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Phylogenetic analysis of the SSU rRNA gene from the piscine diplomonad *Spironucleus torosus* (Diplomonadida: Hexamitinae)

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Abstract. Previous studies have recorded *Spironucleus torosus* Poynton et Morrison, 1990 from several species of gadoid fishes, including the only freshwater gadoid, the burbot *Lota lota* (L.). Two morphologically different isolates of *S. torosus* have been described (elongate and pyriform). Both have been found in saltwater, while only the elongate has been found in freshwater. To address the conspecificity of the two morphs of *S. torosus*, and to identify the source of *S. torosus* in burbot in Norway, we have sequenced the small subunit ribosomal RNA (SSU rRNA) gene from 43 isolates of *S. torosus* from six species of gadoid fishes sampled at 15 localities in Norway, Sweden and the Baltic Sea. Phylogenetic analyses of the SSU rRNA gene sequence data recovered two major clades, one containing mainly isolates from burbot, while the other contained isolates from marine gadoid fishes only. The genetic distance (based on 25 nucleotide substitutions in 789 base pairs) separating the two assemblages was not large enough to consider the two groups separate species. *Spironucleus torosus* isolated from burbot displayed limited genetic variation in the small subunit ribosomal RNA (SSU rRNA) gene along the post-Pleistocene migration route of its host. The present study is the first report of *S. torosus* in tusk *Brosme brosme* (Ascanius), whiting *Merlangius merlangus* (L.), and fourbeard rockling *Enchelyopus cimbrius* (L.).

Four species of enteric diplomonad flagellates from gadoid fishes in Europe have previously been described by light microscopy. Early French studies reported *Hexamita capellani* Lavier, 1936 from poor cod *Trisopterus minutus* (L.), and *Hexamita motellae* Alexeieff, 1910 and *Hexamita phycidis* Lavier, 1936 from shore rockling *Gaidropsarus mediterraneus* (L.) (Alexeieff 1910, Lavier 1936). More recently, a Finnish (Fagerholm and Bylund 1997) study reported *Hexamita salmonis* (Moore, 1922) (valid name *Spironucleus salmonis* Poynton, Fard, Jenkins et Ferguson, 2004) from Atlantic cod *Gadus morhua* (L.) and burbot *Lota lota* (L.), the only freshwater gadoid, from the brackish waters of the Baltic Sea.

The first detailed study of enteric diplomonads from gadoids was done by Poynton and Morrison in 1990. Using transmission and scanning electron microscopy (TEM and SEM) they described *Spironucleus torosa* [sic] Poynton et Morrison, 1990 from the lower intestine of Atlantic cod and haddock *Melanogrammus aeglefinus* (L.) sampled in Canadian waters. Subsequent ultrastructural studies confirmed that *S. torosus* was also found in cod and saithe *Pollachius virens* (L.) (Sterud 1998a), and in burbot (Sterud 1998b) in Norwegian waters.

In the study by Poynton and Morrison (1990), two different morphs of *S. torosus* were recognized, one pyriform and one elongate. Both morphs were found in the Canadian cod and haddock. However, only the pyriform morph was found in the Norwegian cod and saithe (Sterud 1998a), and only the elongate morph was found in the burbot (Sterud 1998b). Sterud identified some ultrastructural differences between the *S. torosus* isolated from Norwegian marine gadoids and burbot, such as number of microtubules in flanges surrounding the posterio-lateral depressions, in the funis and in the infranuclear bands. However, since both morphs had been found in marine gadoids sampled in Canada, these variations were considered intraspecific. Sterud suggested that sequence data from the SSU rRNA gene could help to resolve any genetic differences between the two morphs, and possibly verify the Baltic Sea as the origin of *S. torosus* found in burbot in Norway.

Recognizing the limits of morphological approaches, Sterud (1998b) also noted that the parasite-host records based on only light microscopy need to be checked. He questioned the report by Fagerholm and Bylund (1997) of *H. salmonis* in cod and burbot from the Baltic Sea, and also the description of *H. capellani*, *H. motellae* and *H. phycidis* and recommended that their conspecificity with *S. torosus* should be investigated.

