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NOTE

Specific PCR for *Myxobolus arcticus* SSU rDNA in juvenile sockeye salmon *Oncorhynchus nerka* from British Columbia, Canada

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ABSTRACT: A PCR for the specific detection of the salmon brain parasite *Myxobolus arcticus* (Pugachev and Khokhlov, 1979) was developed using primers designed to amplify a 1363 base pair fragment of the small subunit rDNA. The assay did not amplify DNA from 5 other *Myxobolus* species or from 7 other myxozoan species belonging to 5 other genera. For juvenile sockeye salmon *Oncorhynchus nerka* (Walbaum) collected from Chilko Lake, British Columbia (BC), Canada, in 2011, the prevalence by PCR was 96%, in contrast to 71% by histological examination of brain tissue. In 2010, the histological prevalence was 52.5%. Sequence identity between *M. arcticus* from Chilko Lake and other sites in BC ranged from 99.7 to 99.8% and was 99.6% for a Japanese sequence. In contrast, an *M. arcticus* sequence from Norway shared 95.3% identity with the Chilko Lake sequence, suggesting misidentification of the parasite. Chilko Lake sockeye salmon were previously reported free of infection with *M. arcticus*, and more research is required to understand the processes involved in the local and global dispersion of this parasite.

KEY WORDS: Myxozoan · Diagnostics · Salmonid · Chilko Lake · Small subunit

INTRODUCTION

Myxobolus arcticus is a myxozoan parasite of the central nervous system in salmonids from the North Pacific Ocean and Bering Sea (Urawa et al. 2011). It has been identified in sockeye salmon *Oncorhynchus nerka* in coastal British Columbia (BC), Canada (Rutherford et al. 1992, Kent et al. 1993, Yokoyama et al. 2006, Urawa et al. 2011), and in masu salmon *O. masou* in Japan (Awakura et al. 1995). Histological data suggest that *M. arcticus* also occurs in Chinook salmon *O. tshawytscha* (Urawa et al. 1998).

Infection with *M. arcticus* causes limited pathogenicity and physiological effects on its host, as assessed by growth and tissue chloride levels (Moles & Heifetz 1998). Moles & Heifetz (1998) found that swimming speed was reduced among infected salmon swum to exhaustion relative to uninfected fish. The parasite is acquired by exposure of juvenile salmon to actinospores while in fresh water, and infections persist in adult salmon (Margolis 1982, Quinn et al. 1987, Rutherford et al. 1992, Kent et al. 1993).

Historically, *M. arcticus* was used as a biological tag for anadromous salmon stocks in BC (Margolis 1982, Bailey et al. 1988, Moser 1991, Rutherford et al. 1992, Moles & Heifetz 1998). Diagnosis was based on histological analysis, and the prevalence of infection among watersheds ranged from low (0 to 3.0%) to high (79 to 100%; Quinn et al. 1987). Histology, while precise, is limited by low sensitivity, low specificity (sectioned tissue must contain intact pathogens at an identifiable stage of development) and can be labour

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and time-intensive. Furthermore, *M. arcticus* may exhibit variation in myxospore morphology that is dependent on the host species (Nagasawa et al. 1994, Urawa et al. 2009). Urawa et al. (2009) found morphological differences in the widths of myxospores sourced from Arctic char *Salvelinus alpinus* and white-spotted char *S. leucomaenis* when compared to myxospores from Pacific salmon (*O. keta* and *O. nerka*). In contrast, molecular detection methods are specific and sensitive, leading to more accurate diagnoses, independent of spore morphology. Moreover, at least 6 species of *Myxobolus* have been reported from the salmonid central nervous system, emphasising the importance of a species-specific PCR assay.

Previous efforts to develop molecular tools for the detection of *M. arcticus* have yielded relatively non-specific primers which amplified within the small subunit ribosomal DNA (SSU-rDNA) region of several *Myxobolus* species (Urawa et al. 2009). In contrast, the rDNA internal transcribed spacer (ITS) regions typically contain regions of higher intra-species sequence variability (Hillis & Dixon 1991, Cunningham 1997), making them less valuable targets for diagnostics. The purpose of the present study was to develop PCR primers that specifically targeted SSU-rDNA of *M. arcticus* from sockeye salmon in Chilko Lake, BC.

