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Myxobolus groenlandicus n. sp. (Myxozoa) distorting skeletal structures and musculature of Greenland halibut *Reinhardtius hippoglossoides* (Teleostei: Pleuronectidae)

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ABSTRACT: A specimen of Greenland halibut *Reinhardtius hippoglossoides* (Walbaum, 1792) caught on the west coast of Greenland (Qasigiannguit) was found to possess serious pathological changes in the body musculature. A series of cartilaginous cylindrical structures organized symmetrically at the position of the proximal pterygiophores had changed the musculature and produced irreversible distortions (cavities and holes) in the fillet of the processed fish, leaving it with no value for the industry. Histopathological investigation showed that these structures consisted of hypertrophic cartilage containing numerous myxospore-producing plasmodia. Morphometric and molecular analyses of the parasites showed that both spore morphology and rDNA sequences complied with characteristics of the genus *Myxobolus*, but no full affiliation with a known species could be found. The parasite is a previously undescribed species, and the name *Myxobolus groenlandicus* n. sp. is assigned to this new myxobolid.

KEY WORDS: Greenland halibut · Greenland · *Myxobolus* · Myxozoa · Pathogenicity · Muscle · Fillet

INTRODUCTION

Greenland halibut *Reinhardtius hippoglossoides* (Walbaum, 1792) is an economically important fish species with an Arctic distribution from Greenland waters through the Northwest, North, and Northeast Atlantic to the Barents Sea and may be found in the North Pacific as well (Whitehead et al. 1986). It occurs at water depths of 200 to 2000 m and is caught using long lines by the local population of Greenland. Several parasitological investigations on this host species have been performed, mainly in order to elucidate distribution of sub-stocks. Lists of protozoan and metazoan parasites recovered from this host have been presented by Margolis & Arthur (1979), Scott & Bray (1989), Arthur & Albert (1993, 1994), and Boje et al. (1997), and several myxozoan species have been recorded. None of these have been associated with overt pathological conditions of the fish. Recorded species such as *Ceratomyxa drepanopsettae*, *C. ramosa*, and *Myxidium incurvatum* occupy mainly the gall bladder, and *Myxoproteus reinhardtii*, *Ortholinea divergens*, and *Schulmania quadriolobata* use the urinary bladder as their microhabitat in this host species (Arthur & Albert 1994).

During July 2010, a Greenland halibut was caught by a local fisherman in Disko Bay on the west coast of Greenland and was subsequently slaughtered, gutted, and partly processed on shore at Qasigiannguit. An unusual occurrence of circular hard structures in the fillets was noticed and the fish was therefore brought into the laboratory and frozen for later diagnostic work by the use of morphometric and molecular techniques. This investigation showed the presence of a previously undescribed myxozoan in the affected fish tissue.

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MATERIALS AND METHODS

Fish

One specimen of Greenland halibut *Reinhardtius hippoglossoides* (Walbaum, 1792) was caught on long lines with hooks by a local fisherman and brought ashore for processing. Fish body length (snout to tail fin margin) was 55 cm and the fish body weight (gutted) was 1.7 kg.

Catch location

The fish was caught at Disko Bay on the west coast of Greenland at Qasigiannguit (Christianshåb) (70° N, 52° W).

Macroscopic inspection

The staff at the local processing unit noted series of symmetrically arranged cylindrical hard structures and holes in the fillet, and it was therefore removed, freeze-stored, and air-transported frozen to the University of Copenhagen, where it was defrosted and subjected to further investigation.

Wet preparation of spores

Whitish plasmodia (macroscopically visible) were removed by a sterile scalpel from the cylindrical cartilage structures, located in muscle tissue, and smeared onto a microscope slide with sterile tap water and cover-slipped for light-microscopy studies. Spores were studied and photographed at 1000× magnification under a Leica DMLB microscope. The slides were then dried, ethanol-preserved, stained with Giemsa, and finally embedded in DePeX lipophilic mounting medium (Gurr®, BDH Laboratory Supplies).

Histology

Parts of the cartilaginous cylindrical structures were fixed in neutral 4% formalin for 1 mo (4°C) and processed for histology by dehydration, paraffin embedding, sectioning (4 µm), and mounting on microscope slides. Following de-paraffinization, sections were stained with Mayer's hematoxylin or Giemsa and then embedded with the media Aquamount and DePeX (Gurr®), respectively. Sections were studied and photographed at 40 to 1000× magnification under a Leica DMLB microscope.

