Vol. 62: 93–96, 2004 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published November 23

SSU rRNA gene sequence reveals two genotypes of *Spironucleus barkhanus* (Diplomonadida) from farmed and wild Arctic charr *Salvelinus alpinus*

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ABSTRACT: *Spironucleus barkhanus* isolated from the blood of Arctic charr *Salvelinus alpinus* from a marine fish farm were genetically compared with *S. barkhanus* isolated from the gall bladder of wild Arctic charr. The wild Arctic charr were caught in the lake used as the water source for the hatchery from which the farmed fish originated. Sequencing of the small subunit ribosomal RNA gene (SSU rDNA) from these 2 populations showed that the isolates obtained from farmed and wild Arctic charr were only 92.7% similar. Based on the sequence differences between these isolates, it is concluded that the parasites isolated from the farmed fish have not been transmitted from wild Arctic charr in the hatchery's fresh water source. It is therefore most likely that the farmed fish were infected by *S. barkhanus* after they were transferred to seawater. *S. barkhanus* isolated from diseased farmed Arctic charr were 99.7% similar to the isolates obtained from diseased farmed Chinook (Canada) and Atlantic salmon (Norway). The high degree of sequence similarity between *S. barkhanus* from farmed Arctic charr, Chinook and Atlantic salmon indicates that systemic spironucleosis may be caused by specific strains/variants of this parasite. The genetic differences between the isolates of farmed and wild fish are of such magnitude that their conspecificity should be questioned.

KEY WORDS: *Spironucleus* · Salmonids · SSU rDNA

INTRODUCTION

There have been several reported cases of severe systemic spironucleosis caused by the hexamitid flagellate *Spironucleus barkhanus* (Sterud, Poppe & Mo, 1997) in farmed Atlantic salmon *Salmo salar* L. in Norway (Mo et al. 1990, Poppe et al. 1992, Poppe & Mo 1993). The most recent outbreak of this disease occurred in Arctic charr *Salvelinus alpinus* (L.) (originally described as *Salmo*) in a marine fish farm in northern Norway, and in this case the parasites were even found intracellularly in the liver, kidney and spleen (Sterud et al. 2003). Although this was the first published case of spironucleosis in farmed Arctic charr, the same fish farm had experienced previous outbreaks of this disease (Sterud et al. 2003). The hatchery that delivered the charr smolts to the affected marine farm is located in a water system where wild Arctic charr harbour *S. barkhanus* (Sommerhaug & Brun 1995, Sterud et al. 2003). Fish infected with *S. barkhanus* have never been observed within the hatchery itself (Sommerhaug & Brun 1995). Thus the possibility exists that the fish in the affected marine farm had acquired the parasites from a different source. In this study we compared approximately 1150 base pairs (bp) of the small subunit ribosomal RNA gene (SSU rDNA) sequences of *S. barkhanus* obtained from wild Arctic charr from the hatchery water source with *S. barkhanus* isolated from the diseased fish studied by Sterud et al. (2003). The SSU rDNA sequences of *S. barkhanus* isolated from diseased farmed Chinook salmon *Oncorhynchus tshawytscha*, Canada (Walbaum, 1792), and Atlantic salmon, Norway, were also included in the comparison.

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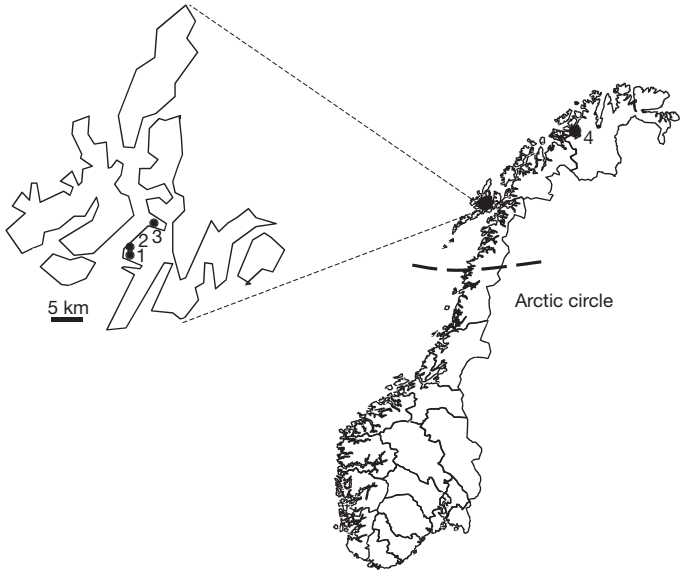