MATERIALS AND METHODS

Fish

In May of 2010 and 2011, juvenile sockeye salmon were randomly collected from smolt fences on the Chilko River near the outlet of Chilko Lake (51.3° N, 124.1° W). Fish caught in 2010 had a mean (±SD) weight of 8.23 ± 4.58 g (N = 40; range: 2.47–17.97 g) and a fork length of 99 ± 18 mm (range: 67–131 mm). For histological analysis, fish caught in 2011 had a weight of 7.18 ± 4.85 g (N = 40; range: 3.53–19.19 g) and a fork length of 96 ± 19 mm (range: 79–137 mm). For molecular analysis, 25 additional fish randomly sampled in 2011 had a weight of 7.85 ± 4.5 g (range: 3.15–18.75 g) and a fork length of 100 ± 18 mm (range: 79–134 mm).

Histological assessment of fish

Brain samples from sockeye salmon collected in 2010 and 2011 were preserved in 10% neutral buffered formalin. Fixed tissues were processed for routine histological examination of haematoxylin and eosin-stained 5 µm sections. Histological preparations from 40 specimens per year were examined and photographed using a Leica DM 2500 light microscope, a PL-B686 digital camera and µScope software (Pixelink).

DNA sequencing and *Myxobolus arcticus*-specific PCR primer design

Twenty-five whole brains from 2011 were preserved in 95% EtOH. DNA from all brain samples was extracted using DNeasy Tissue kits (Qiagen) as per the manufacturer's instructions. Using a minimum of 5 brain samples from 2011, overlapping segments of *M. arcticus* SSU-rDNA were amplified using the primer pairs Myxgen4F & ERIB10, 18E & ACT1r, and ACT3f & MYX4r (Hillis & Dixon 1991, Barta et al. 1997, Kent et al. 2000, Hallett & Diamant 2001). PCR products were amplified in 20 µl reactions containing 1× Platinum *Taq* buffer (Invitrogen), 0.2 µM dNTPs, 0.2 µM of each forward and reverse primers, 0.5 U of Platinum *Taq* and 2 µl of template DNA. Reactions were subjected to 95°C for 3 min followed by 40 cycles of 45 s at 95°C, 45 s at 55°C (for all primer combinations) and 1 min at 72°C; with a final extension at 72°C for 7 min. PCR products were visualized on 1.5% agarose gels stained with SYBR Safe (Invitrogen). Samples were purified with ExoSAP-IT (USB) prior to sequencing in 10 µl reactions containing 1 µl of purified PCR product, 1× Big Dye Terminator mix (Applied Biosystems), 1× Big Dye Terminator buffer (Applied Biosystems) and 0.5 µl of a 3.2 pmol sequencing primer solution. PCR primers described above were used as sequencing primers. Sequencing reactions were purified using the Dye EX 2.0 Spin kit (Qiagen) and run on an ABI 3130xl genetic analyser. Sequences were viewed and edited using Sequencher 5.1 (Gene Codes). Segments from each PCR product were assembled into a full-length SSU-rDNA contig for *M. arcticus*-specific primer design.

The SSU-rDNA contig was aligned with sequences from other species of *Myxobolus* obtained from GenBank (*M. squamalis,* U96495; *M. fryeri,* EU346372; *Myxobolus* sp. KAB2001A, AF378342; *Myxobolus* sp. ASH, AM042702; *M. neurotropus*, DQ846661; *M. insidiosus,* EU346376; *M. murakami*, AB469984 and AB469985; *M. kisutchi*, AB469988) using ClustalW2 with a gap penalty of 10 and gap extension penalty of 0.1. Five regions of high nucleotide divergence were

identified among the aligned sequences. Forward and reverse primers were designed from these regions using Oligo7 software (Molecular Biology Insights Version 7.57). Primers MarctF1 (5′-AGT GGT TAA CGC TTC TGA TGT-3′) and MarctR2 (5′-CCA ACC TCA CAA TCA ACG ACA-3′) were selected based on low annealing temperature (T_m) differences and on the length of the expected PCR product. PCR conditions were as described above with the exception of a T_m of 53°C.