Molecular analysis

Plasmodia were removed from the cylindrical cartilaginous structures and genomic DNA was purified from approximately 25 mg tissue using a QIAmp® DNA Mini Kit (Qiagen) with the protocol for 'DNA purification from tissues' according to the manufacturer's instructions. Two overlapping PCR amplicons covering the 18S rDNA to 28S rDNA of myxospореans were produced using PCR amplification. Sequences of Primer Set 1 were forward primer MyxospecF (TTC TGC CCT ATC AAC TWG TTG; Fiala 2006) and reverse primer NLR1694 (TCT YAG GAY CGA CTN AC; Van der Auwera et al. 1994). Primer Set 2 was forward primer NLF1050 (AAT CGA ACC ATC TAG TAG CTG G; Bartosova et al. 2009) and reverse primer NLR3113 (GTC TAA ACC CAG CTC ACG TTC CCT; Van der Auwera et al. 1994). The PCRs were done in 60 µl reactions using 6 µl of genomic DNA, 1 mM of dNTPs, 1.5 mM $MgCl_2$, and 1 unit of Biotaq™ DNA Polymerase (Bioline) in NH_4 reaction buffer. In order to achieve higher specificity in the PCR, a touchdown procedure was applied: pre-denaturation at 95°C for 5 min; 15 cycles of denaturation at 95°C for 30 s, annealing at various temperatures for 30 s (see below), elongation at 72°C for 3 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 3 min; and finally, post-elongation at 72°C for 5 min. The annealing for the touchdown regime was 2 cycles at 57°C, 2 cycles at 55°C, 2 cycles at 54°C, 3 cycles at 53°C, 3 cycles at 52°C, and 3 cycles at 51°C, with 15 cycles in all. Aliquots of 5 µl of the products were analyzed using electrophoresis on 1.5% agarose and visualized using ethidium bromide staining. PCR products were purified using an Illustra GFX PCR DNA and Gel Purification Kit (GE Healthcare) according to the manufacturer's instructions. Sequencing of the overlapping PCR products were performed using primer walking at Macrogen. The internal transcribed spacer 2 (ITS2) of the rDNA region was identified by using the web-based annotation tool ITS2 Database (http://its2.bioapps.biozentrum.uni-wuerzburg.de) (Eddy 1998, Keller et al. 2009). This tool searches for the end of the 5.8S RNA gene (5.8S rDNA) and the start of the 28S RNA gene (28S rDNA).

Phylogenetic analysis

A phylogenetic analysis was performed on a selection of 18S rDNA sequences that comprised the new sequence and additional sequences from members of the '*Myxobolus* clade' (Fiala 2006), including similar, more recent sequences identified using a basic local alignment search tool (BLAST) (Altschul et al. 1997). This selection also contained 2 sequences of myxozoans that are taxonomically distinct from but genetically closely related to *Myxobolus* spp. (*Ortholinea orientalis* and *Myxobilatus gasterostei*). *Sphaerospora oncorhynchi* and *Myxidium lieberkuehni* functioned as outgroups. We tried to include only sequences that were longer than or almost as long as the new sequence (1643 bp) in order to obtain a more robust alignment. Sequences (54 in total) were aligned using Clustal X v. 2.0 (Larkin et al. 2007), and a few obviously misaligned positions were edited manually. Very variable regions of the alignment were located and removed using Gblocks software (Castresana 2000), with parameters set for a less stringent rDNA alignment (minimum number of sequences for a flanking position: 28; minimum length of a block: 5; allow gaps in half positions), leaving 1444 positions in the final alignment (including inserted gaps). A Bayesian phylogenetic tree was constructed with MrBayes v. 3.2 (Huelsenbeck & Ronquist 2001) using a generalized time-reversible (GTR) substitution model with gamma-distributed rate variation across sites (GTR + I + Γ), as suggested as the best-fit model in MrModeltest v. 2.3 (Nylander 2004). Four simultaneous Monte-Carlo Markov chains were run from random trees for a total of 1 000 000 generations in 2 parallel runs. A tree was sampled every 100 generations, and a total of 2500 trees were discarded as 'burn-in' upon checking for stationarity by examining the log-likelihood curves over generations. A consensus tree (50% majority rule) was constructed from the post-burn-in trees, and posterior probabilities were calculated in MrBayes. Clades in the resulting phylogenetic tree were labeled using the system employed in a recent analysis (U-taynapun et al. 2011).

