



Geographical variation in spore morphology, gene sequences, and host specificity of *Myxobolus arcticus* (Myxozoa) infecting salmonid nerve tissues

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ABSTRACT: *Myxobolus arcticus* Pugachev and Khokhlov, 1979 is a freshwater myxosporean parasite infecting the nerve tissues of salmonid fishes throughout the Pacific region of Far East Asia and North America. The principal fish host is sockeye salmon *Oncorhynchus nerka* in North America and masu salmon *O. masou* in Japan. Actinospores of *M. arcticus* were isolated from the lumbricolid oligochaetes *Lumbriculus variegatus* and *Stylodrilus heringianus* in Japan and Canada, respectively. Morphological comparisons indicated that Japanese actinospores from *L. variegatus* have significantly shorter caudal projections than Canadian isolates from *S. heringianus*, whereas the corresponding myxospores are indistinguishable. Transmission experiments showed that sockeye salmon were rarely susceptible to the Japanese actinospores, while masu salmon are highly susceptible to this parasite. Sequences of 4560 base pairs of the ribosomal RNA (rRNA) gene, including small subunit (SSU) and internal transcribed spacer (ITS) regions, from Japanese and Canadian isolates had a high similarity over 99.9%, suggesting that they may be conspecific. However, the biological data indicate that they are at least distinct strains. *M. arcticus* may be geographically isolated due to the specific homing migration of the anadromous fish hosts and has specialized its morphology and host selection for its local environment in the ongoing process of differentiation, potentially leading to speciation.

KEY WORDS: *Myxobolus arcticus* · Actinospore · Morphology · SSU rRNA · ITS rRNA · Susceptibility · Geographical variation · Salmonid

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INTRODUCTION

Myxosporean parasites of fish with known life cycles, approximately 4 % of species, have an alternate actinospore phase in annelids to fulfill the characteristic biphasic life cycles (Kent et al. 2001, Yokoyama 2003, Canning & Okamura 2004). An exception of this is the marine myxosporean *Enteromyxum leei*, which can transmit directly among fish hosts (Diamant 1997). Thus, the taxonomy of myxosporeans

should, when possible, also utilize the morphology of the actinospore stage as well as that of the corresponding myxospores that are traditionally used alone. Lom & Dyková (2006) suggested that the specific identification of myxozoans also requires additional information such as molecular data, host and tissue tropisms, and geographical distribution.

Myxobolus arcticus Pugachev and Khokhlov, 1979 is a freshwater myxosporean infecting the central nerve tissues of salmonid fishes in the North Pacific

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coasts of Far East Asia and North America. The principal fish host is masu salmon *Oncorhynchus masou* in Japan (Awakura et al. 1995) and sockeye salmon *O. nerka* in Canada and Alaska (Bailey & Margolis 1987, McDonald & Margolis 1995, Moles & Jensen 2000). The alternate invertebrate hosts are the aquatic oligochaetes *Lumbriculus variegatus* in Japan (Urawa & Awakura 1994, Yokoyama & Urawa 1997) and *Stylodrilus heringianus* in Canada (Kent et al. 1993), both classified in the family Lumbriculidae. *M. arcticus* has been used as a biological tag to identify the stock origins of salmon in open seas (Moles et al. 1990, Margolis 1998, Urawa et al. 1998) and has been associated with reduced swimming performance in host fish (Moles & Heifetz 1998).

Various regions of the ribosomal RNA (rRNA) gene (small subunit [SSU], internal transcribed spacer [ITS] regions, and large subunit [LSU] are being increasingly utilized to study both inter- and intra-specific relationships of myxozoans (Fiala 2006, Whipps & Kent 2006, Bartošová et al. 2009). Recently, variations of myxosporean spore morphology and SSU rDNA gene sequence were compared among *Myxobolus* species infecting salmonid nerve tissues (Urawa et al. 2009). The purpose of the present study was to determine the geographical variation in spore morphology and fish host susceptibility to the actinospore stage of *M. arcticus* in Japan and Canada. In addition, rRNA gene sequence data were used to demonstrate the genetic similarities between geographically distinct isolates of *M. arcticus*.

