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Mycobacterium salmoniphilum infection in burbot *Lota lota*

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ABSTRACT: Burbot *Lota lota* sampled from lakes Mjøsa and Losna in southeastern Norway between 2005 and 2008 were found to be infected with *Mycobacterium salmoniphilum* at a culture-positive prevalence of 18.6 and 3.3%, respectively. The condition factor (CF) of mycobacteria-affected fish sampled from Mjøsa in 2008 was lower than the average CF of total sampled fish the same year. Externally visible pathological changes included skin ulceration, petechiae, exopthalmia and cataract. Internally, the infections were associated with capsulated, centrally necrotic granulomas, containing large numbers of acid-fast bacilli, found mainly in the mesenteries, spleen, heart and swim bladder. Mycobacterial isolates recovered on Middlebrook 7H10 agar were confirmed as *M. salmoniphilum* by phenotypical investigation and by partial sequencing of the 16S rRNA, *rpoB* and *Hsp65* genes as well as the internal transcribed spacer (ITS1) locus. This study adds burbot to the list of fish species susceptible to piscine mycobacteriosis and describes *M. salmoniphilum* infection in a non-salmonid fish for the first time.

KEY WORDS: Mycobacteriosis · *Mycobacterium salmoniphilum* · Multi-gene sequencing · Phylogeny · Pathology · Burbot · *Lota lota*

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INTRODUCTION

Burbot *Lota lota*, the sole member of its genus, is the only truly freshwater gadiform (cod-like) fish, having a circumpolar distribution above latitude 40°N (Cohen et al. 1990).

In recent years, burbot populations have been declining in both North America and Europe, with industrial pollution speculated as a possible cause (Pulliainen et al. 1992, Stapanian et al. 2010). Little is known of the natural pathogens of burbot and of the susceptibility of this fish species to pathogenic agents. To the best of our knowledge, the only relevant published work is that of Polinski et al. (2010) in which they investigated the virulence of infectious haematopoetic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), *Aeromonas salmonicida*, *Flavobacterium psychrophilum* and *Renibacterium salmoninarum* in burbot. No overt disease was demonstrated.

The present study was performed as part of a larger investigation into the effects of environmental pollution on fish health in Lake Mjøsa. As preliminary sampling revealed mycobacteriosis in several burbot containing extremely high levels of polybrominated diphenyl ethers (PBDEs) from this lake, sampling was extended to compare the mycobacterial infection status of Lake Mjøsa burbot with burbot from nearby Lake Losna which display background levels of PBDE (Mariussen et al. 2008).

While piscine mycobacteriosis is common in warmer waters (Nigrelli & Vogel 1963, Hedrick et al. 1987), this disease appears to be relatively uncommon in Europe, particularly in freshwater. Incidences of marine mycobacteriosis in Europe include reports from mackerel *Scomber scombrus* (presumptive) in the northeast Atlantic (MacKenzie 1988), wild cod in Danish coastal waters (Dalsgaard et al. 1992) and sea-farmed Atlantic salmon *Salmo salar* (Bruno et al. 1998, Zerihun et al.

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2011b). As far as we are aware, the present report represents the first documentation of mycobacteriosis in a wild, native, northern European fish species and the first report of *Mycobacterium salmoniphilum* infection in a non-salmonid freshwater fish species.

MATERIALS AND METHODS

Fish samples. From 2005 to 2008, 58 burbot were sampled from Lake Mjøsa and 30 from Lake Losna. Sampling of the fish was conducted as part of a study of the effects of persistent organic pollutants (POPs) on burbot in both lakes.

Lake Losna is located upstream and in the same water catchment as Lake Mjøsa, which is the largest lake in terms of surface area and the fourth deepest in Norway (Fig. 1). The studied fish from Lake Mjøsa were caught in the vicinity of Vingrom; those from Lake Losna were caught close to the head of the river Gudbrandsdalslågen. The fish populations are physically separated by a dam. In both lakes the fish were captured live in fyke nets set at a depth of approximately 10 to 20 m in Lake Losna and 20 to 40 m in Lake Mjøsa. The nets were emptied after 10 d and the fish transported live in iced water to the Norwegian Veterinary Institute (NVI), Oslo, for examination. In addition to burbot, 10 pike *Esox lucius* were sampled from Lake Mjøsa.

