetermined bases (Table 3). Both the 529-base pair and 661-base pair fragments were identical to relevant segments of the SSU rRNA gene sequence of *Myxobolus cerebralis* at 99% of their nucleotides, and both fragments shared more sequence identity (i.e. more identical nucleotides) with

Table 3. Percent sequence identity between the (a) 529-base pair and (b) 661-base pair fragments of the partial SSU rRNA gene sequence of the myxosporean from Atlantic salmon *Salmo salar* and known SSU rRNA gene sequences from a selection of myxosporeans (*Myxobolus cerebralis*, *M. arcticus*, *M. neurobius*, *M. insidiosus*, *Henneguya doori*, *Ceratomyxa shasta* and *M. squamalis*)

(a)	529-bp	<i>M. cere</i>	<i>M. arc</i>	<i>M. neur</i>	<i>M. insid</i>	<i>H. door</i>	<i>C. shast</i>	<i>M. squa</i>
529-bp	100	99.6	93.6	93.4	93.0	71.5	63.7	61.5
<i>M. cere</i>		100	93.2	93.0	92.7	71.1	63.8	58.0
<i>M. arc</i>			100	98.7	97.6	71.1	64.6	60.3
<i>M. neur</i>				100	97.4	70.5	65.0	56.6
<i>M. insid</i>					100	69.9	62.5	58.6
<i>H. door</i>						100	62.9	64.5
<i>C. shast</i>							100	55.9
<i>M. squa</i>								100
(b)	661-bp	<i>M. cere</i>	<i>M. arc</i>	<i>M. neur</i>	<i>M. insid</i>	<i>H. door</i>	<i>C. shast</i>	<i>M. squa</i>
661-bp	100	99.4	89.5	91.5	89.3	85.4	80.7	75.1
<i>M. cere</i>		100	89.0	91.1	89.0	85.0	80.2	74.8
<i>M. arc</i>			100	97.6	94.5	82.0	80.0	71.6
<i>M. neur</i>				100	96.5	83.7	80.5	72.5
<i>M. insid</i>					100	82.7	80.5	70.9
<i>H. door</i>						100	77.7	80.9
<i>C. shast</i>							100	82.1
<i>M. squa</i>								100