MATERIALS AND METHODS

Spore collection

Japanese actinospores of *Myxobolus arcticus* were collected from *Lumbriculus variegatus* from the Chitose River in Hokkaido (42° 48' 29" N, 141° 34' 09" E). Canadian actinospores were collected from *Stylodrilus heringianus* from Sproat Lake on southwest Vancouver Island, British Columbia (49° 17' 25" N, 124° 55' 10" W). Collection of actinospores was in accordance with Yokoyama et al. (1991). Briefly, oligochaetes were individually placed in 48-well plates and kept at 8 to 9°C overnight. Actinospores released from the oligochaetes within 24 h were collected and used as described below. Mature myxospores of *M. arcticus* were collected from the medulla oblongata and spinal cord of masu salmon in the Chitose River and sockeye salmon in Sproat Lake.

Spore observation

Actinospores and myxospores (n = 30 to 50 each) were fixed with 10% neutral buffered formalin and measured with an ocular micrometer under a compound microscope. Terminology used in the spore descriptions follows the guidelines of Lom & Arthur (1989), Lom et al. (1997), and Lom & Dyková (2006). The difference in measurements between Canadian and Japanese spores was tested by using a Mann-Whitney *U*-test, and $p < 0.05$ was set as the level of significance.

Molecular analyses

Myxospores and actinospores of Japanese and Canadian *Myxobolus arcticus* were collected as described above and stored frozen until required. All samples were taken in duplicate, from 2 fish and 2 oligochaetes, and all PCR reactions were performed in triplicate with DNA sequencing performed in both directions. DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) following the manufacturer's tissue protocol and used as template DNA for PCR reactions. Small subunit ribosomal DNA (SSU rDNA) of the myxosporeans was amplified using a combination of previously described universal and specific primers (Freeman et al. 2008, Urawa et al. 2009). Partial LSU sequences were amplified from extracted actinospores, to minimize host DNA interference, using the primers NLF-184, NLR-1270, and NLR-1694 and PCR conditions described by van der Auwera et al. (1994) and Bartošová et al. (2009). From the initial LSU sequences obtained, a specific LSU reverse primer, Ma28-320r 5' TAC CAC CCA CTT CAG ACT GC 3', was used in combination with the semi-specific (binds to other nerve/cartilage-infecting *Myxobolus* spp.) SSU forward primer 18-1830f 5' ACA GGT CTG TGA TGC CCT AAG 3' to enable the amplification of the ITS regions. PCR conditions were 95°C for 4 min followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1 min, with a final extension of 72°C for 7 min. In addition, 2 further specific forward LSU primers (Ma28-310f 5' TGG GAA TGC AGT CTG AAG TG 3' and Ma28-1330f 5' ACT GCA AAC CGG TGA TAA CG 3') were used in combination with the reverse primers NLR-1270 and NLR-1694 to confirm and extend the initial LSU sequence reads.

The PCR products obtained were purified using a PCR purification kit (Qiagen), and the resulting DNA was used in direct sequencing reactions using BigDyeTM Terminator v3.1 chemistry and a 310 capil-

lary DNA sequencer (Applied Biosystems). Contiguous sequences were constructed manually and compared to related *Myxobolus* spp. infecting salmonid nervous tissues, including isolates of *M. arcticus* from Canada (EU346378) and Japan (AB353130) using ClustalX (Thompson et al. 1997) and edited using the BioEdit sequence alignment editor (Hall 1999). Percentage divergence matrices were constructed in ClustalX using the neighbor-joining method (Saitou & Nei 1987). Phylogenetic analyses were performed using maximum parsimony in PAUP*4.0 beta10 using default settings and 1000 bootstrap replicates (Swofford 2002).

***In vitro* assay of reaction of actinospores to fish mucus**

Skin mucus was collected from 6 species of healthy salmonid fish (masu salmon, sockeye salmon, chum salmon *Oncorhynchus keta*, pink salmon *O. gorbuscha*, rainbow trout *O. mykiss*, and brown trout *Salmo trutta*), and smeared onto a clean glass slide. Actinospore suspensions of Japanese *Myxobolus arcticus* were immediately added to the mucus and examined for release of sporoplasms by phase-contrast microscopy (Yokoyama et al. 1993). The reactivity of actinospores to fish mucus was determined on the basis of the sporoplasm release from at least 150 spores. An actinospore suspension without the addition of fish mucus served as a negative control.