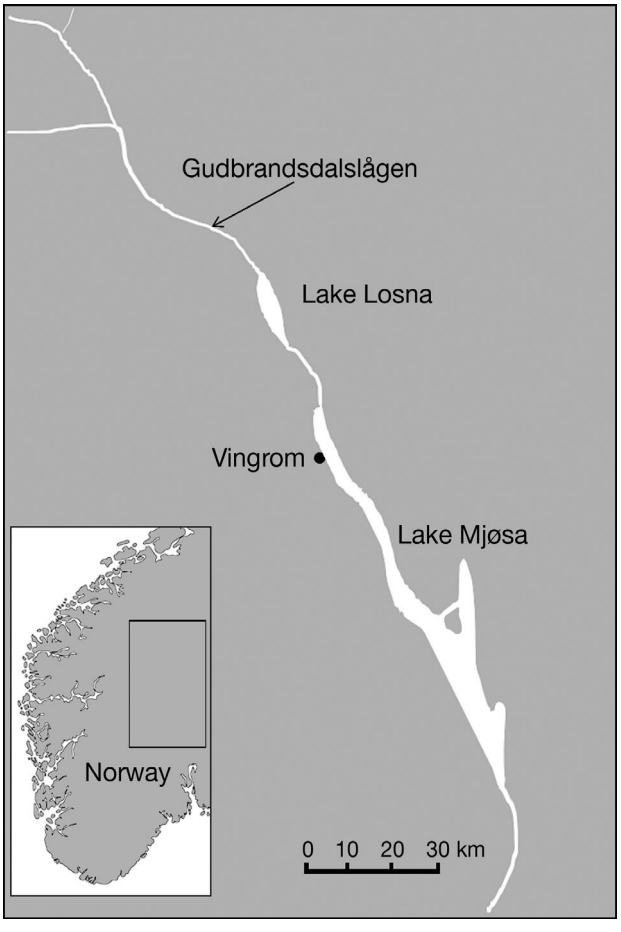

Fig. 1. Sampling sites Lake Mjøsa and Lake Losna, located in eastern Norway. Mjøsa is the largest lake in Norway and Lake Losna is part of the Gudbrandalslågen river catchment, which is the major inflow to Lake Mjøsa

Gross examination. All sampled fish were weighed, measured, and condition factor (CF) was calculated as $CF = W \times 100 \times L^{-3}$, where W is body weight (g) and L is length (cm) (Begenal & Tesch 1978). External and internal macroscopic lesions observed during aseptic necropsies were recorded.

Histopathology. Samples for histopathological examination were taken mainly from fish with macroscopically visible pathological changes in one or more organs. A total of 55 fish, comprising 11 fish from Losna and 44 from Mjøsa, were examined. Tissue samples (head kidney, liver, spleen, heart, mesenteries and intestine, gills, muscle and skin and other organs with visible pathological lesions) were fixed in 10% buffered formalin for routine paraffin embedding and sectioning. Tissue sections were then examined using light microscopy after staining with haematoxylin and eosin (H&E) (Luna 1968). Parallel sections were stained with Ziehl-Neelsen (ZN) stain.

Immunohistochemistry. Tissue sections taken from 15 fish with macroscopically visible granulomas were immunostained using genus-specific *Mycobacterium* polyvalent antisera as described by Zerihun et al. (2011a).