Fig. 1. Map of Norway showing the location of Fiskfjordvatn lake (1), hatchery (2), Arctic charr farm (3) and Alta (4)

MATERIALS AND METHODS

Approximately 100 wild anadromous and resident Arctic charr were caught by gill nets in the Fiskfjordvatn lake in Nordland County (northern Norway), the water source for the hatchery (Fig. 1). The fish were killed by a blow to the head and shipped on ice to the National Veterinary Institute (NVI) in Oslo. Thirty fish were examined for the presence of *Spironucleus barkhanus*. The content of the gall bladders was aseptically collected using syringes and examined in a phase contrast microscope at 100× magnification. Varying amounts of bile containing hexamitid flagellates from 6 fish were preserved separately in 5 ml tubes containing 96% ethanol. Parasites isolated from blood and muscle tissue of 2 farmed Arctic charr (Fig. 1) studied by Sterud et al. (2003) had been preserved separately in 5 ml tubes containing 96% ethanol and were included in the study. Ethanol preserved parasites from a systemically infected farmed Atlantic salmon were also included in the study. This sample was isolated from a fish farm in Alta in 2002 (Fig. 1). Genomic parasite DNA was isolated from the ethanol preserved material using the QIAamp DNA Mini Kit (Qiagen). Ethanol preserved bile (1 ml) was resuspended in 13 ml phosphate-buffered saline (PBS) and pelleted by centrifugation at 2000 × *g* for 10 min. The PBS was discarded and the pellet resuspended in 200 µl of PBS. DNA extraction was performed according to the QIAamp DNA Mini Kit protocol. DNA was eluted in 200 µl of elution buffer AE.

Cultivation of the parasites from the wild Arctic charr was attempted. Bile from 4 fish was used to inoculate 4 separate 13 ml polystyrene tubes containing 9 ml of *Tritrichomonas foetus* growth medium. The tubes were incubated at 5°C and checked daily for the presence of live parasites by microscopy.

***Tritrichomonas foetus* growth medium.** A stock solution containing 250 ml concentrated meat broth, 1% Bacto Peptone (Difco), 51 mM sodium chloride, 27.8 mM maltose (Merck), 5.7 mM L-cysteine HCl (Sigma) and 0.1% agar no. 1, (Oxoid) was prepared. The stock solution was divided into 50 ml bottles and sterilised at 120°C for 20 min and stored at 4°C. Before use, 5 ml of inactivated horse serum and ascorbic acid were added to make a final concentration of 17 mM ascorbic acid.

PCR and sequencing of the SSU rDNA. The SSU rDNA fragment from *Spironucleus barkhanus* isolated from both wild and farmed Arctic charr was amplified using primers designed from the sequence of *S. barkhanus* SSU rDNA, GenBank accession number U93084 (Keeling & Doolittle 1997). The sequences of the primers used in sequencing are shown in Table 1.

The 50 µl reaction mix contained 3 µl of template DNA, 500 µM of each primer, 200 µM of each dNTP, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9 at room temperature) and 2.5 U *Taq* DNA polymerase (Amersham). The following thermal parameters were used to amplify the SSU rDNA fragments: initial denaturation at 95°C for 5 min, then 35 cycles of denatura-