RESULTS

Gross pathology

No abnormalities were observed when the fish was inspected from the skin side, but when screening the fillets, a total of 36 abnormal and enlarged cylindrical cartilage structures were found distributed symmetrically in linear rows (Fig. 1A) located at the position of the proximal pterygiophores (1 to 2 cm from the fin base), which support the fin rays (both dorsally and ventrally). The cylinders (Fig. 1B), with a height of 0.5 to 1.0 cm and a diameter of 1.1 to 1.3 cm, were cartilaginous, with a central depression or cavity. The cylinder wall contained numerous white plasmodia (Fig. 1C). The cylinders had replaced the body musculature and were found loosely attached in the frozen fillet, where they could easily be removed.

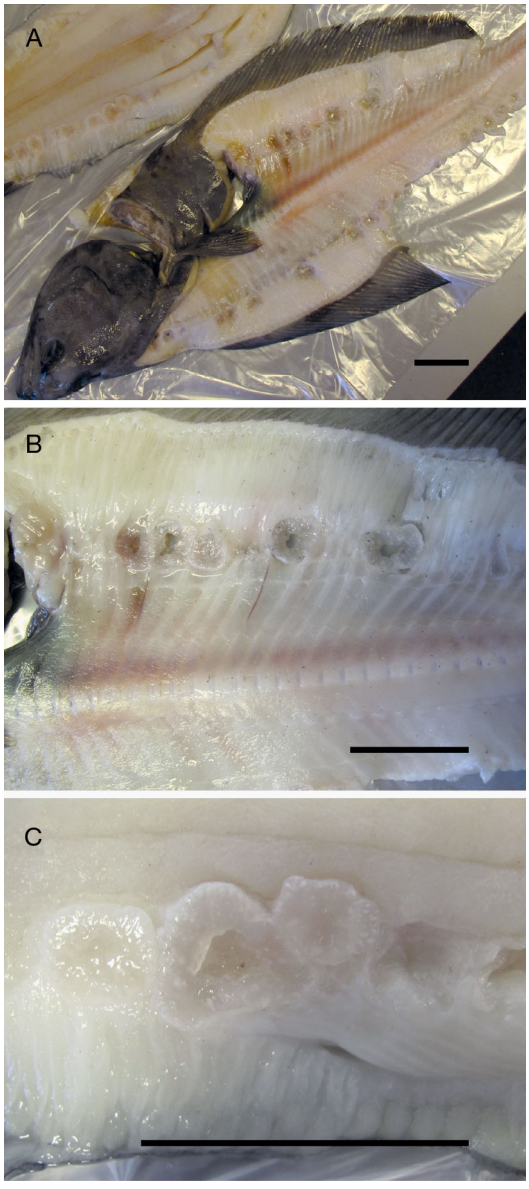

Fig. 1. *Myxobolus groenlandicus* n. sp. infecting *Reinhardtius hippoglossoides*. Gross pathology. (A) Distribution of cylindrical cartilage structures along the fillet. (B) Cylindrical cartilaginous structures penetrating the muscular tissue. (C) Whitish sporogonic plasmodia in cartilaginous cylindrical structures in muscle tissue. Scale bars = 50 mm

When the cylinders were removed, distortions (cavities) were observed in the body musculature (fillet).

Histopathology

The most prominent cartilage cylinders were sectioned. They consisted of enlarged cartilage tissue with severe hypertrophy of chondrocytes. Within each cylinder, numerous sporogenic plasmodia were observed (Fig. 2A,B) in cavities. Some plasmodia were partly branched (Fig. 2C,D). Freshly prepared smears from thawed tissue contained numerous spores (Fig. 3).

Spore morphology

Fresh spores (recovered from frozen material) matched the diagnostic features of the genus *Myxobolus* structure (Lom & Arthur 1989, Lom & Dykova 1992, Eiras et al. 2005).