Bacteriology. Blocks of tissue (approximately 0.2 g) from kidney, spleen, mesenteries and other organs were taken from all necropsied fish. These tissues were placed into sterile tubes with metal beads (Bertin Technologies) and 1 ml of Butterfield's phosphate buffer (PB) was then added and homogenised using a MagNA Lyser® (Roche). Blood agar with 2% NaCl (BAS), without salt (BA), and Middlebrook 7H10 Agar supplemented with Bacto Middlebrook oleic acid-albumin-dextrose-catalase enrichment (MDA) were spread-plated with 0.1 ml homogenate. A series of dilutions (10^1 to 10^3) were prepared from selected tissue homogenates to avoid overgrowth by other bacteria. BA and MDA plates were incubated at 22°C and BAS plates at 15°C. MDA plates were incubated for 8 wk and examined twice weekly. BA and BAS plates were incubated for 10 d. Bacterial isolates were characterised using accepted methods (Kent & Kubica 1985, Lutz 1992), including Gram and ZN staining. Isolates were inoculated from MDA onto MacConkey agar plates with crystal violet, urea agar and Löwenstein Jensen (LJ) medium, incubated at 28°C for up to

1 mo and checked at least weekly. Recommended positive and negative controls were included in each test. Urease, nitrate reduction, citrate utilisation and iron uptake were also measured and results were validated using positive and negative controls as previously described (Lutz 1992).

DNA extraction. Approximately half of the 10^1 tissue homogenate (500 µl) prepared for bacterial culture was placed into FastProtein™ Blue (Epicentre Biotechnologies) and, following the addition of 500 µl PB, homogenized twice for 45 s each at 3779 × *g* using a MagNA Lyser® (Roche). Two full inoculating loops (10 µl each) of bacterial cells were emulsified in 1 ml PB, transferred into FastProtein™ Blue and homogenized as described above. DNA was extracted from tissue and bacterial homogenates using QuickExract™ DNA Extraction kit (Epicenter® Biotechnologies) and procedures recommended by the manufacturer.

Real-time PCR. *Mycobacterium*-specific real-time PCR was carried out on spleen tissue from all sampled fish as described by Zerihun et al. (2011a). DNA extracted from uninfected fish tissues were used as negative controls and were consistently negative. All samples were analysed in duplicate.

Sequence analysis. The 16S rRNA gene of the obtained isolates from plated cultures was amplified using PCR and primers as described by Weisburg et al. (1991). Sequencing of the PCR products was performed using the same primer sets and additional sequencing primers V1, V2, V3, V4, V5 and V6 (Neefs et al. 1993).

Partial sequences of the ITS1, *rpoB* and *Hsp65* genes were amplified and sequenced using primers and procedures described previously (Steingrube et al. 1995, Roth et al. 2000, Adékambi et al. 2003, Gomila et al. 2007). Negative and positive controls were included for each set of amplification. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), and sequencing was performed using the ABI BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit and the ABI PRISM® 3100 Genetic Analyser (Applied Biosystems). Sequence fragments obtained in this study were compared with other database entries using BLAST search analysis (Altschul et al. 1997) and deposited in the National Center for Biotechnology Information (NCBI) database with the following accession numbers: for *Hsp65*, HM638432 to HM638438; ITS1, HM638439 to HM638445; *16S rRNA*, HM638446 to HM638452 and *rpoB*, HM63638453 to HM638459.

Phylogenetic analysis. Contiguous sequences (NVI6590 to NVI6594, NVI6608 and NVI6609) were assembled using the Sequencher program (Gene Codes Corporation). DNA sequences of 16S rDNA, *rpoB* and *Hsp65* were aligned in CLUSTAL_X (Thompson et al. 1997) with related sequences, mainly type strains retrieved from GenBank as included by Whipps et al. (2007). A neighbour-joining phylogenetic tree was generated using the Kimura 3-parameter model in PAUP* version 4.0b10 (Swofford 1998). The strains *Mycobacterium tuberculosis* H37Rv and *M. leprae* TN were used as outgroups. Ambiguous and/or missing characters were excluded from the analysis.