Recent studies have shown that there are genetically distinct, but morphologically indistinguishable, species in both *Spironucleus* (Jørgensen and Sterud 2004, 2006) and *Giardia* (Thompson and Monis 2004). Using the molecular tools developed in these previous studies, we wanted to answer the following questions put forward by Sterud (1998b). 1) Are the morphologically distinct

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S. torosus from Norwegian cod and burbot the same species? 2) Is the Baltic Sea the origin of *S. torosus* found in burbot in lakes in Norway? 3) What are the genetic differences between isolates of *S. torosus* from burbot along its post-Pleistocene migration route from the Baltic Sea into Norway taken some 5,000–7,000 years ago? 4) What is the true identity of the hexamitid flagellate, described as *H. salmonis* (syn. *S. salmonis*) by Fagerholm and Bylund (1997), from cod and burbot in the Baltic Sea?

We have therefore sequenced part of the SSU rRNA gene from *S. torosus* isolated from six species of gadoid fishes sampled at 15 localities in Norway, Sweden and the Baltic Sea. The SSU rDNA sequences we obtained were included in phylogenetic analyses to study the relationship between isolates of *S. torosus*.

MATERIALS AND METHODS

Sampling. *Spironucleus torosus* was collected from a total of 43 individual fish: tusk *Brosme brosme* (Ascanius) (n = 2), fourbeard rockling *Enchelyopus cimbrius* (L.) (n = 1), cod *Gadus morhua* (L.) (n = 11), burbot *Lota lota* (L.) (n = 26), whiting *Merlangius merlangus* (L.) (n = 1), and saithe *Pollachius virens* (L.) (n = 2). Fish sampled in the Baltic Sea, localities B1–B4 (Fig. 1), were caught by trawling, whereas fish from the other 11 localities were caught by angling. A list of fish and their sample localities, along with the GenBank accession numbers assigned to the resulting *S. torosus* sequences, are given in Table 1.

Isolation of DNA, PCR, and sequencing. All fish were killed by a sharp blow to the head. The lower part of the intestine (the rectum) was removed, cut open and preserved in 96% ethanol. One ml of ethanol-preserved intestinal contents were used for isolation of parasite DNA as described by Saghari Fard et al. (2007) using the QIAamp DNA Stool Mini Kit (Qiagen) protocol. Initial amplifications of the SSU rRNA gene were done using primers Spiro-1f (5'-AAG ATT AAG CCA TGC ATG CC-3') and Spiro-2r (5'-GCA GCC TTG TTA CGA CTT CTC-3') according to Jørgensen and Sterud (2004). This PCR produced multiple bands, and therefore had to be cloned using the TOPO TA cloning kit (Invitrogen) before sequencing. Twenty clones were picked, sequenced, and used in a BLAST search to identify the *Spironucleus* SSU rDNA sequence.

The sequence with the closest match to other *Spironucleus* SSU rDNA from fish was identified and used to construct a new primer set, Torosa-f (5'-CTC TTG AGT GAG GTA GTC ACC AGC-3') and Torosa-r (5'-GTG TCC GAA TAA CTC ACC AAA ACC-3') which produced specific products. A set of internal sequencing primers were also constructed to ensure overlapping sequences in both directions, Torosaseq-f (5'-CCT GGG GGA TTA CGC TTG CA-3') and Torosaseq-r (5'-TCA CTC GTC GGT CAA TCC TT-3'). The primers Spiro-5 and Spiro-6 described by Jørgensen and Sterud (2004) were also used for sequencing. All PCR products were sequenced using the DYEnamic ET dye terminators, and analysed on a MegaBACE (1000) analysis system (GE Healthcare). All sequencing products were purified using Autoseq™ G-50 columns (GE Healthcare).