Myxobolus groenlandicus n. sp. (Figs. 3 & 4)

Diagnosis

Plasmodia were polysporous, round to branched, with a diameter of 1.0 to 1.5 mm. Spores were round in frontal view, with a length of 10.31 ± 0.74 µm (mean ±SD; range: 8.5 to 11.0 µm), width of 10.11 ± 0.70 µm (range: 9.1 to 11.2 µm) (n = 32), and thickness of 6.21 ± 0.77 µm (range: 4.9 to 7.1 µm) (n = 29). In the lateral view they were oval to lemon-shaped, and in the apical view, lemon-shaped. Polar capsules were pyriform, with a length of 4.37 ± 0.44 µm (range: 4.0 to 5.1 µm) and width of 2.53 ± 0.64 µm (range: 2.1 to 4.1 µm) (n = 15); the polar filament showed 5 to 6 coils, and the extruded polar filament length was 24.92 ± 1.98 µm (range: 21.0 to 29.1 µm) (n = 13). An intercapsular appendix was absent.

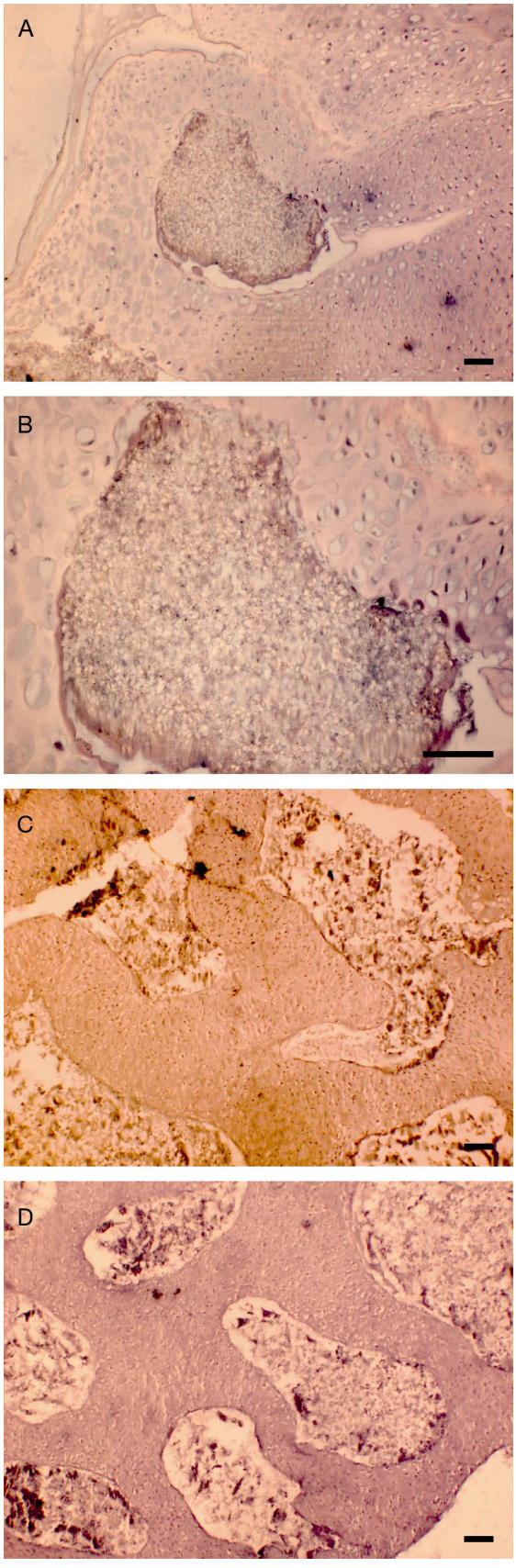

Fig. 2. *Myxobolus groenlandicus* n. sp. infecting *Reinhardtius hippoglossoides*. Histopathology. (A) Sporogonic plasmodium located in infection focus in hypertrophic cartilage. A 4 µm paraffin-embedded section of infected tissue. Haematoxylin-stained. (B) Spores produced in plasmodia in hypertrophic cartilage. Paraffin-embedded and sectioned tissue. Hematoxylin-stained. (C) Branched plasmodium in hypertrophic cartilage. Paraffin-embedded and sectioned tissue. Giemsa-stained. (D) Multiple infection foci in hypertrophic cartilage. Paraffin-embedded and sectioned tissue. Giemsa-stained. Scale bars = 100 µm