Transmission experiments

To test the susceptibility of masu salmon to Japanese actinospores of *Myxobolus arcticus*, juvenile masu salmon were exposed to actinospores collected from *Lumbriculus variegatus* in the Chitose River for 1 h at concentrations of 0, 10, 100, 200, 500, and 1000 spores fish⁻¹. Juvenile chum, sockeye, pink, and amago salmon *Oncorhynchus masou ishikawae*, rainbow trout, and brown trout were also exposed to actinospores at concentrations of 200 to 1000 spores fish⁻¹ for 1 h. These fish and unexposed control fish were transferred to 15 l tanks, and reared with running well water at 10.5°C. After 4 mo, the medulla oblongata was collected from each fish and smeared on a glass slide. These slides with cover slips were examined to confirm the presence of *M. arcticus* spores under a microscope at a magnification of 200×.

RESULTS

Morphological comparison

Morphological characteristics of actinospores of Japanese *Myxobolus arcticus* were similar to those of Canadian *M. arcticus* in having an anchor-like tri-actinomyxon form with protruding tips of polar capsules and plasmodial sporoplasm. Japanese actinospores had significantly shorter caudal processes than Canadian ones, while there were no other considerable differences in morphometrics or in the number of sporozoites within the sporoplasm between the 2 actinospore isolates (Table 1, Fig. 1). The myxospores of the isolates from Canada and Japan were pyriform in valvular view and morphologically indistinguishable (Table 1).

Molecular comparison

SSU rDNA sequence data of 2025 nucleotide bases for each isolate determined in the present study

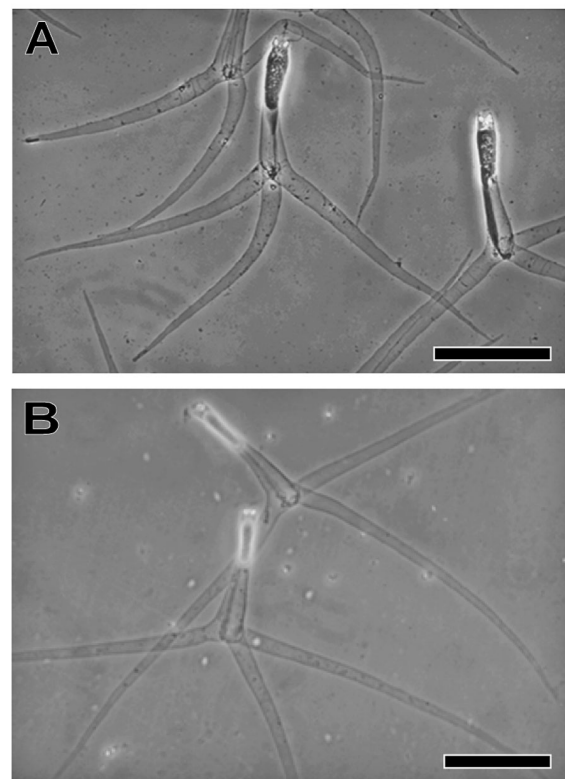


Fig. 1. *Myxobolus arcticus*. (A) Japanese actinospores released from *Lumbriculus variegatus* in the Chitose River, Hokkaido, and (B) Canadian actinospores from *Stylogdrilus heringianus* in Sproat Lake on Vancouver Island, British Columbia. Scale bars = 0.1 mm

Table 1. *Myxobolus arcticus*. Morphological comparison of myxospores and actinospores from Japan (Chitose River, Hokkaido) and Canada (Sproat Lake, British Columbia). Mean length and width are shown in μm (range in parentheses). NS: not significant ($p > 0.05$)