RESULTS

Gross pathology

Of 58 fish examined from Lake Mjøsa, 20 showed visceral granulomas while 38 showed both visceral granulomas and external lesions. The CF of mycobacteria-affected fish sampled from Mjøsa in 2008 (0.75) was lower than the average CF (0.84) of all fish sampled in Mjøsa the same year. Externally visible pathological changes included skin ulceration, keratitis, petechiae, exophthalmia, vertebral deformity and cataract. Internally, greyish-white nodules (1 to 4 mm) were prominent on mesenteries and occasionally in the spleen, liver and heart. One fish showed a large (>20 mm) grey-brown nodule on the swim bladder (Fig. 2A). A swollen spleen (splenomegaly) was observed in 13 fish from Mjøsa, with some of these fish displaying inflammatory processes of the spleen capsule and adhesion to the mesentery (Fig. 2B). Plerocercoid cysts of the pike tapeworm *Triaenophorus nodulosus* were observed in the viscera, mainly on mesenteries and serosa of the gastro-intestinal tract (GIT) of the majority of fish sampled from Mjøsa. Fish sampled from Losna (n = 30) did not show macroscopically visible pathological changes.

Histopathology

Granulomas were identified in the visceral organs of 33 out of 44 (75%) fish from Mjøsa and 2 out of 11 (18.2%) fish from Losna. Granulomas were mainly observed in the mesenteries and occasionally in the heart, spleen, liver and wall of the GIT (Fig. 3A–D). ZN positive bacilli were visible within granulomas (Fig. 3D) only in culture and real-time PCR positive fish, although not all granulomas from such fish displayed ZN positive bacilli. Several ZN negative tissue sections from culture and PCR positive fish stained positively by immunohistochemistry (IHC). None of the tissue sections from fish taken from Losna stained positively with either ZN or immunostaining.

The observed granulomas appeared to be of the reticuloendothelial (RE) type. Granulomas displayed RE cells and necrotic debris in the centre circumscribed by layers of spindle and epithelioid cells. The

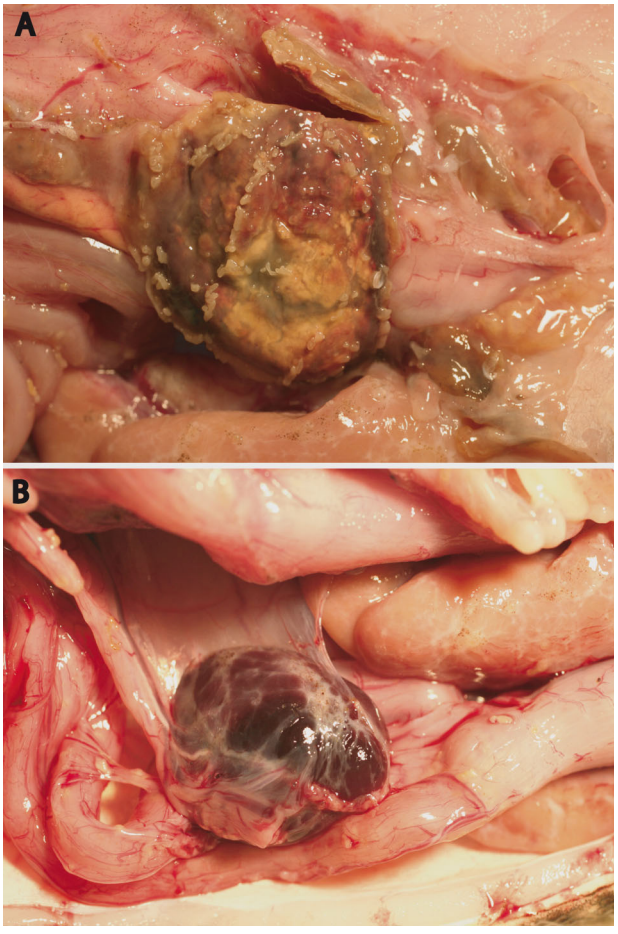

Fig. 2. *Lota lota*. Burbot sampled from Lake Mjøsa with gross pathology attributed to mycobacterial infection: (A) greyish-brown granulomatous process (>20 mm) on the outer surface (serosa) of the swim bladder and (B) swollen spleen (splenomegaly) with inflammatory process and adhesion to the surrounding mesenteries

outermost zone was encapsulated by thick layers of fibrous tissues (Fig. 3C).