M.cerebralis>	1	AGATATACGC	10	TTTTCTCTAA	20	GACTAAGCCA	30	TGCATGTTNA	40	AGTTCATACG	50
M.neurobius >	-89										-40
M.arcticus >	-89										-40
unknown cons>	-414										-365
M.cerebralis>	51	TAGTAAACG	60	TGAGACTGCG	70	GACGGCTCAG	80	TAAATCAGTT	90	ATCATCTATT	100
M.neurobius >	-39							T		ATCATCTATT	11
M.arcticus >	-39							T		ATCATCTATT	11
unknown cons>	-364										-315
M.cerebralis>	101	TGTTTGTCTA	110	CCCATTGGAT	120	AACNNNGG	130	AAATCTAGTG	140	CTAATACATG	150
M.neurobius >	12	TGTTTGTCTA	110	CCCATTGGAT	120	AACCGTGGGN	130	AA-TCTAGNG	140	CTAATACATG	150
M.arcticus >	12	TGTTTGTCTA	110	CCCATTGGAT	120	AACCGTGGGA	130	AAATCTAGAG	140	CTAATACATG	150
unknown cons>	-314										-265
M.cerebralis>	151	CAGTTTTGGG	160	G?AGCGTTAA	170	AACTGTCTCA	180	CGGCATTAT	190	TGGACATAAC	200
M.neurobius >	62	CAGTTTTGGG	160	NTAGCGT-AA	170	GT-TGTCTCA	180	CGGCATTAT	190	TGGACATAAC	200
M.arcticus >	62	CAGTTTTGGG	160	ATAGCGT-AA	170	GT-TGTCTCA	180	CGGCATTAT	190	TGGACATAAC	200
unknown cons>	-264										-215
M.cerebralis>	201	CAACTACCGG	210	TGCATTGGGT	220	TACGCTGATG	230	T-ACCGAGTA	240	AGGTCAATCT	250
M.neurobius >	112	CAACTACCGG	210	CGNACAGCT	220	T--GCTGATG	230	CGA-CGCGTA	240	AGGTCAATCT	250
M.arcticus >	112	CAACTACCGG	210	CGNACAGCT	220	T--GCTGATG	230	CAAC-CGTA	240	AGGTCAATCT	250
unknown cons>	-214										-165
M.cerebralis>	251	AGATAACTTT	260	ACTCATCGTA	270	TGG-CCTATA	280	GCCGGCCACG	290	TTTCAATTGA	300
M.neurobius >	162	AGATAACTTT	260	ACTCATCGTA	270	TGG-CCTATA	280	GCCGGCCACG	290	TTTCAATTGA	300
M.arcticus >	162	AGATAACTTT	260	ACTCATCGTA	270	TGG-CCTATA	280	GCCGGCCACG	290	TTTCAATTGA	300
unknown cons>	-164										-115
M.cerebralis>	301	ATTCTGCGCC	310	TATTAACCTAG	320	TGGTAGTAT	330	AGTAGCCTAC	340	CAGGGTTGCG	350
M.neurobius >	212	ATTCTGCGCC	310	TATTAACCTAG	320	TGGTAGTAT	330	AGTAGCCTAC	340	CAGGGTTGCG	350
M.arcticus >	212	ATTCTGCGCC	310	TATTAACCTAG	320	TGGTAGTAT	330	AGTAGCCTAC	340	CAGGGTTGCG	350
unknown cons>	-114										-65
M.cerebralis>	351	AGGGGTACG	360	GGGAATCAGG	370	GTTTCGATTCC	380	GGAGAGGGAG	390	CCTGAGAAAC	400
M.neurobius >	262	AGGGGTACG	360	GGGAATCAGG	370	GTTTCGATTCC	380	GGAGAGGGAG	390	CCTGAGAAAC	400
M.arcticus >	262	AGGGGTACG	360	GGGAATCAGG	370	GTTTCGATTCC	380	GGAGAGGGAG	390	CCTGAGAAAC	400
unknown cons>	-64										-15
M.cerebralis>	401	GGCTACCACA	410	TCCATGG-AA	420	GGCAGCAGGC	430	GCGCAAAATTA	440	CCCAATCCAG	450
M.neurobius >	312	GGCTACCACA	410	TCCATGG-AA	420	GGCAGCAGGC	430	GCGCAAAATTA	440	CCCAATCCAG	450
M.arcticus >	312	GGCTACCACA	410	TCCATGG-AA	420	GGCAGCAGGC	430	GCGCAAAATTA	440	CCCAATCCAG	450
unknown cons>	-14										36
M.cerebralis>	451	ACACTGGG-A	460	GGTGGTACG	470	AGAAGTACTA	480	AGTGGTGGCC	490	CTTAGGGTCG	500
M.neurobius >	362	ACACTGGG-A	460	GGTGGTACG	470	AGAAGTACTA	480	AGTGGTGGCC	490	CTTAGGGTCG	500
M.arcticus >	362	ACACTGGG-A	460	GGTGGTACG	470	AGAAGTACTA	480	AGTGGTGGCC	490	CTTAGGGTCG	500
unknown cons>	37	ACACTGGG-A	460	GGTGGTACG	470	AGAAGTACTA	480	AGTGGTGGCC	490	CTTAGGGTCG	86
M.cerebralis>	501	CAGCTTGGA	510	ATGGACGTAA	520	TTTAAGTAAT	530	TCGATGAGTA	540	ACAACCTGGAG	550
M.neurobius >	412	CAGCTTGGA	510	ATGGACGTAA	520	TTTAAGTAAT	530	TCGATGAGTA	540	ACAACCTGGAG	550
M.arcticus >	412	CAGCTTGGA	510	ATGGACGTAA	520	TTTAAGTAAT	530	TCGATGAGTA	540	ACAACCTGGAG	550
unknown cons>	87	CAGCTTGGA	510	ATGGACGTAA	520	TTTAAGTAAT	530	TCGATGAGTA	540	ACAACCTGGAG	136
M.cerebralis>	551	GGCAAGTCTG	560	GTGCCAGCAG	570	CGCGGGTAAT	580	TCCAGCTCCA	590	GTAGCGTATT	600
M.neurobius >	462	GGCAAGTCTG	560	GTGCCAGCAG	570	CGCGGGTAAT	580	TCCAGCTCCA	590	GTAGCGTATT	600
M.arcticus >	462	GGCAAGTCTG	560	GTGCCAGCAG	570	CGCGGGTAAT	580	TCCAGCTCCA	590	GTAGCGTATT	600