Table 1. Sequence and position of PCR and sequencing primers used in this study

Primer name	Primer sequence (5'–3')	Position relative to GenBank accession no. U93084
Spiro-1f (PCR)	AAGATTAAGCCATGCATGCC	18–37
Spiro-2r (PCR)	GCAGCCTTGTTACGACTTCTC	1429–1449
Spironucleosis-1f (PCR)	TCATTTATCAGTGGTTAGTACATGC	88–112
Spironucleosis-2r (PCR)	TTCAAGCCTAACCACGACAAG	1219–1239
Spiro-3r (sequencing)	CATTGGGYAATYTYCGCGCCT	336–356
Spiro-4f (sequencing)	GAYTCYGGAGAVTGRGCAYGAG	285–306
Spiro-5r (sequencing)	TCCGTCAATMCYTTMAAGTTTC	856–877
Spiro-6f (sequencing)	AAGRYTGAAACTTKAARGKATTGACGG	850–876

tion at 95°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 45 s. After 35 cycles an extension step at 72°C for 4 min was included to ensure complete elongation of all products. All PCR products were purified using the Qiaquick PCR purification kit (Qiagen). Both strands of PCR products were sequenced using the PCR-primers and the internal sequencing primers for the SSU rDNA fragments. All products were sequenced using BigDye™ chemistry (Applied Biosystems) and analysed on an ABI Avant 3100 (Applied Biosystems).

Alignment/similarity. The AlignX component of Vector NTI Suite 8 software package (Informax) was used to align the SSU rDNA sequences. The alignment was manually checked for misaligned bases. Two SSU rDNA sequences from *Spironucleus barkhanus* (ATCC 50330, isolated from farmed Chinook salmon) accession numbers U93083.1 and U93084.1 were retrieved from GenBank and included in the alignment (types 1 and 2).

RESULTS

Primers Spiro-1f and Spiro-2r amplified a single fragment of parasite DNA from the wild fish isolate, but gave non-specific products when used on DNA extracted from the farmed fish isolate. These non-specific products comprised both a *Spironucleus* fragment (verified by sequencing) and fragments believed to be from fish host DNA. Specific amplification of *Spironucleus* DNA from farmed fish material was accomplished by using the primers Spironucleosis-1f and Spironucleosis-2r. These primers were also used on *Spironucleus barkhanus* isolated from wild fish but no products were obtained.

A gene fragment of approximately 1400 bp of the SSU rDNA of *Spironucleus barkhanus* was amplified, using primers Spiro-1f and Spiro-2r, from DNA isolated from both wild and farmed fish. A gene fragment of approximately 1200 bp of the SSU rDNA of *S. barkhanus* was amplified with primers Spironucleosis-1f and Spironucleosis-2r from DNA purified from blood and necrotic muscle tissue from 1 diseased farmed Atlantic salmon and 2 diseased farmed Arctic charr. An axenic culture of *S. barkhanus* was established from one of the wild charr. The culture was maintained for 2 mo prior to termination. DNA was isolated 3 times during this period and 1400 bp of the SSU rDNA were sequenced each time, but no variation was observed.

A sequence similarity table (Table 2) was constructed based on the alignment of 1154 positions of the *Spironucleus barkhanus* SSU rDNA sequences isolated from wild Arctic charr, farmed Arctic charr and farmed Atlantic salmon (GenBank accession numbers AY646679, AY677181 and AY677182, respectively)

DISCUSSION

Our results show that *Spironucleus barkhanus* isolated from systemically infected farmed Arctic charr is genetically different from *S. barkhanus* isolated from wild Arctic charr, but very similar to *S. barkhanus* isolated from farmed Atlantic salmon and Chinook salmon with systemic spironucleosis. Our results strongly suggest that the farmed Arctic charr have not been infected in the fresh water hatchery by parasites transmitted from wild relatives in the same water system. This conclusion is supported by the fact that *S. barkhanus* has not been found within the hatchery. The low degree of genetic variation between parasites isolated from the farmed Arctic charr, Atlantic salmon and Chinook salmon also supports this conclusion. Lake Fiskfjordvatn has a population of brown trout *Salmo trutta* L. in which hexamitid flagellates have not been found (Sommerhaug & Brun 1995, pers. obs.). There is also three-spined stickleback *Gasterosteus aculeatus* L. in the lake but these have never been examined for hexamitids. Nevertheless, based on the