PCR specificity and screening of sockeye salmon

The primer pairs MarctF1 & MarctR2, Myxgen4F & ERIB10, 18E & ACT1r, and ACT3f & MYX4r were screened against a panel of myxozoan species obtained from infected salmon tissues (t) or spore preparations (p): *M. fryeri* and *M. kisutchi* (mixed infection, p), *M. insidiosus* (p), *M. cerebralis* (p), *Henneguya zschokkei* and *H. salminicola* (mixed infection, p), *Chloromyxum thymalli* and *C. fluviatile* (mixed infection, p), *Kudoa thyrsites* (t), *Parvicapsula minibicornis* (t), *M. squamalis* (t) and *Ceratonova shasta* (t). Each sample contained a minimum of 2.3 ng DNA μl^{-1}. Generic SSU rDNA primers 18E and 18G (Hillis & Dixon 1991) were used to confirm DNA quality. PCR screening of the 25 sockeye brain samples from Chilko Lake was performed as described above and products were visualized on a 1.5% agarose gel. Sequence alignments and percent identity matrices were constructed in CLUSTAL OMEGA (European Bioinformatics Institute) (see Table 2).

RESULTS AND DISCUSSION

A 1900 bp *Myxobolus arcticus* SSU-rDNA contig (GenBank accession number KM870563) served as a template from which a 1363 bp product was amplified by the PCR primers MarctF1 and MarctR2 (Table 1). These primers failed to amplify DNA from 5 other species of *Myxobolus* and from 7 species of myxozoans belonging to 5 genera. *M. murakamii* and *M. neurotropus* were not available for testing. *M. neurotropus* SSU-rDNA (DQ846661) showed the greatest sequence identity in the primer-binding regions, and it is unknown whether our primers would amplify a product from this species. The 3 other primer pairs tested each amplified SSU-rDNA from multiple species of Myxozoa (Table 1). Thirteen other primer pairs tested were not included in this analysis because their products were either too small to sequence or yielded non-specific products (Appendix Table A1).

In 2010, 21 of 40 Chilko Lake samples (52.5%; 95% confidence interval [CI] = 37%, 68%) were histologically positive for brain myxozoans and in 2011, 9 of 40 samples were unsuitable for analysis, and 22 of the remaining 31 (71.0%; CI = 55%, 87%) were positive (Fig. 1). By PCR, a product was amplified from 24 of 25 (96%; CI = 88%, 100%) salmon in 2011 and BLAST analysis of resulting sequences confirmed these to be *M. arcticus* by comparison with sequences in GenBank (HQ113227 and EU346378). The 2011 data suggest that our PCR assay was more sensitive than histology in the detection of *M. arcticus*.

Analysis of the Chilko Lake *M. arcticus* small SSU-rDNA sequence revealed high identity to geographically diverse *M. arcticus* isolates (Table 2). We found >99.7% similarity to 2 sequences from Sproat Lake, Vancouver Island, BC: 1 from 1998 (EU346378) and 1 from 2009 (HQ113227) (Ferguson et al. 2008, Urawa et al. 2011). We found 99.6% identity to an *M. arcticus* isolate from Japan (AB353128). Differences among these sequences are restricted to a small number of locations. At position 689 (relative to GenBank accession numbers HQ113227 and AB353128), the 2 other BC sequences and the Japanese sequence have an 'S' (cytosine or guanine), whereas the Chilko Lake sequence has a 'C' at that location. At position 179, a 'C' occurs in all BC sequences and a

Table 1. Comparison of amplification profiles from other myxozoan species with primer pairs able to detect *Myxobolus arcticus*. Expected DNA product amplification is positive (+) or negative (–)

Species DNA	Primer pair			
	Myxgen4F & ERIB10	ACT3F & MYX4R	18E & ACT1R	MarctF1 & MarctR2
Kudoa thyrsites	–	–	+	–
Parvicapsula minibicornis	+	–	+	–
Henneguya zschokkei & *H. salminicola*	+	–	+	–
Chloromyxum thymalli & *C. fluviatile*	+	+	+	–
Myxobolus cerebralis	+	+	+	–
Myxobolus kisutchi & *M. freyeri*	+	+	+	–
Myxobolus insidious	+	+	+	–
Myxobolus squamalis	+	+	+	–
Ceratonova shasta	–	–	+	–
Myxobolus arcticus	+	+	+	+