Location	Myxospores				Actinospores				No. of sporozoites						
	Fish host	Spore Length	Spore Width	Polar capsule Length	Polar capsule Width	Oligochaete host	Style Length	Style Width		Caudal process Length	Caudal process Width	Spore body Length	Spore body Width		
Chitose River, Hokkaido, Japan	Masu salmon	14.1 (13.3–15.6)	8.7 (7.8–9.4)	8.4 (7.5–9.0)	3.1 (2.6–3.5)	30	<i>Lumbriculus variegatus</i> (Lumbriculidae)	86 (72–97)	19 (11–22)	229 (193–254)	13 (11–16)	40 (35–48)	10 (8–11)	62	30
Sproat Lake, BC, Canada	Sockeye salmon	14.2 (12.9–15.6)	8.6 (7.8–9.8)	8.1 (7.3–9.2)	3.4 (2.5–4.0)	50	<i>Stylodrilus heringianus</i> (Lumbriculidae)	95 (66–117)	20 (16–23)	323 (241–372)	14 (12–18)	40 (29–52)	11 (10–14)	62	30
Significance		NS	NS	NS	NS	NS	NS	NS	NS	$p < 0.01$	NS	NS	NS	NS	NS

were deposited in GenBank: actinospores of Japanese *Myxobolus arcticus* (accession number AB353128) from *Lumbriculus variegatus* collected in the Chitose River; actinospores of Canadian *M. arcticus* (HQ-113228) from *Stylodrilus heringianus* collected in Sproat Lake; and myxospores of Canadian *M. arcticus* (HQ113227) from sockeye salmon collected in Sproat Lake.

Partial SSU, complete ITS1, 5.8S, and ITS2, and partial LSU regions of the rRNA gene were successfully amplified and sequenced for actinospores and infected oligochaetes from both Japan and Canada, respectively. Sequences of 2754 bp were generated for both isolates of *Myxobolus arcticus* and submitted to GenBank: actinospores of Japanese *M. arcticus* (JN003830) from *Lumbriculus variegatus* collected in the Chitose River and actinospores of Canadian *M. arcticus* (JN003829) from *Stylodrilus heringianus* collected in Sproat Lake.

The combined complete SSU, ITS1, 5.8S, ITS2, and partial LSU of the rRNA gene gave a total sequence read of 4560 bp from both Japanese and Canadian isolates of *Myxobolus arcticus*.

SSU rDNA

The sequence from actinospores of Japanese *Myxobolus arcticus* (AB353128) is identical to the corresponding myxospore sequence from masu salmon (AB353130, Urawa et al. 2009). Likewise, the sequence data from Canadian actinospores (HQ-113228) and myxospores (HQ113227) are identical. A single nucleotide base change is the only difference between the Japanese and Canadian isolates examined in this study, which gives an overall percentage similarity over 99.9%, demonstrating a very high gene similarity (Table 2). Furthermore, another isolate of *M. arcticus* (EU346378, Ferguson et al. 2008) from British Columbia, Canada, has 100% identity to both the Japanese and Canadian isolates obtained in this study (Table 2), albeit over a shorter (1130 bp) and more conserved part of the gene. In the phylogenetic analysis, all Japanese and Canadian isolates of *M. arcticus* form a robustly supported clade (Fig. 2).

ITS regions and LSU rDNA

The sequence from Japanese actinospores (JN003830) is over 99.9% similar to the sequence obtained from infected Canadian oligochaetes (JN003829). There are

Table 2. *Myxobolus* spp. Percentage identities (above diagonal) of small subunit rDNA of selected species infecting salmonid neural tissues with the number of comparable bases used in the analyses (below diagonal)