unknown cons>	137	GGCAAGTCTG	560	GTGCCAGCAG	570	CGCGGGTAAT	580	TCCAGCTCCA	590	GTAGCGTATT	186
M.cerebralis>	601	TTAAAGTTGC	610	TGCGTTTAAA	620	ACGCTCGTAG	630	TTGGATCACG	640	CAGTGTAAAGT	650
M.neurobius >	512	TTAAAGTTGC	610	TGCGTTTAAA	620	ACGCTCGTAG	630	TTGGATCACG	640	CAGTGTAAAGT	650
M.arcticus >	512	TTAAAGTTGC	610	TGCGTTTAAA	620	ACGCTCGTAG	630	TTGGATCACG	640	CAGTGTAAAGT	650
unknown cons>	187	TTAAAGTTGC	610	TGCGTTTAAA	620	ACGCTCGTAG	630	TTGGATCACG	640	CAGTGTAAAGT	236
M.cerebralis>	651	TGGTAGGCTG	660	ATCGAATGGT	670	GCTACTAACT	680	GCTCCAGGTA	690	TGAATTTCTA	700
M.neurobius >	562	TGGTAGGCTG	660	ATCGAATGGT	670	GCTACTAACT	680	GCTCCAGGTA	690	TGAATTTCTA	700
M.arcticus >	562	TGGTAGGCTG	660	ATCGAATGGT	670	GCTACTAACT	680	GCTCCAGGTA	690	TGAATTTCTA	700
unknown cons>	237	TGGTAGGCTG	660	ATCGAATGGT	670	GCTACTAACT	680	GCTCCAGGTA	690	TGAATTTCTA	286
M.cerebralis>	701	ATTCACTGTT	710	GGACTAGTGT	720	GGCTCTTTT	730	AGTTATTGCG	740	CAATTTACAC	750
M.neurobius >	612	ATT-ATTACT	710	GGACTAGTGT	720	GGCTCTTTT	730	AGTTATTGCG	740	CAATTTACAC	750
M.arcticus >	612	ATT-ATTACT	710	GGACTAGTGT	720	GGCTCTTTT	730	AGTTATTGCG	740	CAATTTACAC	750
unknown cons>	287	ATTCACTGTT	710	GGACTAGTGT	720	GGCTCTTTT	730	AGTTATTGCG	740	CAATTTACAC	336
M.cerebralis>	751	TACTTACGCG	760	TAAGGATGGC	770	AGTTG-CCTT	780	TAGTGCCTCG	790	ATTGCCGTGT	800
M.neurobius >	662	TACTTACGCG	760	TAAGGATGGC	770	AGTTGACCTT	780	TAGTGCCTCG	790	ATTGCCGTGT	800
M.arcticus >	662	TACTTACGCG	760	TAAGGATGGC	770	AGTTGACCTT	780	TAGTGCCTCG	790	ATTGCCGTGT	800
unknown cons>	337	TACTTACGCG	760	TAAGGATGGC	770	AGTTGACCTT	780	TAGTGCCTCG	790	ATTGCCGTGT	386
M.cerebralis>	801	-CTTACGGAG	810	TGTGCCCTTGA	820	ATAAATCAGA	830	GTGCTCAAAG	840	CAGGCTTTTG	850
M.neurobius >	712	-CTTACGGAG	810	TGTGCCCTTGA	820	ATAAATCAGA	830	GTGCTCAAAG	840	CAGGCTTTTG	850
M.arcticus >	712	-CTTACGGAG	810	TGTGCCCTTGA	820	ATAAATCAGA	830	GTGCTCAAAG	840	CAGGCTTTTG	850
unknown cons>	387	-CTTACGGAG	810	TGTGCCCTTGA	820	ATAAATCAGA	830	GTGCTCAAAG	840	CAGGCTTTTG	436
M.cerebralis>	851	CTTGAATCTT	860	AATAGCATGG	870	AACGAANAAT	880	TGTGTAGTAG	890	TGTGTTGTGA	900
M.neurobius >	762	CTTGAATCTT	860	AATAGCATGG	870	AACGAANAAT	880	TGTGTAGTAG	890	TGTGTTGTGA	900
M.arcticus >	762	CTTGAATCTT	860	AATAGCATGG	870	AACGAANAAT	880	TGTGTAGTAG	890	TGTGTTGTGA	900
unknown cons>	437	CTTGAATCTT	860	AATAGCATGG	870	AACGAANAAT	880	TGTGTAGTAG	890	TGTGTTGTGA	486
M.cerebralis>	901	CATATAGCGA	910	TCGGCTTTTG	920	ACTGAATGTT	930	ATT-CAGTTA	940	CAGCATACAG	950
M.neurobius >	812	CATATAGCGA	910	TCGGCTTTTG	920	ACTGAATGTT	930	ATT-CAGTTA	940	CAGCATACAG	950
M.arcticus >	812	CATATAGCGA	910	TCGGCTTTTG	920	ACTGAATGTT	930	ATT-CAGTTA	940	CAGCATACAG	950
unknown cons>	487	CATATAGCGA	910	TCGGCTTTTG	920	ACTGAATGTT	930	ATT-CAGTTA	940	CAGCATACAG	536
M.cerebralis>	951	CACCAACCCAC	960	CAATAACCGGA	970	TGTTGGTTTCC	980	GTATTGGGGT	990	GATGATTAAA	1000
M.neurobius >	862	CACCAACCCAC	960	CAATAACCGGA	970	TGTTGGTTTCC	980	GTATTGGGGT	990	GATGATTAAA	1000
M.arcticus >	862	CACCAACCCAC	960	CAATAACCGGA	970	TGTTGGTTTCC	980	GTATTGGGGT	990	GATGATTAAA	1000
unknown cons>	537	??????????	960	CAATAACCGGA	970	TGTTGGTTTCC	980	GTATTGGGGT	990	GATGATTAAA	586
M.cerebralis>	1001	AGGAGCGGTT	1010	GGGGGCATTG	1020	GTATTTGGCC	1030	GCGAGAGGTG	1040	AAATTCCTAG	1050
M.neurobius >	912	AGGAGCGGTT	1010	GGGGGCATTG	1020	GTATTTGGCC	1030	GCGAGAGGTG	1040	AAATTCCTAG	1050
M.arcticus >	912	AGGAGCGGTT	1010	GGGGGCATTG	1020	GTATTTGGCC	1030	GCGAGAGGTG	1040	AAATTCCTAG	1050
unknown cons>	587	??????????	1010	GGGGGCATTG	1020	GTATTTGGCC	1030	GCGAGAGGTG	1040	AAATTCCTAG	636