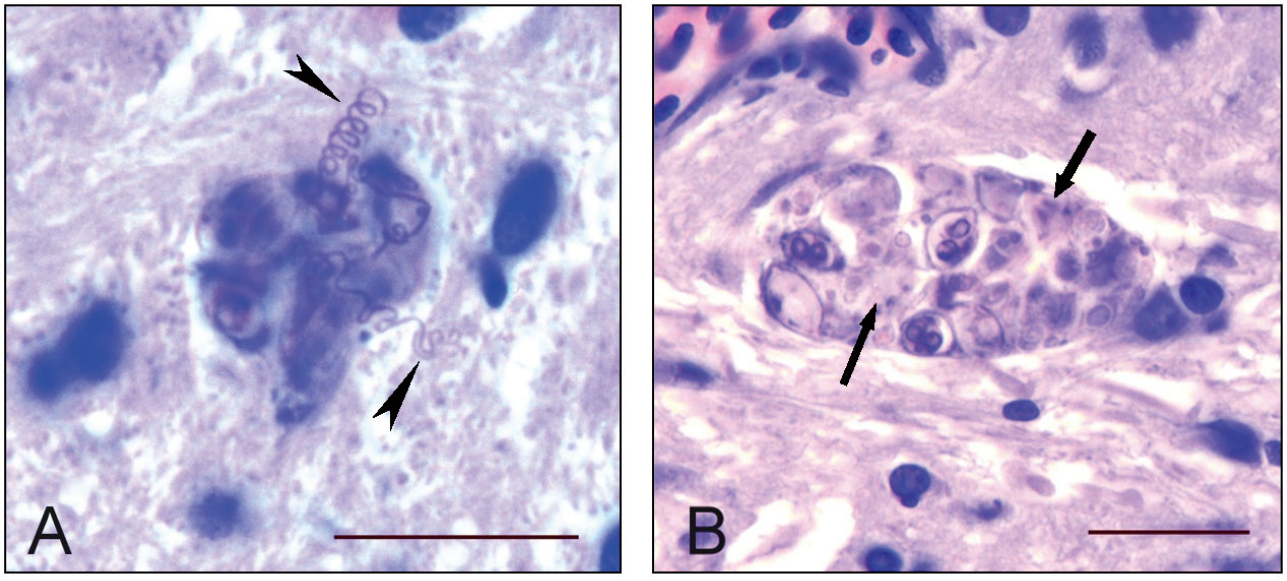

Fig. 1. Micrographs of histological sections of sockeye salmon *Oncorhynchus nerka* brain infected with *Myxobolus arcticus*. (A) Extrusion of polar filaments (arrowheads) may have been triggered during tissue sampling or fixation. (B) Plasmodium contains spores and presporogonic stages (arrows). Haematoxylin and eosin. Scale bars = 20 µm

'T' in the Japanese sequence, as noted by others but without reporting the base location (Urawa et al. 2011). At positions 910 and 1682, differences occur between all BC sequences and the Japanese sequence. The high identity among *M. arcticus* sequences is consistent with intraspecific sequence differences of 98.9% documented for *Parvicapsula minibicornis* SSU-rDNA sequences among 197 samples (Atkinson et al. 2011). Similarly, no variation was detected among SSU-rDNA sequences from 14 *Ceratonova shasta* samples obtained from salmon in the Klamath River (Atkinson & Bartholomew 2010). Identity between the Chilko Lake *M. arcticus* sequence and those from 5 other species ranged from 88 to 96% (Table 2). Falling into this range at 95.3% was a 1745 bp sequence, identified as *M. arcticus*, from a river in northern Norway (AB469992). Comparison of these 2 sequences revealed 4 distinct regions which contained 89.9% (62 of 69) of the differences. We conclude that the Norwegian sequence AB469992 is misidentified as *M. arcticus*. There is no obvious explanation for the high sequence similarities observed among sequences from Chilko Lake, other locations in BC and from Japan. The transmission biology of *M. arcticus* suggests that genetic homogenization of parasite populations will only occur in fresh water.

