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A parvicapsulid (Myxozoa) infecting *Sprattus sprattus* and *Clupea harengus* (Clupeidae) in the Northeast Atlantic uses *Hydroides norvegicus* (Serpulidae) as invertebrate host

Marianne K oie¹, Egil Karlsbakk^{2,3}, Ann-Cathrine B ardsgj are Einen² and Are Nylund³

¹Marine Biological Laboratory, University of Copenhagen, Helsing r, Denmark;

²Institute of Marine Research, Bergen, Norway;

³Department of Biology, University of Bergen, Norway

Abstract: A myxosporean producing actinospores of the tetractinomyxon type in *Hydroides norvegicus* Gunnerus (Serpulidae) in Denmark was identified as a member of the family Parvicapsulidae based on small-subunit ribosomal DNA (SSU rDNA) sequences. Myxosporean samples from various Danish and Norwegian marine fishes were examined with primers that detect the novel myxosporean. *Sprattus sprattus* (Linnaeus) and *Clupea harengus* Linnaeus (Teleostei, Clupeidae) were found to be infected. The sequences of this parvicapsulid from these hosts were consistently slightly different (0.8% divergence), but both these genotypes were found in *H. norvegicus*. Disporic trophozoites and minute spores of a novel myxosporean type were observed in the renal tubules of some of the hosts found infected through PCR. The spores appear most similar to those of species of *Gadimyxa* K oie, Karlsbakk et Nylund, 2007, but are much smaller. The actinospores of the tetractinomyxon type from *H. norvegicus* have been described previously. In GenBank, the SSU rDNA sequences of Parvicapsulidae gen. sp. show highest identity (82%) with *Parvicapsula minibicornis* Kent, Whitaker et Dawe, 1997 infecting salmonids (*Oncorhynchus* spp.) in fresh water in the western North America. A phylogenetic analysis places *P. minibicornis* and Parvicapsulidae gen. sp. in a sister clade to the other parvicapsulids (*Parvicapsula* spp. and *Gadimyxa* spp.).

Keywords: Myxozoa, Parvicapsulidae, *Clupea harengus*, *Sprattus sprattus*, actinospores, life cycle, Denmark, Norway

Many two-host life cycles of freshwater myxozoans are known (see Yokoyama et al. 2012), but only five marine life cycles have been elucidated (K oie et al. 2004, 2007, 2008, Rangel et al. 2009, Karlsbakk and K oie 2012). These represent members of the genera *Ceratomyxa* Th elohan, 1892; *Ellipsomyxa* K oie, 2003; *Gadimyxa* K oie, Karlsbakk et Nylund, 2007; and *Sigmo-myxa* Karlsbakk et K oie, 2012, all belonging to the marine clade of Myxosporea (Fiala 2006). These develop actinospores of the tetractinomyxon type in their polychaete hosts. Recently, a markedly different actinosporean type, unicapsulactinomyxon, was discovered in the polychaete *Diopatra neapolitana* Delle Chiaje from Portugal (Rangel et al. 2011). Based on its SSU rDNA sequence, this unidentified myxosporean is most closely related to *Enteromyxum* spp., which also belong to the marine clade of Myxosporea.

In this study we present evidence suggesting that the marine polychaete *Hydroides norvegicus* Gunnerus (Polychaeta, Serpulidae) from the northern  resund, Denmark, is the invertebrate host of a novel parvicapsulid parasite infecting the urinary system of sprat *Sprattus*

sprattus (Linnaeus) and Atlantic herring *Clupea harengus* Linnaeus.

MATERIALS AND METHODS

From Denmark, 200 specimens of sprat *Sprattus sprattus* (10–15 cm in total length) and 100 specimens of the Atlantic herring *Clupea harengus* (15–20 cm in length) were examined. The fishes were trawled in the northern  resund (55 58'N, 12 40'E) at 15–20 m, September–December 2007 and 2008. From Norway, samples were taken from sprat from Hardanger (60 24'56"N, 6 31'29"E), western Norway (N = 2; 9.5–12.5 cm in length) in August 2008 and from Vefsnfjorden, northern Norway (six sprat 6.4–8.2 cm long; two herring 12.8–13.1 cm long) (65 56'36"N, 12 42'12"E) in November 2011. The latter specimens of the two clupeids were caught in the same trawl haul. Some fish were examined fresh, but most were frozen immediately upon capture.