		1060	1070	1080	1090	1100	
M.cerebralis>	1051	ACCGGCCAAG	GACTAACGAA	TGCGAAGGCA	TTTGCACAGA	CCGCGTCGCT	1100
M.neurobius >	962	ACCGGCCAAG	GACTAACGAA	TGCGAAGGCA	TTTGCACAGA	CCGCGTCGCT	1011
M.arcticus >	962	ACCGGCCAAG	GACTAACGAA	TGCGAAGGCA	TTTGCACAGA	CCGCGTCGCT	1011
unknown cons>	637	??????????	??????????	??????????	TTTGCACAGA	CCGCGTCGCT	686
		1110	1120	1130	1140	1150	
M.cerebralis>	1101	TAATCAAGAA	CGATAGTGG-	AGGTTTCAAG	ACGATCAGAT	ACCGTCCTAG	1150
M.neurobius >	1012	TAATCAAGAA	CGATAGTGGG	AGGTTTCAAG	ACGATCAGAT	ACCGTCCTAG	1061
M.arcticus >	1012	TAATCAAGAA	CGATAGTGGG	AGGTTTCAAG	ACGATCAGAT	ACCGTCCTAG	1061
unknown cons>	687	TAATCAAGAA	CGATAGTGGG	AGGTTTCAAG	ACGATCAGAT	ACCGTCCTAG	736
		1160	1170	1180	1190	1200	
M.cerebralis>	1151	TTCCCACTGT	AAACTATGCC	GACCCGGGAT	CAGCATGAAG	CTCTATATAC	1200
M.neurobius >	1062	TTCCCACTGT	AAACTATGCC	GACCCGGGAT	CAGCATGAAG	CTCTATATAT	1111
M.arcticus >	1062	TTCCCACTGT	AAACTATGCC	GACCCGGGAT	CAGCATGAAG	NTCTATATAT	1111
unknown cons>	737	TTCCCACTGT	AAACTATGCC	GACCCGGGAT	CAGCATGAAG	CTCTATATAC	786
		1210	1220	1230	1240	1250	
M.cerebralis>	1201	GCTTATGT	GGGCCCCCTG	GGAAACCTCA	AGTTTTTCGG	TTACGGGGAG	1250
M.neurobius >	1112	GCTTATGT	GGGCCCCCTG	GGAAACCTCA	AGTTTTTCGG	TTACGGGGAG	1161
M.arcticus >	1112	GCTTATGT	GGGCCCCCTG	GGAAACCTCA	AGTTTTTCGG	TTACGGGGAG	1161
unknown cons>	787	GCTTATGT	GGGCCCCCTG	GGAAACCTCA	AGTTTTTCGG	TTACGGGGAG	836
		1260	1270	1280	1290	1300	
M.cerebralis>	1251	AGTATGGTCA	CAAGGCTGAA	ACTTAAAGGA	ATTGACGGAA	GGGCACCACC	1300
M.neurobius >	1162	AGTATGGTCC	CAAGGCTGAA	ACTTAAAGGA	ATTGACGGAA	GGGCACCACC	1211
M.arcticus >	1162	AGTATGGTCC	CAAGGCTGAA	ACTTAAAGGA	ATTGACGGAA	GGGCACCACC	1211
unknown cons>	837	AGTATGGTCC	CAAGGCTGAA	ACTTAAAGGA	ATTGACGGAA	GGGCACCACC	886
		1310	1320	1330	1340	1350	
M.cerebralis>	1301	AGGAGTGGAG	CCTGCGGCTT	AATTTGACTC	AACACGGGAA	AACCTACCAG	1350
M.neurobius >	1212	AGGAGTGGAG	CCTGCGGCTT	AATTTGACTC	AACACGGGAA	AACCTACCAG	1261
M.arcticus >	1212	AGGAGTGGAG	CCTGCGGCTT	AATTTGACTC	AACACGGGAA	AACCTACCAG	1261
unknown cons>	887	AGGAGTGGAG	CCTGCGGCTT	AATTTGACTC	AACACGGGAA	AACCTACCAG	936
		1360	1370	1380	1390	1400	
M.cerebralis>	1351	GTCCGGACAT	CAATAGGATA	GACAAAGACTG	ATAGATCTTT	-CTTGATATG	1400
M.neurobius >	1262	GTCCGGACAT	CAATAGGATA	GACA-GACTG	ATAGATCTTT	TCTTGATATG	1311
M.arcticus >	1262	GTCCGGACAT	CAATAGGATA	GACA-GACTG	ATAGATCTTT	-CTTGATATG	1311
unknown cons>	937	GTCCGGACAT	CAATAGGATA	GACA-GACTG	ATAGATCTTT	-CTTGATATG	986
		1410	1420	1430	1440	1450	
M.cerebralis>	1401	ATGGATAGTG	GTGCATGGCC	GTCTCTAGTT	CGTGGAGTGA	TCTGTCAAGC	1450
M.neurobius >	1312	ATGGATAGTG	GTGCATGGCC	GTCTCTAGTT	CGTGGAGTGA	TCTGTCAAGC	1361
M.arcticus >	1312	ATGGATAGTG	GTGCATGGCC	GTCTCTAGTT	CGTGGAGTGA	TCTGTCAAGC	1361
unknown cons>	987	ATGGATAGTG	GTGCATGGCC	GTCTCTAGTT	CGTGGAGTGA	TCTGTCAAGC	1036
		1460	1470	1480	1490	1500	
M.cerebralis>	1451	TAATCCCGGT	AACG-AACGA	GAACTTATTC	TCCATTGTGAT	GAGCGGAAG	1500