Table 2. Percent similarity matrix among small subunit ribosomal DNA sequences from 11 *Myxobolus* isolates. BC: British Columbia, Canada

Location	GenBank accession	Name	Table ID	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Chilko Lake, BC	KM870563	*M. arcticus*	(1)	100										
Norway, south	AB469987	*M. neurobius*	(2)	94.6	100									
Norway, north	AB469992	*M. arcticus*	(3)	95.3	93.6	100								
BC	EU346378	*M. arcticus*	(4)	99.8	95.2	94.7	100							
Japan	AB353128	*M. arcticus*	(5)	99.6	95.4	94.4	99.8	100						
Sproat Lake, BC	HQ113227	*M. arcticus*	(6)	99.7	95.5	94.5	100	99.8	100					
Idaho, USA	DQ846661	*M. neurotropus*	(7)	96.5	94.6	92.7	95.1	95.8	95.9	100				
Washington, USA	AB469988	*M. kisutch*	(8)	96.5	94.9	93.8	96.5	96.6	96.8	95.7	100			
California, USA	MSU96495	*M. squamalis*	(9)	88.1	88.1	86.1	91.6	88.7	88.8	88.6	88.9	100		
Oregon, USA	EU346376	*M. insidiosus*	(10)	92.5	92.1	90.8	91.8	92.6	92.7	91.8	92.9	95.8	100	
Oregon, USA	EU346372	*M. fryeri*	(11)	92.5	92.0	90.7	91.6	92.5	92.6	92.0	92.8	95.2	99.5	100

However, the homing fidelity of spawning sockeye salmon is not conducive to mixing among spawning populations and their parasites in fresh water.

M. arcticus is widely distributed among sockeye salmon populations in the North Pacific Ocean. In Alaska, USA, the prevalence ranges from 10 to 90% (Moles & Heifetz 1998, Moles & Jensen 2000). Earlier reports from BC misidentified myxobolids in sockeye salmon brain as *M. neurobius* (McDonald & Margolis 1995). *M. arcticus* (as *M. neurobius*) was found in juvenile salmon from 3 northern lakes within the Fraser River watershed: Quesnel Lake (62% prevalence), Bowron Lake (66% prevalence) and Francois Lake (2% prevalence) by microscopic examination of histological slides or pepsin digests (Bailey & Margolis 1987). Between 1966 and 1985, those authors were unable to detect the parasite in several other Fraser River watersheds including Chilko Lake, Weaver Creek, Pitt River, Adams River, Horsefly River and Stellako River. Our finding of *M. arcticus* in a high percentage of Chilko Lake sockeye salmon suggests either that the methods used earlier lacked sufficient sensitivity to detect the infections or that the parasite was introduced to Chilko Lake within the last 25 yr via an unknown process. More thorough surveys are required to evaluate the complete host range of this parasite. In particular, detection of *M. arcticus* infections in Chilko Lake suggests that a more comprehensive survey of juvenile sockeye salmon is required to shed light on the extent of infections throughout the Fraser River watershed.

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Appendix

Table A1. Seventeen oligonucleotide PCR primer combinations assessed for amplification of small subunit ribosomal DNA from *Myxobolus arcticus* and other myxozoan parasites (see 'Results and Discussion'). Of these, 13 combinations did not generate a sufficiently large or specific product, and were not included for further analysis

Forward primer	Reference	Paired with reverse primer(s)	Reference
Used in further analyses			
18e	Hillis & Dixon (1991)	ACT1r	Hallett & Diamant (2001)
Myxgen4f	Kent et al. (2000)	ERIB10	Barta et al. (1997)
ACT3f	Hallett & Diamant (2001)	MYX4r	Hallett & Diamant (2001)
MarctF1	Present study	MarctR2	Present study
Not used in further analyses			
18e	Hillis & Dixon (1991)	Mb1000rev	Urawa et al. (2009)
ERIB1	Barta et al. (1997)	ACT1r	Hallett & Diamant (2001)
MX5	Andree et al. (1998)	MX3	Andree et al. (1998)
Myxgen1f	Kent et al. (1998)	Mb1000rev	Urawa et al. (2009)
Myxgp2f	Kent et al. (1998)	ACT1r	Hallett & Diamant (2001)
LIN3F	Lin et al. (1999)	10LINR	Lin et al. (1999)
MYX1f	Hallett & Diamant (2001)	MX3	Andree et al. (1998)
		MYX4r	Hallett & Diamant (2001)
		ACT1r	Hallett & Diamant (2001)
ACT3f	Hallett & Diamant (2001)	MX3	Andree et al. (1998)
Mb1000fwd	Urawa et al. (2009)	18g′	Hillis & Dixon (1991)
		MX3	Andree et al. (1998)
		18gM	Freeman et al. (2008)