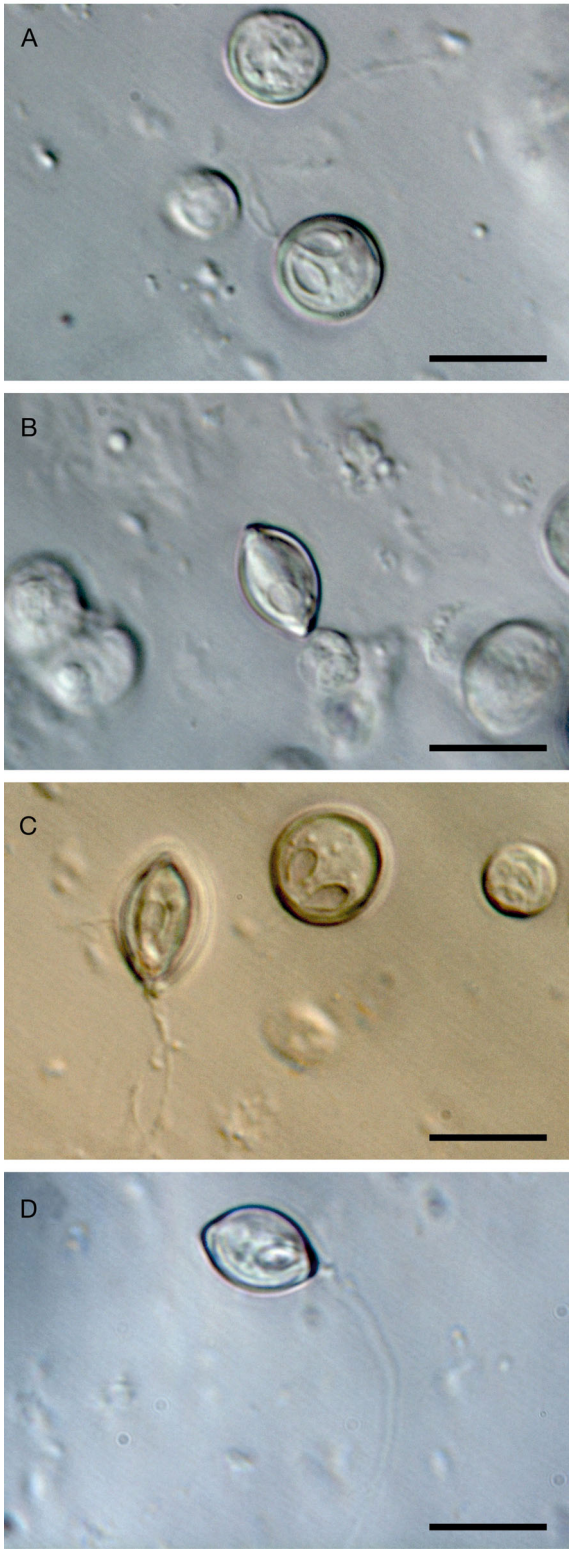

Fig. 3. *Myxobolus groenlandicus* n. sp. Wet mounts of spores (defrosted). (A) Frontal view. Polar capsules with everted polar filaments. (B) Apical view showing lemon shape. (C) Lateral and frontal views. (D) Lateral view showing everted polar filaments. Scale bars = 10 µm

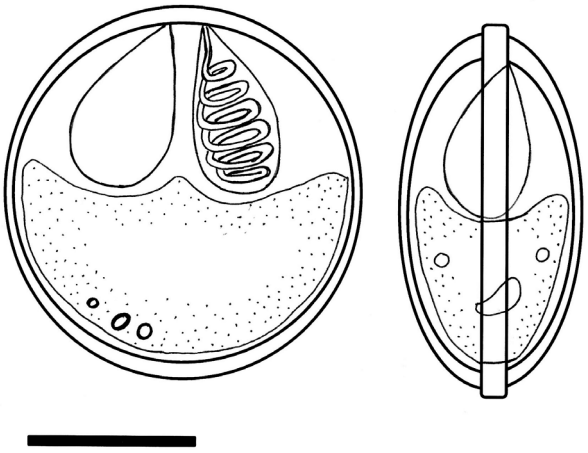

Fig. 4. *Myxobolus groenlandicus* n. sp. Line drawing. Scale bar = 5 µm

Taxonomic summary

Type host: Greenland halibut *Reinhardtius hippoglossoides* (Walbaum, 1792).