M.neurobius >	1362	TAATCCCGGT	AACG-AACGA	GAACTTATTC	TCCATTGTGAT	GAGCGGAAG	1411
M.arcticus >	1362	TAATCCCGGT	AACG-AACGA	GAACTTATTC	TCCATTGTGAT	GAGCGGAAG	1411
unknown cons>	1037	TAATCCCGGT	AACG-AACGA	GAACTTATTC	TCCATTGTGAT	GAGCGGAAG	1086
		1510	1520	1530	1540	1550	
M.cerebralis>	1501	AGATAGTGTA	GCTGATCAT	TGTTTCGGCG	AT-TCTCAAG	TTATCTCTAT	1550
M.neurobius >	1412	AGATAGTG--	GCTGATCAT	TGTTTCGGCG	ATAT-TCAAG	TTAT-C-ATC	1461
M.arcticus >	1412	AGATAGTG--	GCTGATCAT	TGTTTCGGCG	ATAT-TCAAG	TTAT-C-ATC	1461
unknown cons>	1087	AGATAGTGTA	GCTGATCAT	TGTTTCGGCG	AT-TCTCAAG	TTATCTCTAT	1136
		1560	1570	1580	1590	1600	
M.cerebralis>	1551	GAAGGCAGTG	TTTGGGATTT	TAGTGT-GAA	A-ATAGAGT	TGTTGCGAGG	1600
M.neurobius >	1462	GAAGGCAGTG	TTTGGGATTT	TAGTGT-GAA	ATATAAGAG-	-GTTGCGAGA	1511
M.arcticus >	1462	GAAGGCAGTG	TTTGGGATTT	TAGTGT-GAA	ATATAAGAG-	-GTTGCGAGA	1511
unknown cons>	1137	GAAGGCAGTG	TTTGGGATTT	TAGTGT-GAA	A-ATAGAGT	TGTTGCGAGG	1186
		1610	1620	1630	1640	1650	
M.cerebralis>	1601	ACGGGATTA	ACCTC-TTACT	GGTGGCAAT	TGTACTAGAC	CTGACTTTGT	1650
M.neurobius >	1512	ACGGGATTA	CCCATC-CT	GGTAGCAAT	TGTACTAAT	CTAATTTTGT	1561
M.arcticus >	1512	ACGGGATTA	CCCATC-CT	GGTAGCAAT	TGTACTAAT	CTAATTTTGT	1561
unknown cons>	1187	ACGGGATTA	ACCTC-TTACT	GGTGGCAAT	TGTACTAGAC	CTGACTTTGT	1236
		1660	1670	1680	1690	1700	
M.cerebralis>	1651	TGGCATACCC	TTCCGTTTATA	CGCTGTTCNA	CT-ACCCA-G	TGGAGCAGTG	1700
M.neurobius >	1562	TGGCATACCC	TTCCGTTTATA	CGCTGTTCNA	CTTACCAAG	TGGAGCAGTG	1611
M.arcticus >	1562	TGGCATACCC	TTCCGTTTATA	CGCTGTTCNA	CTTACCAAG	TGGAGCAGTG	1611
unknown cons>	1237	TGGCATACCC	TTCCGTTTATA	CGCTGTTCNA	CT-ACCCA-G	TGGAGCAGTG	1286
		1710	1720	1730	1740	1750	
M.cerebralis>	1701	TGTCATGGAG	AGACTGTGAG	GTATATATCC	AAGCTCAATG	AAGCAAGGCC	1750
M.neurobius >	1612	TGTCATGGAG	AGACTGTGAG	G.....			1661
M.arcticus >	1612	TGTCATGGAG	AGACTGTGAG	G.....			1661
unknown cons>	1287	TGTCATGGAG	AGACTGTGAG	GTATATATCC	AAGCTCAATG	AAGCAAG...	1336
		1760	1770	1780	1790	1800	
M.cerebralis>	1751	ATAACAGTCT	TGTGATGCC	TAAGATGTCC	TGGGCTGCAC	GCGCGCTACA	1800
M.neurobius >	1662						1711
M.arcticus >	1662						1711
unknown cons>	1337						1386
		1810	1820	1830	1840	1850	
M.cerebralis>	1801	ATGATGGTGA	CAGCGAGTTT	CTAGGTCGAG	AGACCTGGGC	AATCTTGTA	1850
M.neurobius >	1712						1761
M.arcticus >	1712						1761
unknown cons>	1387						1436
		1860	1870	1880	1890	1900	
M.cerebralis>	1851	TCACCATCGT	GATGGGGATT	GACCATTGTA	ATTGGTTCAT	GAAATAGGAA	1900
M.neurobius >	1762						1811
M.arcticus >	1762						1811
unknown cons>	1437						1486
		1910	1920	1930	1940	1950	
M.cerebralis>	1901	TTCCCTGTAG	GCACACTTAA	TTAGAGTGTG	CCGAACGAGT	CCCTGCCCTT	1950
M.neurobius >	1812						1861
M.arcticus >	1812						1861
unknown cons>	1487						1536
		1960	1970	1980	1990	2000	
M.cerebralis>	1951	TGTA.....					2000
M.neurobius >	1862						1911
M.arcticus >	1862						1911
unknown cons>	1537						1586