Species (host & location) accession no.	1	2	3	4	5	6	7	8	9	10
1 <i>M. arcticus</i> (<i>Lumbriculus variegatus</i> , Chitose River, Japan) ^a AB353128		100	100	99.95	99.95	97.05	97.01	95.78	93.85	93.54
2 <i>M. arcticus</i> (<i>Oncorhynchus masou</i> , Chitose River, Japan) AB353130	1970		100	99.95	99.95	97.05	97.01	95.79	93.80	93.55
3 <i>M. arcticus</i> (<i>O. nerka</i> , British Columbia, Canada) EU346378	1128	1130		100	100	96.81	97.26	95.4	93.88	93.62
4 <i>M. arcticus</i> (<i>O. nerka</i> , Sproat Lake, Canada) HQ113227	1970	1972	1130		100	97.10	97.06	95.84	93.85	93.60
5 <i>M. arcticus</i> (<i>Stylodrilus herringianus</i> , Sproat Lake, Canada) ^a HQ113228	1970	1972	1130	1972		97.10	97.06	95.84	93.85	93.60
6 <i>M. kisutchi</i> (<i>O. kisutch</i> , Washington, USA) AB469988	1966	1968	1128	1968	1968		97.00	95.53	93.59	93.65
7 <i>M. neurotropus</i> (<i>O. mykiss</i> , Idaho, USA) DQ846661	1970	1972	1130	1972	1972	1969		95.84	93.96	93.96
8 <i>M. neurobius</i> (<i>Salmo trutta</i> , Bergen, Norway) AB469987	1969	1971	1130	1971	1971	1968	1972		93.34	93.39
9 <i>M. murakamii</i> (<i>O. masou ishikawae</i> , Hiroshima, Japan) AB469984	1966	1968	1128	1968	1968	1967	1969	1968		97.21
10 <i>M. fryeri</i> (<i>O. kisutch</i> , Oregon, USA) EU346370	1966	1968	1128	1968	1968	1967	1969	1968	1971	

^aGene sequence obtained from the oligochaete host

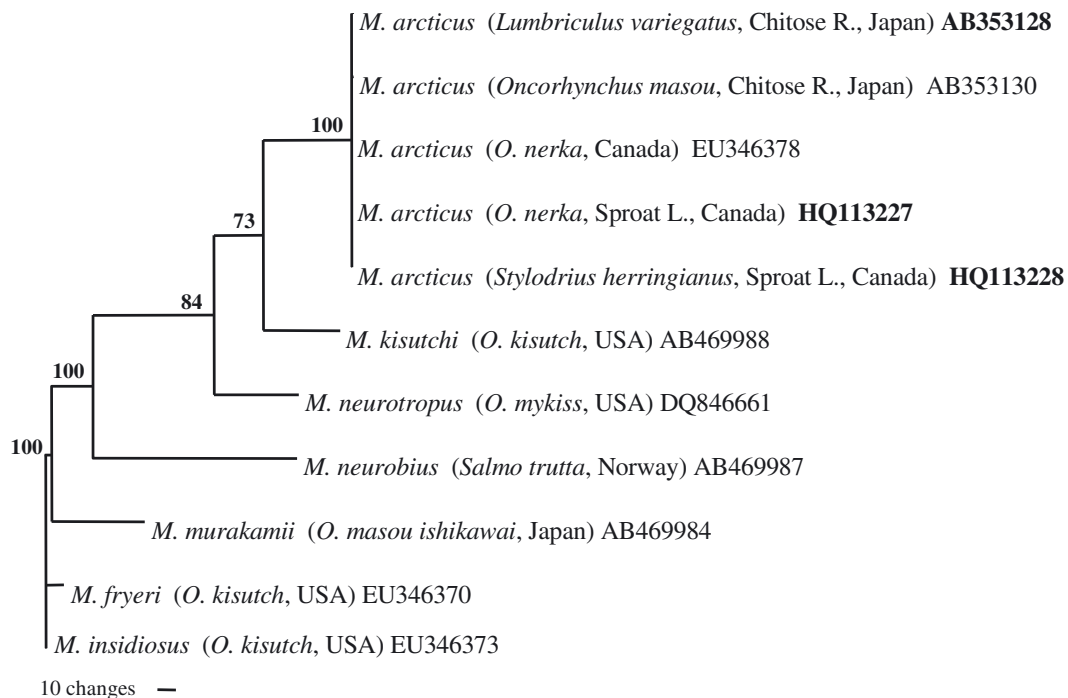
Fig. 2. *Myxobolus* spp. Maximum parsimony phylogram based on small subunit rDNA sequences of *Myxobolus* spp. infecting oligochaetes and salmonid nervous tissues. Host, location and GenBank accession numbers also shown. Bootstrap values shown at the nodes are based on 1000 resamplings. The tree is rooted with *M. insidiosus*, a related myxosporean infecting salmonid muscle. The number of nucleotide changes is represented by the scale bar. GenBank accession numbers obtained in the present study are shown in **bold**

Table 3. *Myxobolus arcticus*. Reactivity of Japanese actinospores to the skin mucus of salmonids

Fish	No. actinospores releasing sporoplasms/ no. examined	Reactivity (%)
Masu salmon	89/153	58.2
Chum salmon	55/187	29.4
Pink salmon	160/211	75.8
Sockeye salmon	94/189	49.7
Rainbow trout	62/154	40.3
Brown trout	57/177	32.2
Control	7/187	3.7

no gaps present between the 2 sequences and just 2 nucleotide base changes over the entire region of 2754 bp, which includes both ITS regions.