Granulomas were either diffusely scattered in the mesenteries, liver, spleen and heart or were multiple and coalescent (Fig. 3A). Most granulomas were well defined with a clear demarcation towards normal tissue and were typically layered with a caseonecrotic centre (Fig. 3A–C). ZN staining revealed aggregates of acid-fast bacilli in the central parts of the granulomas (Fig. 3D).

Bacteriology and phenotypical characterisation

Smooth, opaque and creamy colonies with an entire margin were cultivated from the head kidney of 11 out of 59 (18.6%) and 1 out of 30 (3.3%) fish from Mjøsa and Losna, respectively, within 10 d of incubation at 22°C on MDA (Table 1). The same bacteria were also cultivated from homogenates of spleen, mesenteries, liver and heart. Bacterial isolates were Gram and ZN positive and showed biochemical characteristics consistent with a *Mycobacterium* sp. previously isolated from salmonids by Arakawa & Fryer (1984), Bruno et al. (1998) and Zerihun et al. (2011b) and now recognised as *M. salmoniphilum*.

Molecular characterisation

The *Mycobacterium*-specific real-time PCR conducted on spleen tissue samples revealed positive results in 14 out of 40 (35%) fish from Lake Mjøsa and 2 out of 30 (6.6%) from Lake Losna (Table 1).

Using conventional PCR, fragments of the 16S rRNA (~1464 bp), *Hsp65* (422 bp) and *rpoB* (709 bp) genes as well as ITS1 (194 bp) were amplified and sequenced from pure cultures of isolated *Mycobacterium* sp. The 16S rRNA sequences of all isolates were identical and displayed 100% identity with *M. salmoniphilum* type strain ATCC 13758^T (DQ866768) and reference strain NCIMB 13533 (EF535601). Partial *Hsp65* and *rpoB* gene sequences were also identical, with *rpoB* showing 99% and 97% identity with *M. salmoniphilum* NCIMB13533 and ATCC 13758^T, respectively, while *Hsp65* displayed 99% identity with ATCC 13758^T and 98% with NCIMB13533. With the exception of isolates NVI6608 and NVI6609, which displayed 4 bp differences, partial ITS1 sequences were also identical and displayed 98% and 97% identity with *M. salmoniphilum* strains NCIMB13533 and ATCC 13758^T, respectively.

Individual phylogenetic trees were constructed for 16S rRNA, *Hsp65* and *rpoB* genes. All trees supported the phylogenetic topology presented by Whipps et al. (2007) (Fig. 4).

Examination of pike *Esox lucius*

No evidence of mycobacterial infection was found following macroscopic, histological bacteriological, or molecular (real-time PCR) studies of the sampled pike.

DISCUSSION

Mycobacteriosis generally manifests as a sub-acute to chronic disease in both wild and captive fish (Chinabut 1999). The findings in the present study are consistent with the existence of such an infection in burbot from both lakes studied, with a considerably higher prevalence of mycobacteriosis detected in bur-