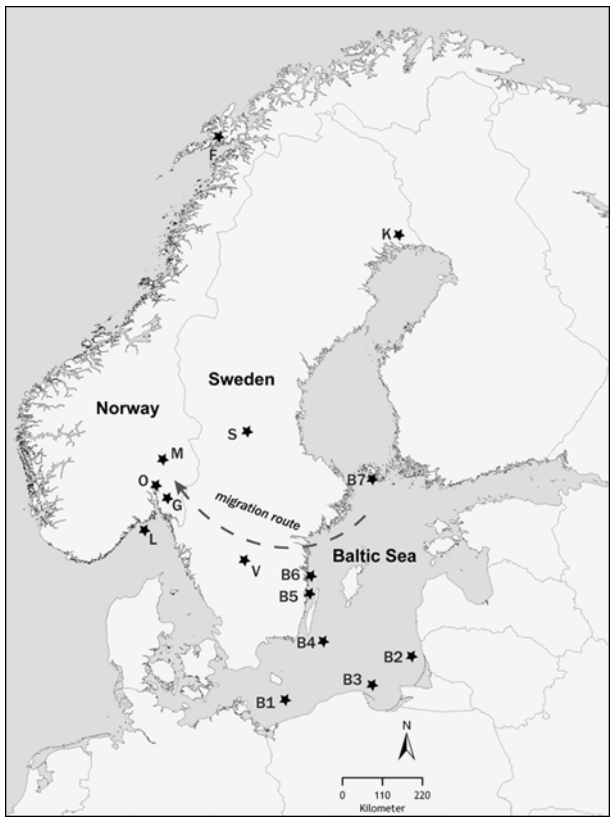

Fig. 1. Map of Northern Europe showing sampling localities. The arrow indicates the post-Pleistocene migration route of burbot. B – Baltic Sea; F – Fiskfjorden; G – Glomma; K – Kalix; L – Langesund; M – Mjøsa; O – Oslofjorden; S – Siljan; V – Vättern.

Cloning. To check for intraspecific sequence variation, PCR products from the SSU rRNA gene of *S. torosus* from each of the six species of fishes (1–4 isolates per species, see Table 1), were subjected to cloning using TOPO TA cloning kit. Varying amounts of clones were picked and the first 500 bp of the cloned PCR product were sequenced in both directions. The number of clones sequenced for each of the 13 cloned isolates is listed in Table 1.

Phylogeny. The SSU rDNA sequences were aligned using BioEdit (Hall 1999) and manually inspected to ensure correct alignment. Gaps, and positions adjacent to gaps, that could not be accurately aligned, were removed. The SSU rDNA sequences from *Spironucleus vortens* isolated from both angelfish *Pterophyllum scalare* (Schultze) and ide *Leuciscus idus* (L.) (GenBank accession numbers U93085 and EF050056, respectively) were used as an outgroup to root the tree. The alignment was subjected to phylogenetic analyses using minimum evolution (ME), maximum parsimony (MP) and maximum likelihood (ML). All analyses were conducted using PAUP* (Swofford 2002). The hierarchical nested likelihood ratio test implemented in Modeltest (Posada and Crandall 2001) was used to select the best-fit model of nucleotide substitution. The ML analysis was based on the K80 model (Kimura 1980) of nucleotide substitution. A gamma-shaped distribution was used to correct for rate variation (8 rate categories) at different sites. The shape parameter of the gamma

Table 1. Samples and sequences of *Spironucleus torosus* sampled from six gadoid species caught in Norway, Sweden and the Baltic Sea. All samples are listed under the name of their host. Samples are named after host and sample localities. For cloned isolates, number of clones sequenced is indicated.