Response of actinospores to skin mucus from different fish species

Sporoplasm release from *Myxobolus arcticus* actinospores occurred by exposure to skin mucus from all salmonids examined in this study (Table 3). The proportion of spores that were released varied widely among fish species (29.4–75.8%) and was significantly higher when compared to unexposed controls (3.7%).

Host susceptibility

Masu and amago salmon were found to be highly susceptible to actinospores of *Myxobolus arcticus* from *Lumbriculus variegatus* in Japan (Table 4). All masu salmon fry were infected with *M. arcticus* myxospores 3 mo after exposure to actinospores at a concentration of 100 to 1000 spores fish⁻¹. Of 15 masu salmon fry exposed at a concentration of 10 actinospores fish⁻¹, 7 (47%) were infected. With the exception of 1 out of 24 chum salmon that were exposed, spore formation did not occur in other salmonid species (pink salmon, sockeye salmon, rainbow trout, and brown trout; Table 4). No infection with *M. arcticus* was found in unexposed control fish.

DISCUSSION

Our transmission experiments demonstrated that Japanese actinospores of *Myxobolus arcticus* isolated from *Lumbriculus variegatus* are highly infectious to masu and amago salmon, but not to other salmonid species including sockeye salmon. Masu and amago salmon occur only in Far East Asia (Kato 1990) and share a close phylogenetic relationship (McKay et al. 1996). Even a concentration of 10 actinospores fish⁻¹ resulted in recoverable myxospores of *M. arcticus* from 7 of 15 masu salmon fry. In addition, all stages of masu salmon, from alevin to smolt stages, were susceptible to infection with *M. arcticus* (S. Urawa unpubl. data). These results indicate the high susceptibility of masu salmon to Japanese actinospores of *M. arcticus*.

The lack of infectivity of sockeye salmon to Japanese *Myxobolus arcticus* is in marked contrast to the Canadian situation, where sockeye salmon are the typical host (Bailey & Margolis 1987, Kent et al. 1993, McDonald & Margolis 1995). Kent et al. (1993) reported that 13 of 15 sockeye salmon fry were infected with *M. arcticus* spores after experimental exposure to Canadian actinospores from *Stylodrilus heringianus* at a concentration of 33 spores fish⁻¹ (Table 4).

Table 4. *Myxobolus arcticus*. Prevalence in juvenile salmonids exposed to actinospores in Japan and Canada for 1 h

Fish	Mean weight (g) of test fish	No. actinospores fish ⁻¹	No. fish infected/tested	Prevalence (%)
Exposed to actinospores from <i>Lumbriculus variegatus</i> in Japan				
Masu salmon	1.32	1000	15/15	100
	1.34	500	10/10	100
	1.43	200	16/16	100
	1.50	100	14/14	100
	1.41	10	7/15	46.7
Amago salmon	1.40	0	0/10	0
	0.20	1000	10/10	100
Chum salmon	0.20	0	0/10	0
	0.49	500	0/19	0
	0.70	200	1/5	25.0
Sockeye salmon	0.70	0	0/9	0
	0.55	900	0/15	0
Pink salmon	0.10	500	0/10	0
	0.20	500	0/10	0
Rainbow trout	0.10	500	0/10	0
	0.20	500	0/26	0
Brown trout	19.25	1000	0/12	0
Exposed to actinospores from <i>Stylodrilus heringianus</i> in Canada^a				
Sockeye salmon	Unknown	33	13/15	86.6
	Unknown	0	0/30	0

^aData from Kent et al. (1993)

The positive reactions of actinospores to skin mucus from various salmonid fishes may appear to be contradictory to the results of the transmission experiment, which demonstrated the high host specificity of *Myxobolus arcticus*. However, the penetration of infectious cells of actinospores into the fish host does not always result in the establishment of infection. It is probable that infectious cells are eliminated by host defense mechanisms or fail to develop into mature myxospores after initial penetration (Yokoyama et al. 2006). Nonspecific responses of actinospores to skin mucus have been reported by many researchers (Yokoyama et al. 1993, Xiao & Desser 2000, Özer & Wootten 2002, Kallert et al. 2005), whereas a specific reaction of *Thelohanellus hovorkai* actinospores to the skin mucus from the natural host (*Cyprinus carpio*) has also been suggested (Yokoyama et al. 2006). These differences may reflect the diversity of the host–parasite relationship among myxosporeans.