Type locality: Disko Bay on the west coast of Greenland at Qasigiannguit (Christianshåb) (70° N, 52° W).

Site of infection: Cartilage structures located in fish muscle in series, 1 to 2 cm from fin base.

Prevalence: Probably low; only one fish found infected among hundreds landed.

Type specimens at Zoological Museum of Copenhagen, Denmark: Holotype ZMUC-Myx-5 and paratypes ZMUC-Myx-6 and ZMUC-Myx-7 were prepared from haematoxylin-stained histological sections (4 µm) of paraffin-embedded (Aquamount, Gurr, BDH Laboratory Supplies) infected cartilage. Paratype ZMUC-Myx-8 was prepared from a slide smear of fresh spore, preserved in 96% ethanol, Giemsa-stained, and embedded in DePeX mounting medium. Molecular analysis (see below): GenBank (accession number: JF694785).

Etymology: The species was named in honour of the diverse marine environment in Greenland.

Molecular analysis

The PCR product obtained with the first primer set was 3667 bp long and covered most of the 18S rDNA; the complete ITS1, 5.8S rDNA, and ITS2; and the 5′ end of the 28S rDNA. The product obtained with the second set of primers was a bit more than 2200 bp long, of which 2196 bp was sequenced. This product contained only part of the 28S rDNA.

In all, 5258 bp was achieved. The entire sequence has been submitted to GenBank (accession number: JF694785). The organisation of the sequence was 1643 bp of the 3′ end of the 18S rDNA; 160 bp ITS1 rDNA from nucleotides 1644 to 1803; 150 bp-long 5.8S rDNA from nucleotides 1804 to 1953; a very short 116 bp-long ITS2 rDNA from nucleotides 1954 to 2069; and finally, 3189 bp of the 5′ end of the 28S rDNA from nucleotides 2070 to 5258. An ambiguous nucleotide Y at Site 983 was confirmed by sequencing of several independent PCR products. When performing a BLAST search, no sequence covering both the 18S rDNA and 28S rDNA was revealed. A BLAST search of the 18S rDNA alone resulted in highest identity to *Myxobolus albi* (89.6%). Taxonomic sampling for myxozoan 28S rDNA sequences is scarce when compared with 18S rDNA sequences. However, the overall similarities with other myxozoan species showed values of 44.81% (*M. cerebralis*), 45.93% (*Henneguya salminicola*), and 43.42% (*H. zschokkei*). The ITS1 rDNA and ITS2 rDNA region sequences did not match any other sequences in GenBank.

Phylogenetic analysis

Most *Myxobolus* spp. clustered into 3 well-supported clades (Clades A, B, and C), plus a minor clade, which is herein referred to as Clade D (Fig. 5). Several other myxozoans also clustered into Clades A, C, and D, such as *Henneguya* spp., *Thelohanellus* spp., *Sphaerospora molnari*, and others. The new sequence clustered with *M. albi* in a long-branched clade with high support, and these 2 species branched together with *M. cerebralis*, *M. acanthogobii*, and *H. salminicola* (Fig. 5), albeit with negligible support. *Ortholinea orientalis* and *M. gasterostei* formed a clade that branched basally to the *Myxobolum* clade, and the most basal *Myxobolus* clade was Clade B, comprising *M. arcticus*, *M. neurobius*, and *M. insidiosus*.

Remarks

The type host Greenland halibut is an entirely marine fish species. No *Myxobolus* species have been recorded in this host before. The relationship to other cartilage-infecting myxozoans is unclear, and the infection site in the host differs from other known cartilage-inhabiting myxozoans in the boreal and Arctic region.