Sample/sequence names (cf. Fig. 1)	Sample localities	GenBank accession number	Number of clones sequenced
Brosme brosme			
B. brosme 1 F	Fiskfjorden	EF173492	5
B. brosme 2 L	Langesund	EF173491	15
Enchelyopus cimbrius			
E. cimbrius 1 B1	Baltic Sea	EF173488	6
Gadus morhua			
G. morhua 1 B4	Baltic Sea	EF173525	8
G. morhua 2 B4	Baltic Sea	EF173523	–
G. morhua 3 B4	Baltic Sea	EF173526	–
G. morhua 4 B4	Baltic Sea	EF173527	4
G. morhua 5 B1	Baltic Sea	EF173518	–
G. morhua 6 B2	Baltic Sea	EF173519	–
G. morhua 7 B3	Baltic Sea	EF173520	–
G. morhua 8 F	Fiskfjorden	EF173521	–
G. morhua 9 O	Oslofjorden	EF173522	17
G. morhua 10 O	Oslofjorden	EF173524	–
G. morhua 11 O	Oslofjorden	EF173510	–
Lota lota			
L. lota 1 B5	Baltic Sea	EF173506	21
L. lota 2 B6	Baltic Sea	EF173509	12
L. lota 3 B7	Baltic Sea	EF173513	–
L. lota 4 C	Kalix	EF173484	–
L. lota 5 C	Kalix	EF173487	–
L. lota 6 C	Kalix	EF173485	3
L. lota 7 C	Kalix	EF173486	–
Lota lota			
L. lota 8 C	Kalix	EF173489	–
L. lota 9 C	Kalix	EF173490	–
L. lota 10 G	Glomma	EF173502	–
L. lota 11 G	Glomma	EF173503	–
L. lota 12 G	Glomma	EF173493	–
L. lota 13 V	Vättern	EF173516	–
L. lota 14 V	Vättern	EF173517	–
L. lota 15 V	Vättern	EF173514	–
L. lota 16 V	Vättern	EF173515	–
L. lota 17 M	Mjøsa	EF173501	19
L. lota 18 M	Mjøsa	EF173507	–
L. lota 19 M	Mjøsa	EF173508	–
L. lota 20 M	Mjøsa	EF173504	–
L. lota 21 S	Siljan	EF173495	–
L. lota 22 S	Siljan	EF173496	–
L. lota 23 S	Siljan	EF173497	–
L. lota 24 S	Siljan	EF173498	–
L. lota 25 S	Siljan	EF173499	–
L. lota 26 S	Siljan	EF173500	–
Merlangius merlangus			
M. merlangus 1 O	Oslofjorden	EF173494	14
Pollachius virens			
P. virens 1 F	Fiskfjorden	EF173512	12
P. virens 2 F	Fiskfjorden	EF173511	3

distribution, α, was estimated to be 0.521454. The transitions versus transversions ratio (Ti/Tv) was estimated to be 1.9. The same settings were used in the ME analysis. The MP analysis was conducted using default settings in PAUP*. A heuristic tree search was performed with 100 random taxa additions for both MP and ML analyses. The tree bisection and reconnection (TBR) algorithm was used for branch-swapping. For the sequenced clones only a ME analysis were conducted. Bootstrap resampling of 500 replications were used for ML analysis, while a 1,000 replications were used for ME and MP analyses to examine the confidence of the nodes within the resultant tree topologies.

RESULTS

Phylogenetic analyses

Minimum evolution and MP analyses based on an alignment of partial (789 bp) SSU rRNA gene sequences from 43 isolates (no clones included) of *S. torosus*, recovered two major clades; A and B (Fig. 2). Clade A contained isolates of *S. torosus* mainly from burbot, while clade B contained isolates of *S. torosus* from only marine gadoids. The genetic distance separating groups A and B were based on 25 substitutions. The genetic distance separating sub-groups a1 and a2 were based on 12 substitutions, while the distance separating sub-groups b1 and b2 were based on three substitutions only.

Maximum likelihood analysis of the same dataset recovered the same groups as presented in Fig. 2; however, the branching order differed. Group a2 appeared basal to a clade containing groups a1 and B. This topology did not receive any support from the ML bootstrap analysis where all the burbot isolates in group a1 appeared as a basal polytomy together with the two other groups, a2 and B. The polytomy was given 100% support. The ML bootstrap support for groups a2 and B are indicated in Fig. 2.