Two *Myxobolus arcticus* isolates utilize different oligochaete species (*Lumbriculus variegatus* in Japan and *Stylodrilus heringianus* in Canada) as the invertebrate host, although both oligochaetes belong to the same family, Lumbriculidae. Thus far, multiple species of oligochaete hosts have been recorded for only 4 myxosporean species: *M. hungaricus* (El-Mansy & Molnár 1997), *M. pseudodispar* (Székely et al. 1999), *Sphaerospora dykova* (as *S. renicola*, Molnár et al. 1999), and *S. truttae* (Özer & Wootten 2000). The former 3 cases were only found by experimental infections, and *S. truttae* had 2 oligochaete hosts (*L. variegatus* and occasionally *Tubifex tubifex*) which cohabited in a farm pond. On the other hand, *M. cerebralis* is specific to one species of oligochaete (*T. tubifex*) despite its wide distribution in the northern hemisphere, and the susceptibility is variable among strains or genotypes of *T. tubifex* (Stevens et al. 2001, Beauchamp et al. 2002).

Actinospore dimension and host susceptibility are different between the Canadian and Japanese isolates examined here, suggestive of distinct biological entities. Yet comparisons of the rRNA gene sequences showed a high similarity of over 99.9%, suggesting a strong taxonomic affinity of these isolates. Variation in actinospore morphology within a species has been also observed elsewhere (Hallett et al. 2002), suggesting considerable diversity of actinospore morphology even within a species. Sequence similarity is high, but more recent speciation events may not be reflected by fixed genetic changes in such a conserved area of the genome. The differences in host specificity are compelling evidence for

distinct species or at least strains of the parasite. Most notably, the isolate from Japan did not experimentally infect a type host, sockeye salmon (Table 4). In spite of these biological data, we hesitate to assign a new species at this time because no material is available for comparison from the type locality. As such, either entity presented here, or possibly neither, is attributable to the original *Myxobolus arcticus* described from the Russian Far East (Pugachev & Khokhlov 1979). The combination of this fact and the high similarity of DNA sequence data allows us to reasonably conclude for the time being that both should be considered *M. arcticus*, but minimally these are unique strains.

Actinospore morphology may be a function of the environment. For example, actinospores of parasites infecting bottom-dwelling fish have short caudal processes, whereas those infecting fish drifting in surface water have larger caudal processes (Longshaw & Feist 2005). We found that Canadian actinospores of *Myxobolus arcticus* have apparently longer projections than Japanese ones. This morphometric variation might occur after major shift of host selection, and may be caused by environmental differences in each region. In Canada, the oligochaete (*Stylodrilus heringianus*) and fish (sockeye salmon) hosts are distributed in a lake, where the long projections may increase the buoyancy of the actinospores and therefore increase the opportunity to lead to infections in fish. In Japan, however, the oligochaete (*Lumbriculus variegatus*) and fish (masu salmon) hosts inhabit a steep stream, where actinospores with long projections may be rapidly swept downstream, resulting in few chances to encounter a salmonid host. Further field and laboratory experiments will be required to test this hypothesis.

Patterns of parasite prevalence and protein polymorphisms have indicated that both sockeye and masu salmon have extremely high precision of homing migration for spawning (Awakura et al. 1982, Okazaki 1986, Quinn et al. 1987). This implies that the geographical segregation of anadromous salmonid hosts provided an opportunity for the onset of divergent evolution of the parasite. In conclusion, we presume that *Myxobolus arcticus* specialized its host selection and actinospore morphology for its local environments in the ongoing process of differentiation, potentially leading to speciation.

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