Table 2. *Spironucleus barkhanus* SSU rDNA sequence similarities (%) between different isolates from farmed Chinook salmon *Oncorhynchus tshawytscha*, farmed Atlantic salmon *Salmo salar*, farmed Arctic charr and wild Arctic charr *Salvelinus alpinus*

	Farmed Chinook salmon, type 1	Farmed Chinook salmon, type 2	Farmed Atlantic salmon	Farmed Arctic charr[a]	Wild Arctic charr[b]
Farmed Chinook salmon, type 1	100	99.65	99.83	99.65	92.98
Farmed Chinook salmon, type 2		100	99.65	99.48	92.72
Farmed Atlantic salmon			100	99.65	92.89
Farmed Arctic charr				100	92.72
Wild Arctic charr					100

[a]No variation among parasites isolated from 2 fish
[b]No variation among parasites isolated from 6 fish

present results we believe that the farmed Arctic charr were infected after their transfer to seawater.

Based on the finding of *Spironucleus barkhanus* (identified by scanning and transmission electron microscopy) in wild anadromous charr, Sterud et al. (1998) hypothesised that these fish could have been the source of infection for Atlantic salmon infected with *S. barkhanus* in 1989 to 1991 (Poppe et al. 1992). Although we have shown that anadromous charr from lake Fiskfjordvatn are unlikely to be the infection source, we cannot reject this hypothesis as we have no genetic data on *S. barkhanus* from Arctic charr from other local populations of Arctic charr in the region. However, the genetic difference between the 2 isolates of *S. barkhanus* strongly suggests that one (or several) other species of anadromous or marine fish is the source of the pathogenic *S. barkhanus* isolate.

The variations seen within the farmed Chinook salmon isolate and similar variations seen in *Spironucleus vortens* Poynton, Fraser, Francis-Floyd, Rutledge, Reed & Nerad, 1995 have been suggested to be due to amplification of different copies of the rRNA gene (Keeling & Doolittle 1997). This may also explain the small genetic variations between the parasites isolated from farmed Artic charr, farmed Atlantic salmon and farmed Chinook salmon in the present study. The flagellate isolated from farmed Chinook salmon (Kent et al. 1992) has never been identified as *Spironucleus barkhanus* in any scientific publication. However Keeling & Doolittle (1996, 1997) suggested that the hexamitid flagellate from farmed Atlantic salmon and farmed Chinook salmon were variations of the same species based on sequencing of the α-tubulin gene. Our results confirm this suggestion based on the SSU rDNA sequences and indicate that systemic spironucleosis is caused by a specific strain of *S. barkhanus*. It was, however, demonstrated by Kent et al. (1992) that the Chinook salmon isolate ATCC 50330 could be transferred to Chinook salmon but not to Atlantic salmon under the same experimental conditions. This discrepancy may be due to differences in other genes than those sequenced to date, indicating the potential of several pathogenic strains of *S. barkhanus*. In Chinook salmon and Arctic charr, infection is located in the vascular system. In Atlantic salmon, the infection appears as parasite-filled abscesses in muscular and sub-cutaneous tissue, in addition to necrotic changes in the kidney, liver and spleen. (Kent et al. 1992, Sterud et al. 2003) These differences may be due to host factors rather than differences between the parasite isolates such as described by Woo (2001).

The differences observed in the SSU rDNA of *Spironucleus barkhanus* from farmed and wild fish are larger than those used to specify other diplomonads (Van Keulen et al. 1998). A separation of *S. barkhanus* into 2 species must however await a closer genetic comparison of additional genes and additional isolates of the parasite.

Acknowledgements. This work was supported by The Research Council of Norway, Grant No. 14656605. The authors would like to thank Duncan Colquhoun, Trygve Poppe, Peter Andreas Heuch and Øivind Øines for critical comments on the manuscript.

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Editorial responsibility: Carey Cunningham, Aberdeen, UK

Submitted: April 5, 2004; Accepted: August 5, 2004
Proofs received from author(s): November 9, 2004