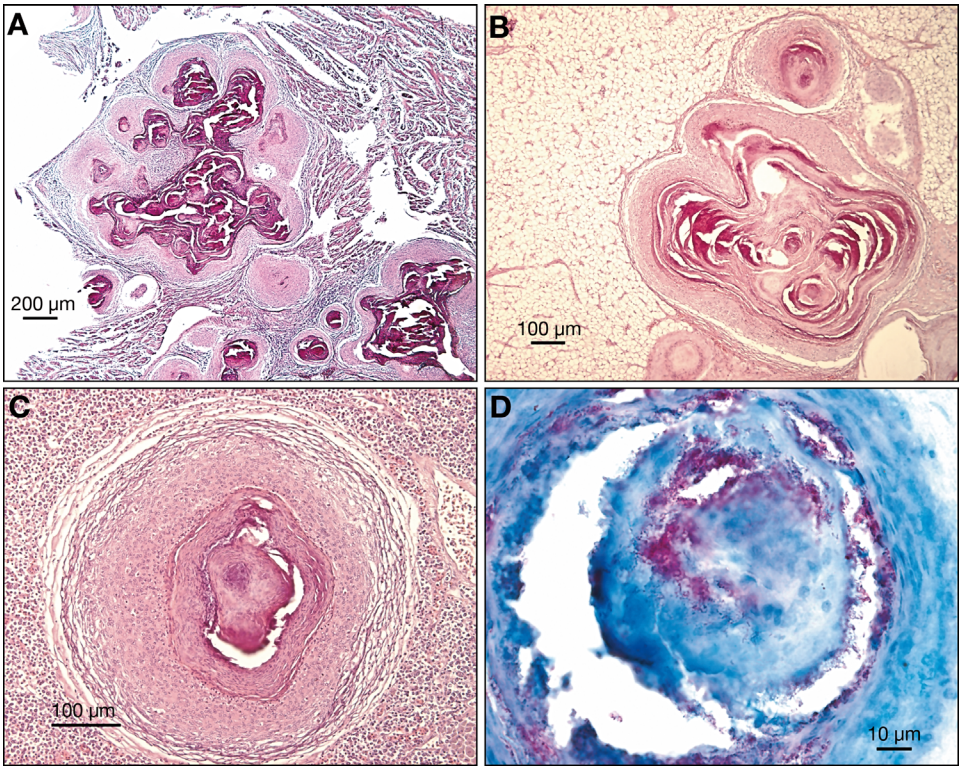

Fig. 3. *Lota lota.* Histological tissue sections of mycobacteria infected burbot sampled from Lake Mjøsa, showing lesions in (A) heart, (B) liver and (C) spleen tissues. The granulomas on these tissues are well encapsulated with thick (multi-layered) tissue and a necrotic centre. (D) shows granulomas in ZN-stained spleen tissue with a large number of acid-fast bacilli

bot from Lake Mjøsa compared to Lake Losna. Infected fish displayed external and internal, macroscopically and histologically detectable lesions consistent with a long standing infection.

Although further variation in environmental conditions must exist between the 2 lakes, the obvious anthropogenic difference relates to the high levels of environmental pollution in Lake Mjøsa compared to Lake Losna. Previous toxicological analysis of burbot from Mjøsa showed that the levels of PBDEs in this species of fish are extremely high, while the levels of PBDEs in burbot from Lake Losna are in the background range (Mariussen et al. 2008). Levels of polychlorinated biphenyls (PCBs) and dichloro-diphenyl-trichloroethane (DDTs) were also 10 and 15 times higher, respectively, in burbot from Lake Mjøsa compared to Lake Losna, while PBDE levels were 200 times higher in Mjøsa (Gregoraszczuk et al. 2008). Lake Mjøsa is surrounded by 3 cities, industrial areas and farmland. Sources of contamination include sewage, industrial effluents and flood waters from the surrounding area (Løvik et al. 2009).

Although it might be tempting to speculate that the difference in mycobacteriosis prevalence (and incidence of granulomas in general) between the 2 separate burbot populations may be caused by immunosuppression related to environmental pollution, especially in view of the strong association between prevalence and pollution levels, it is not possible to conclude this from the present study. More extensive studies are therefore required to confirm or disprove the association between pollution and mycobacterial infection. Mortality attributed to mycobacteriosis in wild finfish populations is difficult and expensive

Table 1. *Lota lota.* Summary of experimental results in burbot sampled from Lakes Mjøsa and Losna. Gross and histopathological examinations relate to the prevalence of visceral granulomatosis. Real-time PCR relates to positive (threshold cycle number <35) *Mycobacterium*-specific real-time PCR on fish spleen tissues. Culture results relate to positive culture of *M. salmoniphilum* on Middlebrook 7H10 from kidney homogenates, and ZN staining relates to observation of acid fast rods in tissue sections. In each case, the number of positive fish out of total fish examined is shown

Sampling area	Gross pathology	Histo-pathology	Real-time PCR	Culture	ZN staining
Lake Mjøsa	20/58	33/44	14/40	11/58	6/33
Lake Losna	0/30	2/11	2/30	1/30	0/2