Clones

Varying amounts of *S. torosus* SSU rDNA clones were sequenced (see Table 1) from 1–4 individual fish of each species. When the cloned sequences were included in the ME analysis, a similar topology to the tree presented in Fig. 2 was recovered (data not shown). However, some differences were observed; some of the clones obtained from the isolate "*G. morhua* 9 O" were recovered in group b1 (previously only b2), and one of the clones obtained from "*B. brosme* 2 L" was found in group a2 (previously only b1). None of the 84 clone sequences from cod, saithe, whiting, fourbeard rockling, and tusk was found within the 55 sequenced clones from burbot and vice versa.

New host-parasite records

The present study is the first report of *S. torosus* in tusk, whiting, and fourbeard rockling. *Spironucleus torosus* was identified in burbot sampled at 7 new localities, one in Norway (M, Fig. 1), three in Sweden (K, V and S, Fig. 1), and three in the Baltic Sea (B5–B7, Fig. 1). In addition, *S. torosus* was also identified in marine gadoids at 7 new localities, three along the

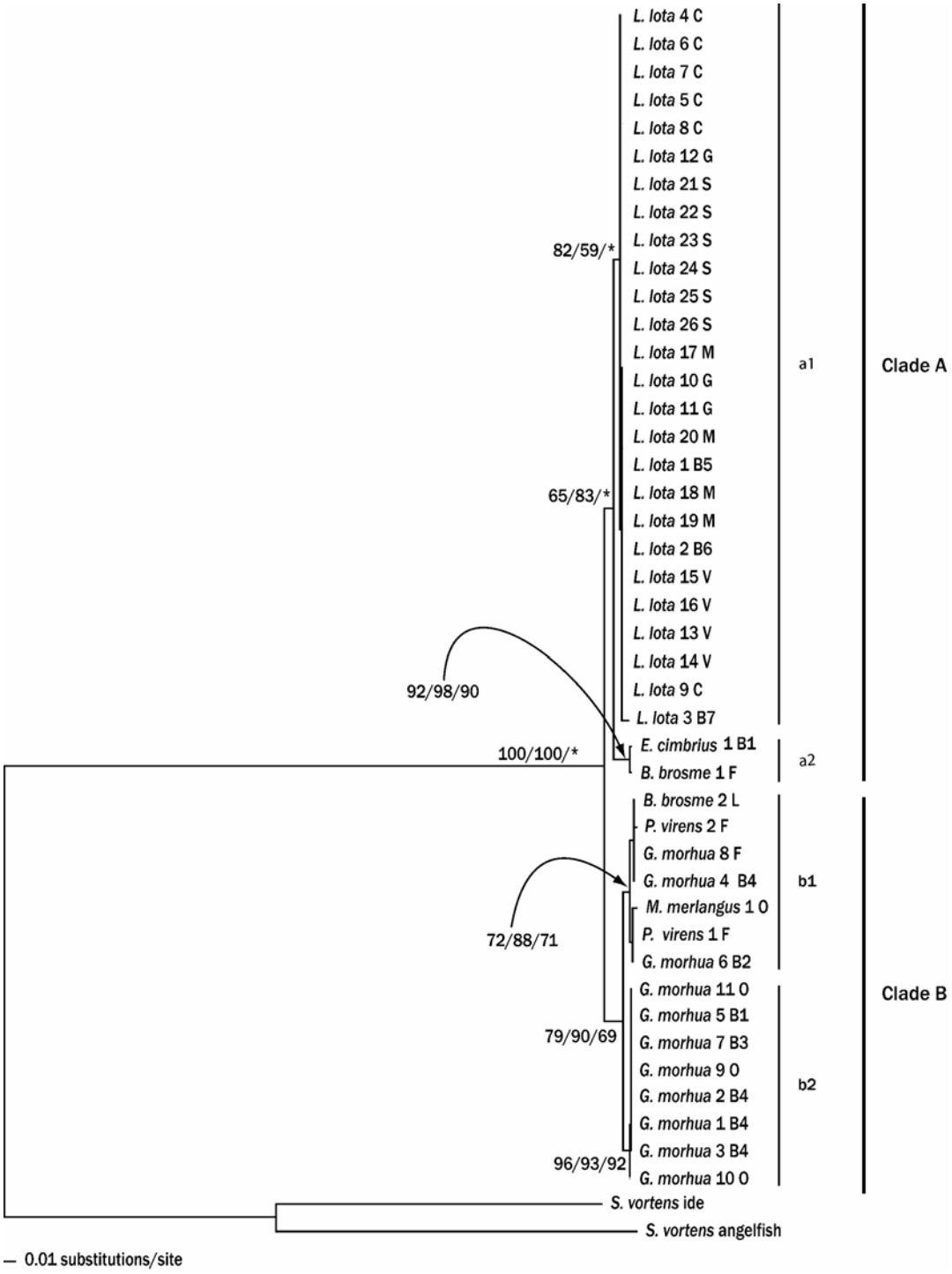

Fig. 2. Minimum evolution analysis of *Spironucleus torosus* isolates based on partial small subunit ribosomal RNA gene sequences. Bootstrap support values calculated using minimum evolution, maximum parsimony and maximum likelihood are indicated at each node, respectively. Branchings different from the minimum evolution analysis are indicated with an asterisk.

Norwegian coast (F, L and O, Fig. 1) and four in the Baltic Sea (B1–B4, Fig. 1), thus expanding our knowledge of the Eastern and Northern geographic range of *S. torosus* that was previously restricted to Halifax in Canada (Poynton and Morrison 1990), the river Glomma and the fjord Oslofjorden in Norway (Sterud 1998a, b). This demonstrates a widespread geographic distribution of this hexamitid flagellate in both burbot and marine gadoids.

DISCUSSION

Phylogenetic analyses of the SSU rDNA sequence data recovered two major clades, A and B (Fig. 2), separated by 25 substitutions. Theses two clades may correspond to the two different morphs of *Spironucleus torosus* (elongate and pyriform shape) (Poynton and Morrison 1990, Sterud 1998a).