DISCUSSION

Based on morphometric comparisons (Eiras et al. 2005) and analyses of available sequences in GenBank, it can be concluded that the myxozoan recovered from the Greenland halibut cartilage is affiliated with other species within the genus *Myxobolus*. More than 800 species within the genus *Myxobolus* have been described to date (Eiras et al. 2005, Molnar et al. 2011), and a number of these match the spore morphology of the Greenland halibut myxozoan of the present study. However, the 18S rDNA sequence we found differed substantially from other species of *Myxobolus*. No morphologically related myxozoans have previously been reported from Greenland halibut, and the parasite isolated in the present study therefore appears to be a previously undescribed species, which we have named *M. groenlandicus* n. sp. Phylogenetic analysis showed that *M. albi* is the nearest known relative to *M. groenlandicus* n. sp. The overall topology of the phylogenetic analysis (Fig. 5) corresponds well with recent analyses of the *Myxobolus* clade (Griffin & Goodwin 2011, Karlsbakk & Køie 2011, U-taynapun et al. 2011), and Clades A, B, and D can also be recognized in a phylogeny of the entire Myxosporea (Fiala 2006). *Myxobolus* is not monophyletic and this fact has already been well documented (Fiala 2006). The long-branched position of *M. groenlandicus* n. sp./*M. albi* shown here is congruent with a previous study on the phylogenetic position of *M. albi* (Picon-Camacho et al. 2009). In that study, the position of *M. albi* was basal to what corresponds to Clade A, whereas the present study suggests that *M. groenlandicus* n. sp./*M. albi* may belong to a sister clade of Clades A, C, and D. However, a large number of myxozoan 18S rDNA sequences are available today, and relative positions of clades are dependent on the number and lengths of sequences included in the analysis. In the future, more and longer sequences of species closely related to *M. groenlandicus* n. sp. and *M. albi* may help resolve the exact phylogenetic position of these 2 species. The ambiguous nucleotide at Site 983 may be due to the fact that the ribosomal genes exist as multiple gene arrays and is the result of differences in these paralogous copies rather than of differences between alleles (Whipps et al. 2004, Whipps & Kent 2006).

The name *Myxobolus groenlandicus* n. sp. was assigned to this new myxobolid. This is the first record of a myxozoan having a severe impact on the musculature and hence the product quality of Greenland halibut. It was shown that the distorting abnor-