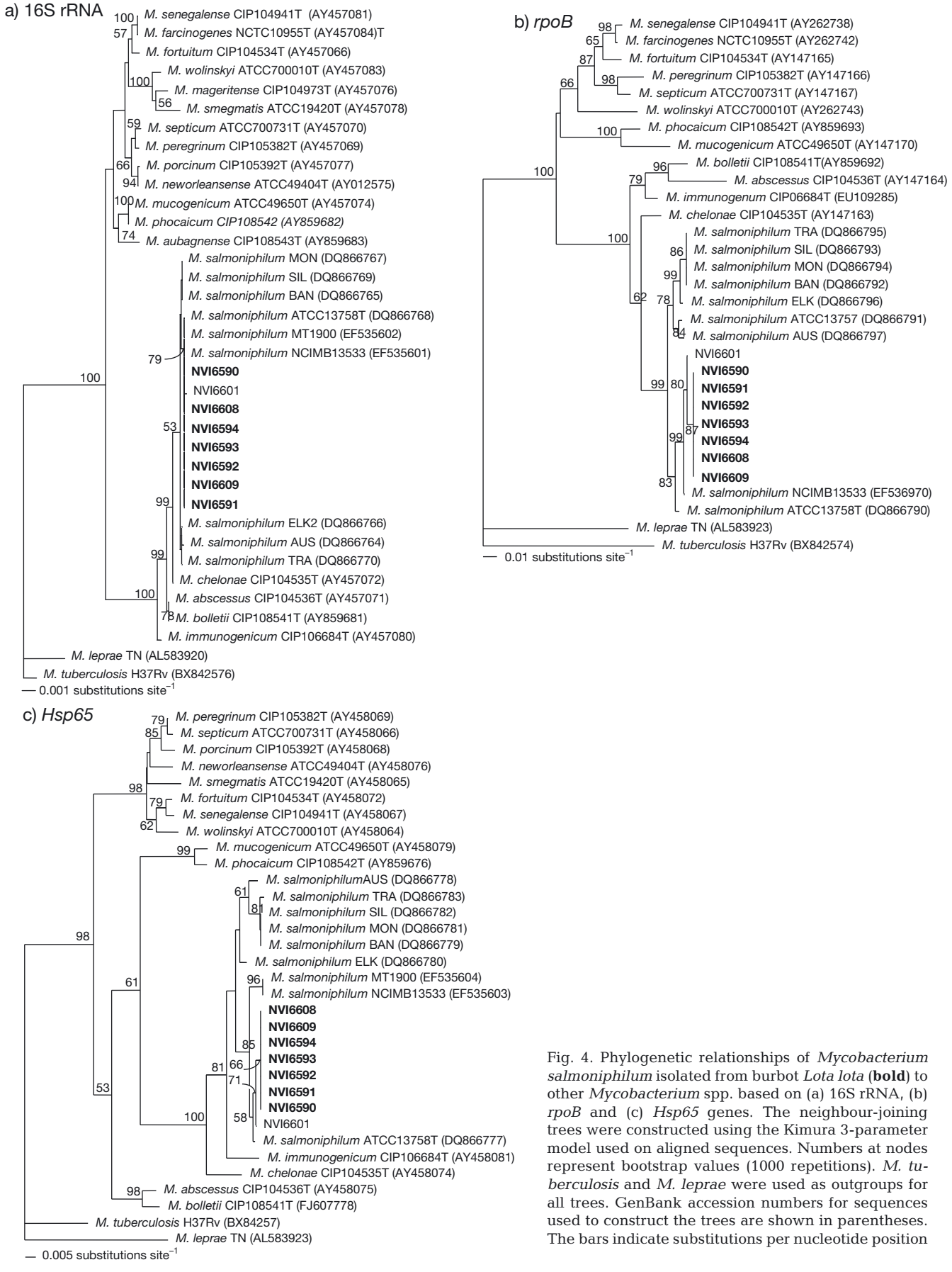

Fig. 4. Phylogenetic relationships of *Mycobacterium salmoniphilum* isolated from burbot *Lota lota* (**bold**) to other *Mycobacterium* spp. based on (a) 16S rRNA, (b) *rpoB* and (c) *Hsp65* genes. The neighbour-joining trees were constructed using the Kimura 3-parameter model used on aligned sequences. Numbers at nodes represent bootstrap values (1000 repetitions). *M. tuberculosis* and *M. leprae* were used as outgroups for all trees. GenBank accession numbers for sequences used to construct the trees are shown in parentheses. The bars indicate substitutions per nucleotide position