The samples *L. lota* 1, *L. lota* 2 and *L. lota* 3 were sampled at localities in the Baltic Sea (B5–B7, Fig. 1) where sympatric populations of burbot and cod exist (brackish water), enabling exchange of parasites between these hosts. All burbot samples (n = 3) and clones (n = 33) contained the same genotype of *S. torosus* (group a1) as did the burbot sampled in freshwater lakes in Norway (7 samples and 19 clones) and Sweden (16 samples and 3 clones). We were not able to sample marine gadoids at these localities; however, *S. torosus* was isolated from cod (7 samples and 12 clones) caught at other locations in the Baltic Sea. These samples did not contain any of the genotypes found in burbot, indicating that the burbot and cod we sampled in the Baltic Sea was infected by distinct genetic lineages of *S. torosus*, which do not cross-infect.

One isolate from fourbeard rockling (*E. cimbrius* 4 B4, Baltic Sea) and one isolate from tusk (*B. brosme* 1F, Northern Norway) contained a genotype (group a2) of *S. torosus* that was closely related to the burbot genotype (group a1), only separated by 12 substitutions. Cloning of the tusk isolate, *B. brosme* 2L, showed that genotype a2 was present in one of 15 clones while the other 14 clones were all genotype b1. This may reflect the observation by Poynton and Morrison (1990) that some marine gadoids contained both of the two morphs of *S. torosus*. In contrast, the observed variation may only reflect the natural genetic variation in *S. torosus* caused by adaptation to different hosts. A solution to the "genotype/morphology problem" could be to combine fluorescent in situ hybridisation (FISH) and SEM. Differently marked probes designed to specifically hybridize with SSU rDNA genotypes from each of the two major clades (recovered in the present phylogenetic analyses) could then be applied to faecal samples from both burbot and marine gadoids. The signal from the two probes would indicate the presence of one or two genotypes in the sample. A parallel of each sample should subsequently be checked using SEM to verify the presence of one or two morphs.