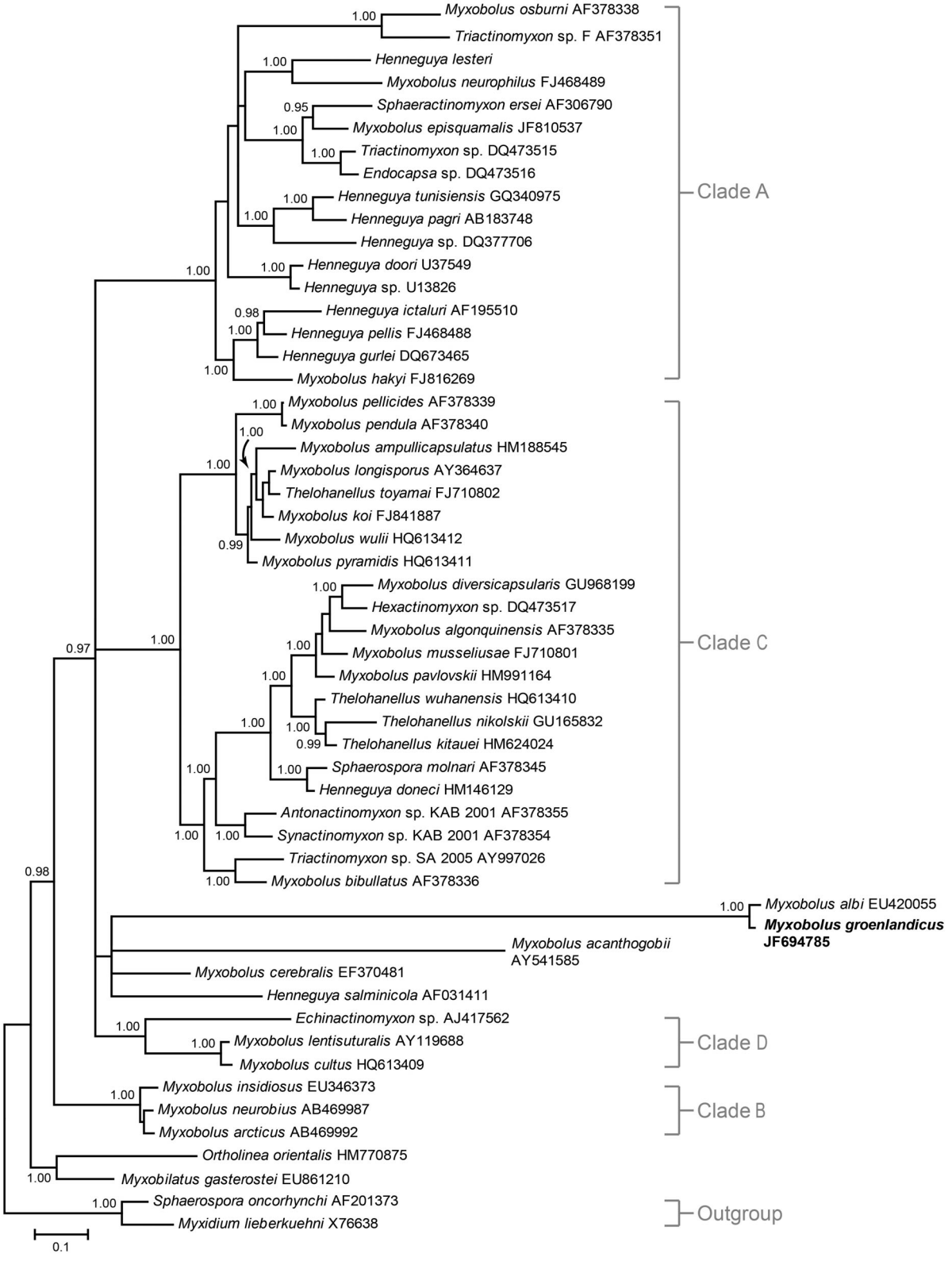

Fig. 5. Phylogenetic tree of the *Myxobolus* clade based on Bayesian inference of a 1444 bp-long alignment comprising 54 myxozoan 18S rDNA sequences. *M. groenlandicus* n. sp. is shown in **bold**. Numbers next to nodes are posterior probabilities; values <0.95 are not shown. Nodes with support <0.50 are collapsed. GenBank accession numbers are shown following each taxon name. Clades A to C are labeled according to U-taynapun et al. (2011)

mal structures had their basis in infected cartilage structures located in 2 rows under the fin rays of the host. It could not be confirmed that the abnormalities were hypertrophic pterygiophores, but this would explain the symmetric and linear organization of the disturbances. Several myxozoan species invade cartilage structures and elicit hypertrophy or proliferation of inflammatory cells in the affected tissue (Lom & Dykova 1992, Feist & Longshaw 2006). Recently, *M. albi* infecting gill cartilage structures of goby was described by Picon-Camacho et al. (2009). *M. groenlandicus* n. sp. also shows a preference for cartilaginous tissues, and the closest resemblance when performing a BLAST search on the 18S rDNA is in fact *M. albi* (Picon-Camacho et al. (2009). However, as shown by those authors, cartilage invaders do not seem to make up a taxonomic or phylogenetic entity. Several distantly related myxozoans may have evolved this trait by convergence. Thus, this morphological and molecular resemblance does not allow conclusions concerning pathogenicity in cartilaginous tissue and phylogeny.

The life cycle of the parasite is unresolved. Recent studies on the life cycle of marine myxozoans, such as *Ellipsomyxa gobii* from the common goby (Køie et al. 2004), *Ceratomyxa auerbachi* from herring (Køie et al. 2008), *Gadimyxa atlantica* from gadids (Køie et al. 2007), and *E. mugilis* (Rangel et al. 2009), have demonstrated that polychaetes serve as intermediate hosts. Even though *Myxobolus groenlandicus* n. sp. is a marine species, it belongs phylogenetically to the *Myxobolus* clade that mainly comprises freshwater species (Fiala 2006). Since marine members of this clade are rare, it will be interesting to obtain and analyze sequences of more marine *Myxobolus* species. Many marine myxozoans depend on polychaetes as alternate hosts, excluding the malacosporean myxozoans that use bryozoans as alternate hosts (Holzer et al. 2007). However, it is interesting to note that, even though *M. groenlandicus* n. sp. is a marine species, it belongs phylogenetically to the freshwater myxozoan clade (Fiala 2006), and based on phylogenetic evidence, one might assume that *M. groenlandicus* n. sp. uses a marine oligochaete as an alternate host. As this is unresolved, future studies should investigate the life cycle of *M. groenlandicus* n. sp. Further, studies should be implemented in order to establish precise and accurate prevalences and intensities of infection in Greenland halibut.

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