Kidney samples for DNA were stored in 96% ethanol. Squash preparations were made from various parts of the kidney and examined at 1000  magnification for myxosporean spores and trophozoites. The Vefsnfjorden samples could not be examined at high magnification due to the movement and vibrations in the research vessel (RV 'Helmer Hansen') at sea. Myxospore

Table 1. Variable and ambiguous positions in partial SSU rDNA sequences of Parvicapsulidae gen. sp. from sprat, herring and *Hydroides norvegicus*. Positions given with reference to JX136956.

Sequence isolate	GenBank Accession No.	Host	Geno- type	Positions														
				320	472	480	505–6	508	519	535–6	547	575–6	1175	1184	1208	1218	1231	1590
HyAug	HQ588150	<i>Hydroides</i>	B	C	G	T	TY	C	A	GG	C	CC	G	A	T	T	A	T
HySep	HQ588151	<i>Hydroides</i>	B	C	G	T	TY	C	A	GG	C	CC	G	A	T	T	A	T
OrSpr	HQ588153	Sprat	B	C	G	T	TY	C	A	GG	C	CY	K	A	T	K	A	T
OrSprNy	HQ588154	Sprat	B	C	G	T	TY	C	A	GG	C	CC	G	A	T	T	A	T
Spr-2	JX136962	Sprat	B	C	G	T	TY	C	A	GG	C	CC	T	A	T	K	A	T
Bri-1	JX136955	Sprat	B	C	G	T	TY	C	A	GG	C	CC	T	A	T	G	A	T
Bri-2	JX136956	Sprat	B	C	G	T	TY	C	A	GG	C	CY	T	A	T	G	A	T
Bri-3	JX136957	Sprat	B	C	G	T	TC	C	A	GG	C	CC	K	A	T	K	A	T
Bri-4	JX136958	Sprat	B	C	G	T	TC	C	A	GG	C	CC	K	A	T	G	A	ou
Bri-5	JX136959	Sprat	B	C	G	T	TY	C	A	GG	C	CC	K	A	T	K	A	T
Bri-6	JX136960	Sprat	B	C	G	T	TT	C	A	GG	C	CY	K	A	T	K	A	T
BriK-2	JX136961	Sprat	B	C	G	T	TY	C	A	GG	C	CC	T	A	T	G	A	ou
Hy07	HQ588152.2	<i>Hydroides</i>	B+S	Y	R	Y	WY	C	W	RK	Y	YC	K	R	Y	D	R	Y
Hy1911	HQ588149	<i>Hydroides</i>	S	T	A	C	AT	C	W	AT	T	TC	T	G	C	A	G	C
Mu-1	JX136963	Herring	S	T	A	C	AT	C	W	AT	T	TC	T	G	C	A	G	C
Mu-2	JX136964	Herring	S	T	A	C	AT	C	W	AT	T	TC	T	G	C	A	R	C
Clm-3	JX136965	Herring	S	T	A	C	AT	A	T	AT	T	TC	ou*	ou	ou	ou	ou	ou

*ou = outside. Additional ambiguous positions: Y at position 516 in Bri-6 only, Y at position 605 in Hy1911 and Mu-1, Y at position 1164 in Mu-1 and Mu-2

measurements are based on six specimens from sprat and four specimens from herring, all from Denmark. Since the rare and small myxospores were not found in stained smears, micrographs of the myxospores are deposited in the Zoological Museum, Copenhagen, Denmark, Catalog No ZMUC-MYX-005 (photo-vouchers).

Specimens of *Hydroides norvegicus* were collected on their common substrate, the bivalve *Aequipecten opercularis* (Linnaeus). The bivalves were dredged in the northern Øresund at 25 m, throughout the years 2001–2008. Some polychaetes were examined immediately upon capture; others were kept in aquaria provided with aerated, filtered seawater from a recirculation water system (30 ‰ S, 10 °C).