to prove conclusively but has been reported (Dalsgaard et al. 1992, Gauthier et al. 2008). While the overall effect on the burbot population of infection with *M. salmoniphilum* is not known, the relatively high prevalence identified in Lake Mjøsa may well have a detrimental effect on the population as a whole.

Despite the small sample size, the negative test results of mycobacterial infection for the 10 pike analysed in the present study, as well as the results of previous pollutant studies (Gregoraszczuk et al. 2008, Mariussen et al. 2008), provide some support for the presumption that burbot, as a predatory and scavenging bottom-dwelling fish (Paakkonen & Marjomaki 2000), is more exposed to persistent environmental pollutants than other types of fish.

The granulomas attributed to mycobacterial infection in the present study were composed of a thick capsule of epithelioid cells surrounding a necrotic centre, some of them with large numbers of acid-fast bacilli present, which is consistent with mycobacteriosis in many other fish species (Colorni et al. 1998, Talaat et al. 1998, 1999, Gauthier et al. 2003).

The prevalence of histologically detectable granulomas was considerably higher than the prevalence of mycobacteriosis in fish studied here. Although mycobacteria were not cultured from all granulomatous lesions in this study, *Mycobacterium salmoniphilum* were cultured only from fish displaying granulomatous lesions, confirming the association of granulomatous lesions with mycobacteria. Granulomas caused by larval stages of the tapeworm *Triaenophorus nodulosus* were detected in a number of fish from Lake Mjøsa, mainly in the mesenteries and walls of the GIT. A number of histological sections displayed presence of the parasite within the granuloma, which could easily be differentiated from those granulomas associated with mycobacteria. The majority of granulomas with negative mycobacterial test results for culture, ZN staining, IHC and real-time PCR was formed in response to parasitic infections, e.g. tissue encapsulated larval stages of *T. nodulosus.*

Granuloma encapsulation has only occasionally been noted in *Mycobacterium salmoniphilum* associated disease in salmonids (Bruno et al. 1998, Zerihun et al. 2011b), yet it appears to be a prominent feature of the disease in burbot. This may indicate that this type of response is more related to host species than mycobacterial species.

Phylogenetic analysis of several genetic loci (16S rRNA, *Hsp65* and *rpoB*) confirmed the identity of all isolates recovered during the study as *Mycobacterium salmoniphilum* (Whipps et al. 2007). However, the variation in ITS1 sequences suggests that more than one clone is involved. Furthermore, our phylogenetic analyses clearly distinguish *M. salmoniphilum* from *M. chelonae,* thus corroborating support by Whipps et al. (2007) for the original proposal of *M. salmoniphilum* as a separate species by Ross (1960), which was not generally accepted at that time. The present study also provides further evidence for *M. salmoniphilum* as a disease-causing agent in teleost fish. To the best of our knowledge, all isolations of *M. salmoniphilum* have been made in association with disease in teleost fish and, until the present report, all isolations were related to salmonid fish species (Ross 1960, Bruno et al. 1998, Whipps et al. 2007, Zerihun et al. 2011b). Therefore, as far as we are aware, the present study is the first report of disease caused by *M. salmoniphilum* in a non-salmonid species. The source of infection in this study is not established.

In conclusion, the present study substantiates burbot, a cold-water fish, as a host species for mycobacteria, and *Mycobacterium salmoniphilum* as a mycobacterial species which can infect fish species other than salmonids. The high level of contamination detected in the fish from Lake Mjøsa may affect the immune system, leading to the increased prevalence of mycobacterial infection. Further investigation will be needed to determine prevalence of *M. salmoniphilum* in other fish species and environmental samples in the 2 lakes.

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