Fig. 4. Alignments of the partial SSU rDNA sequence of the myxosporean (unknown cons) from *Salmo salar* brains with the SSU rDNA sequences of 3 neurotropic myxobolids: *Myxobolus cerebralis*, *M. neurobius*, and *M. arcticus*. Note that unmatched bases are highlighted. -: base position not reported; N: undetermined base, possibly A, T, C, or G; ?: base position not sequenced

sequences from neurotropic and myotropic *Myxobolus* species than with those from epitheliotropic species of *Myxobolus* and *Henneguya* or an enterotropic species of *Ceratomyxa*. For all the myxosporeans of the study group, there was more sequence identity over the 661-base pair region than the 529-base pair region. With the exception of *M. squamalis*, the 529-base pair fragment from the parasite demonstrated more sequence identity with sequences from species of *Myxobolus* than with sequences from *Henneguya doori* or *Ceratomyxa shasta*.

In situ hybridization

Digoxigenin-labeled DNA probes complementary to segments of the SSU rRNA gene of *Myxobolus cerebralis* hybridized with DNA of the parasite in histologic sections of formalin-fixed Atlantic salmon brains. Probes hybridized with trophozoites and spores of *M. cerebralis* in histologic sections of rainbow trout fingerlings, whereas no hybridization signal was detectable in serial histologic sections processed without probes (i.e. negative probe control) (Fig. 5A,B). Probes which