DNA was extracted from kidney pieces or pieces of actinospore-infected *H. norvegicus* using the DNeasy® Tissue Kit protocol for animal tissues (Qiagen, Hilden, Germany). The polymerase chain reaction (PCR) primers used to obtain partial small-subunit (SSU) ribosomal RNA gene (rDNA) sequences were MarF1/RosR2 (annealing temperature 56 °C), MyxF2/Myxgen4R (57 °C) (see Køie et al. 2008). Two sequences were slightly extended at the 5' end using the primers Ecf (see Nylund et al. 2005) and HydrR4 (57 °C). The primer pair HydrF2/Hydr-R4 (54 °C) was designed from the partial SSU rDNA sequence of the myxosporean infecting *H. norvegicus* and used for the examination of kidney pieces of various marine fishes for infections with the parasite. These primers have sequences 5'-ACTAAACTGCCTGTCGGTTAAG-3' and 5'-CCCACAACCAGAACTTACA-3' respectively, and amplify a 267 bp product.

The PCR amplifications were performed in a total volume of 50 µl using 2 µl of template DNA and a reaction mixture consisting of 10-µl 5× PCR buffer, 3 µl 25 mM MgCl₂, 5 µl 10 mM dNTP, 2 µl (10 mM) of the reverse and forward primers, 2 U of thermostable DNA polymerase (GoTaq), and 26 µl dH₂O. The PCR conditions were as previously described (Køie et al. 2008). The PCR products were cleaned with ExoSAP-IT® (Affymetrix Inc.) and then sequenced using the BigDye (3.1) Terminator Se-

quencing kit. The sequencing was done using the amplification primers.

The sequence data were assembled with the Vector NTI Advance™ 11 software (Invitrogen Corp., Carlsbad, CA), and GenBank searches were done with BLAST (2.0). Alignments were obtained with Clustal W (AlignX, Vector NTI). Ambiguous signals occurred in the SSU rDNA sequences. These were registered if the secondary peak was consistently present in multiple reads and showed half or more of the signal of the major signal at that position.

The phylogenetic relationship between Parvicapsulidae gen. sp. and related Myxosporea was examined using Maximum Likelihood (ML) (MEGA 5; 500 bootstrap replicates) (Tamura et al. 2011) and Bayesian inference (BI) (MrBayes 3.1.2; 1 × 10⁶ generations, every 100th tree saved) (Ronquist and Huelsenbeck 2003). Sequences were aligned using AlignX, and manually edited in Genedoc. Hypervariable or ambiguous regions were deleted in order to achieve comparison of homologous positions. In the sequence of *Sinuolinea* sp. (GenBank accession number AF378346) the first 450 bp were removed (see Holzer et al. 2010). The best model (AICc) of nucleotide substitution was GTR I+Γ according to both MEGA5 and jModelTest (0.1.1). *Ceratomyxa* spp. were selected as outgroup based on previous studies (Fiala 2006, Bartošová et al. 2011). Trees with the same topology were obtained with MEGA5 and MrBayes.

The possible association of parasite genotype with fish host species was examined with the Fisher exact test using the Statistica 10 software (StatSoft, Tulsa, USA).

RESULTS

SSU rDNA sequences

Myxosporean partial SSU rDNA sequences were first obtained from tetractinomyxon-infected *Hydroides norvegicus* collected in January 2002 (sequence isolate

Hy1911), and again from three worms collected in August (HyAug) and September 2007 (Hy07 and HySep). Two slightly different sequences (genotypes) were obtained, designated as type B and S, differing at 12 sites (99.2% identity, 1531 sites compared). In addition, ambiguous signals occurred at five sites (positions 506, 516, 576, 1175, 1218) in the type B but not in our type S sequences, and at four sites (positions 519, 605, 1164, 1231) in the type S sequences but not observed in those of type B (Table 1). Isolates HySep and HyAug represented type B, and Hy1911 type S. A fourth sequence from an infected *H. norvegicus* (Hy07) showed ambiguous signals at all the 12 sites used to distinguish the types B and S (a mixed B and S signal) (Table 1).