The genotype of *S. torosus* found in burbot appears to be host specific and was not identified in any of the samples or clones from marine gadoids, and vice versa. Unfortunately some cloning reactions produced no or few *S. torosus* SSU rDNA clones, although cloning experiments were repeated several times. Increased sampling of clones and isolates may have shown that the *S. torosus* genotypes from burbot and other gadoids do cross-infect. Furthermore, the genetic distance separating the two major clades is much shorter than the genetic distance separating other closely related diplomonads (Jørgensen and Sterud 2006). Thus, we consider all isolates/genotypes presented in this study as *S. torosus*. However, sequence data from other genes may change this conclusion.

In a study by Sterud (1998b) it was suggested that data from the SSU rRNA gene could be used to determine if the Baltic Sea was the origin of the *S. torosus* isolate found in burbot in Norwegian lakes. Our genetic data suggest that this is correct, as the sequences obtained from *S. torosus* isolated from burbot sampled in Norway, Sweden and the Baltic Sea are essentially identical (Fig. 2, group a1). No genetic variation in the SSU rRNA gene was observed within *S. torosus* from burbot sampled along its migration route from the Baltic Sea, through Sweden into Norway. However, the SSU rRNA gene probably does not evolve fast enough so that we would able to detect genetic differences between populations only separated by 5,000-7,000 years. Other molecular markers, such as microsatellites need to be employed to further adress this subject.

Sterud (1998b) presented the possibility that *S. torosus* in burbot may have been recently transferred from cod via katadromous eel *Anguilla anguilla* L. or the anadromous Atlantic salmon *Salmo salar* L. and/or trout *Salmo trutta* L. The genetic distance (based on 25 substitutions) separating the isolates from burbot (Fig. 2, group a1) and cod (Fig. 2, group B) rules out this possibility for *S. torosus*.

Sterud (1998b) also suggested that the report of *Hexamita salmonis* (valid name *Spironucleus salmonis*) from cod and burbot in the Baltic Sea by Fagerholm and Bylund (1997) was wrong, and that these hexamitid flagellates were actually *S. torosus*. Fagerholm and Bylund (1997) based their findings on light microscopy only, a method shown to be unsuitable for species identification of diplomonads (Poynton and Sterud 2002). We have in the present study sampled the same species of gadoid fishes as Fagerholm and Bylund (1997) and our data confirm the interpretation made by Sterud (1998b).

Although the present study has resolved the former uncertain identification, some other questions remain about diplomonads in gadoid fishes. We were unfortunately not able to sample shore rockling and poor cod to check the possible conspecificity of their diplomonads *Hexamita motellae* and *H. phycidis* with *S. torosus*. However, we have identified *S. torosus* in three additional species of gadoid fishes (fourbeard rockling, tusk and whiting), thus demonstrating a wide host range of *S. torosus*. This wide distribution of *S. torosus* in gadoids suggests that *H.* motellae *and H. phycidis* are conspecific with *S. torosus*.

We have now demonstrated that *S. torosus* constitutes two major genetic clades. One clade contained isolates from marine fishes only, while the other clade contained mainly isolates of *S. torosus* from burbot. These two clades may correspond to the previously

described pyriform and elongate morphs of *S. torosus*. We hope that future studies combining SEM and FISH methodology will help to answer this question. Based on the limited genetic distance separating the major clades recovered, we did not suggest separate species status of members of any of these clades. To further resolve the phylogenetic relationship within *S. torosus*, analyses of additional genes such as glutamate dehydrogenase and α-tubulin should be undertaken. Increased sampling of marine gadoid species and burbot originating from other glacial refugia, may add valuable information to both gadoid and *S. torosus* phylogeny.

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