Fig. 5. *In situ* hybridization (ISH) of the myxosporean of *Salmo salar* in histologic sections of formalin-fixed paraffin-embedded brains of smolts from 1994, using *Myxobolus cerebralis* SSU rDNA sequence-specific digoxigenin-labeled single-stranded oligonucleotide DNA probes. (A) Positive (i.e. blue) labeling of sporoplasms of spores of *M. cerebralis* in a histologic section from an experimentally infected rainbow trout *Oncorhynchus mykiss* fingerling and (B) similar section, processed for ISH using hybridization solution lacking probes (i.e. negative probe control). Sporoplasms stain only with the red counterstain. (C) Positive labeling of the myxosporean in a histologic section of Atlantic salmon brain from a 1994 smolt using the probes that hybridized with spores of *M. cerebralis* in (A); and (D) similar section, processed for ISH using hybridization solution lacking probes (i.e. negative probe control). Parasites stain only with the red counterstain. Stains: Nitro blue tetrazolium chloride and nuclear fast red. Scale bars = 10 µm

hybridized with DNA of *M. cerebralis* also hybridized with DNA of the myxosporean in histologic sections of Atlantic salmon brains from 1994 (Fig. 5C,D). There was no hybridization in histologic sections of uninfected Atlantic salmon brains from 1997.

When compared to consensus sequences of PCR products from the myxosporean, sequences of the *in situ* probes were complementary to regions within the sequences of products 5335 (i.e. Tr3-17) and 5838 (i.e. Tr3-16). Probe Tr5-16 was located in a region of the *M. cerebralis* SSU rRNA gene 5' to the segment complementary to product 5636.

DISCUSSION

Polymerase chain reaction and hybridization experiments

The amplification and hybridization of DNA from formalin-fixed paraffin-embedded salmonid tissues in this study are consistent with previous findings using human tissue samples (Impraim et al. 1987). Inconsistent amplification of DNA from samples containing the myxosporean parasite may be related to factors such as the absence or paucity of target DNA in small tissue samples, the co-purification of biochemical compounds inhibitory to enzymatic reactions, and the degradation of target DNA by fixation and extraction, each of which has been known to compromise efficient PCR amplification of DNA from formalin-fixed paraffin-embedded human tissue samples (Merkelbach et al. 1997).

Myxosporean proliferative stages, such as the solitary cells, cell doublets, or multicellular stages of *Myxobolus cerebralis* (El-Matbouli et al. 1995), or the multicellular blood-borne stages of *Sphaerospora renicola* (Molnár 1993), often occur in small numbers and are widely dispersed within host tissues, making capture of their DNA difficult. In order to enrich test samples for PCR with target DNA from the parasite, brains from the 1994 epizootic were used in preference to those from the 1995 study, as the former had an increased prevalence and density of infection; tissue sections with a high probability of containing parasite foci were pooled by first examining preceding tissue sections for the presence of the parasite.

Co-purification of residual protein and membrane components contribute to weak or absent amplification signals by inhibiting *Taq* polymerase activity (Jackson et al. 1991, Merkelbach et al. 1997) and may have contributed to the weak amplification signals from experimental samples in this study. The QIAamp DNA extraction system was employed because of its ability to reduce the concentration of inhibitory compounds in extracted DNA, to yield DNA fragments of higher

average length, and to yield higher amounts of DNA as compared to other methods of DNA purification (Merkelbach et al. 1997).

Degradation of target DNA by fixation is the most significant problem in molecular analysis of DNA extracted from paraffin blocks (Mies et al. 1991). Breakpoints in the linear series of bases in DNA prevent oligonucleotide-directed enzymatic amplification of DNA despite primer annealing in the context of an appropriately optimized PCR reagent mixture, thus leading to the occurrence of false negative amplification results (Mies 1994). However, partially degraded low molecular weight DNA from formalin-fixed paraffin-embedded tissue can serve adequately in PCR, provided that yields of DNA from extraction techniques are sufficient and that primer strategies target smaller regions of DNA for amplification (Honma et al. 1993, Mies 1994). Initial attempts at PCR in this study were unsuccessful using primers designed to amplify an 858-base pair fragment of the myxosporean SSU rDNA in either single-round or nested protocols (data not shown). Based on these findings and to compensate for the low molecular weight DNA from the Atlantic salmon brains (some of which had been stored for >3 yr in formalin before being embedded in paraffin), primer pairs were constructed to amplify regions of SSU rDNA in the range of 100 to 500 base pairs. Productive primers in this study generated amplicons that ranged in length from 187 to 287 base pairs.

Detection of amplification products by hybridization with sequence-specific probes confirmed the sequence identity of amplicons with those of SSU rDNA from *Myxobolus cerebralis*, and further *in situ* hybridization (ISH) experiments confirmed that the template DNA for these products was derived from the myxosporean identified in histologic sections of infected Atlantic salmon brains. *In situ* hybridization can detect which cells of a mixed population within a tissue possess a specific nucleic acid sequence (Crabbe et al. 1992), thereby increasing the resolution of sequence recognition from that of the entire tissue, as determined by Southern hybridization, to that of the cellular or subcellular compartment (Gan et al. 1994). *M. cerebralis* SSU rDNA sequence-specific DNA probes hybridized with DNA of cells within multicellular developmental stages of the parasite. Such hybridizations are consistent with the high percent sequence identity determined to exist between amplification products from the myxosporean and the *M. cerebralis* SSU rRNA gene, and they establish the myxosporean as the most probable source of template DNA for PCR products using primers derived from the SSU rDNA sequence of *M. cerebralis*.

In accord with the findings of Antonio et al. (1998), the labeling of *Myxobolus cerebralis* trophozoites in our ISH experiments was more consistent than that

of spores, owing to the fact that spores were more resistant to protease digestion (Antonio et al. 1998). Similarly, ISH involving myxosporean-infected Atlantic salmon brains from 1994 required longer protease digestion times to counter the extensive cross-linking of proteins caused by prolonged formalin fixation, which can significantly limit hybridization signals (Guiot & Rahier 1995).

Sequence alignments and percent sequence identity

Sequence alignments with neurotropic *Myxobolus* species confirmed that the partial SSU rDNA sequence of the myxosporean from the brains of Atlantic salmon is identical to that of *M. cerebralis*. The 1% variation between the partial SSU rDNA sequence of the Atlantic salmon myxosporean and that of *M. cerebralis* is likely attributable to polymerase error and the inclusion of an undetermined base at position 877 (Fig. 4). The partial SSU rDNA sequence of the myxosporean also has high percent sequence identity with the neurotropic *Myxobolus* species *M. arcticus* and *M. neurobius*, as well as with one other member of the genus, *M. insidiosus*, whereas its percent sequence identity is much less with *Henneguya doori* or with *Ceratomyxa shasta*. The percent sequence identity of the Atlantic salmon myxosporean with *M. squamalis* follows the patterns demonstrated by the other *M.* species.

The partial SSU rDNA sequence of the myxosporean from Atlantic salmon comprises 61% of the total *Myxobolus cerebralis* SSU rRNA gene and includes both conserved and variable regions (Fig. 4). However, variable regions of the SSU rRNA gene exist outside of those sequenced and compared from the Atlantic salmon myxosporean and preclude a definitive characterization of the parasite based solely on this sequence data. Judging from those myxosporean species for which SSU rDNA sequences are available, the SSU rDNA sequence data from this investigation indicate that the myxosporean infecting the brains of Atlantic salmon is most likely *M. cerebralis*, which is consistent with previous morphologic assertions based on ultrastructural features of developmental stages (Frasca et al. 1998).

According to Halliday (1976), *Myxobolus cerebralis* has been identified in natural and farm-reared *Salmo salar* in Ireland, although no further details of its occurrence are given. At the marine rearing site where epizootic mortality was described (Rodger et al. 1995), 2 rivers flow into the south end of the lough, each of which has runs of wild Atlantic salmon *S. salar* and anadromous and partially anadromous brown trout *S. trutta*; in addition, a rainbow trout *Oncorhynchus mykiss* hatchery lies on one of these rivers (Frasca et al. 1998). Each of these salmonid populations could poten-

tially serve as a source of *M. cerebralis* to the ecosystems of this geographic region; however, *M. cerebralis* has never been identified by histopathologic examination of farmed Atlantic salmon from these rearing sites during the course of the routine health surveillance regime instituted by the sea-farm in 1980. Fish from wild populations were not examined for evidence of infection by *M. cerebralis* as part of this study.

The assertion that this myxosporean from Atlantic salmon is *Myxobolus cerebralis* is supported by the fact that the multicellular developmental stages of this myxosporean have only been detected in the spinal cord and brain (Rodger et al. 1995), a distribution that is consistent with the migratory route of developmental stages of *M. cerebralis* in rainbow trout (El-Matbouli et al. 1995). However, neither spores nor infection of cartilage by developmental stages have been detected in infected smolts (Rodger et al. 1995, Frasca et al. 1998). Smolts with high-intensity parasite infection may die before cartilage infection and sporogony, as they have been reported to have the most severe pathology (Rodger et al. 1995). The fate of developmental stages in low-intensity infections of smolts has yet to be determined, although smolts can tolerate low-intensity infections without showing neurologic signs (Frasca et al. 1998). The absence of spores and cartilage involvement in infected smolts may also be attributable to normal growth and skeletal ossification of smolts, with replacement of target cartilage by bone thus preventing sporogony.

Implications of molecular characterization

The results of this study indicate that well-established molecular techniques can be successfully applied to formalin-fixed paraffin-embedded salmonid tissues and can generate useful sequence data from proliferative stages of myxosporeans, which may otherwise be difficult to taxonomically characterize based solely on their morphologic features. The sequence data from this myxosporean will be integral to the generation of probes for its detection in salmonid tissues and for the identification of possible alternate hosts.

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