In fishes, a type B sequence was first obtained from a kidney sample of a sprat (sequence isolate OrSpr) collected in northern Øresund in November 2007, and later verified by sequencing from a second kidney sample obtained from the same fish (whole fish stored at -20 °C). This fish was infected with minute myxospores (described below) in the kidney. Sequences from two additional sprat from Denmark infected with the same type minute myxospores were subsequently obtained (isolates OrSprNy, BriK-2). Additional type B sequences were obtained from: (i) a sprat from Hardanger (western Norway) examined because it had myxosporean trophozoites in the Bowman's space of renal corpuscles (isolate Spr-2) and (ii) from 6 kidney samples of sprat collected in Vefsnfjorden, northern Norway (isolates Bri-1 to Bri-6).

A type S sequence was first obtained from a herring infected with the minute type myxospores (isolate Clm-3). A second herring infected with the minute type myxospores was also PCR positive. Subsequently type S sequences were obtained from kidney samples of two juvenile herring from northern Norway (Mu-1 and Mu-2).

Phylogenetic affinities

The sequences of Parvicapsulidae gen. sp. showed highest similarity with sequences of *Parvicapsula minibicornis* Kent, Whitaker et Dawe, 1997 in BLAST searches, and sequence comparisons show 82 and 88% identity with and without gaps, respectively. Fig. 1 shows the phylogenetic position of Parvicapsulidae gen. sp. among other parvicapsulids and selected members of the marine clade of Myxosporea. Parvicapsulidae gen. sp. is placed in a well supported clade together with *P. minibicornis*, *Parvicapsula* sp. genotype X and *Sphaerospora testicularis*. This clade represents a sister group to the other parvicapsulids (Fig. 1).

Morphology of myxospores

No morphological differences were apparent between myxospores from sprat and herring. In total, only 30 myxospores were found in the two herring and three

Fig. 2

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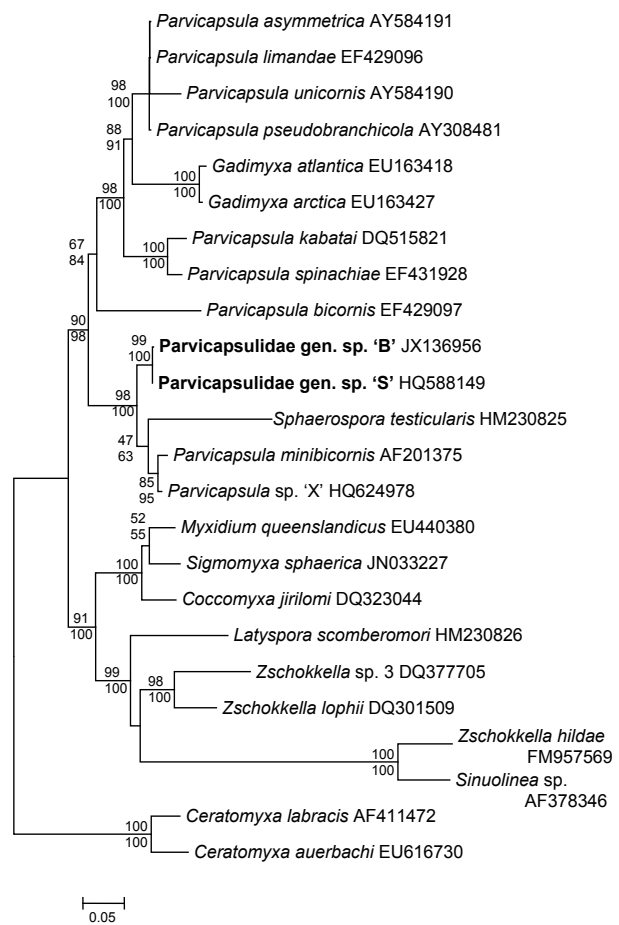


Fig. 1. Phylogenetic position of Parvicapsulidae gen. sp. as inferred from SSU rDNA data by using the maximum likelihood method (MEGA 5) ($lnL = -7713.26$). The analysis involved 24 nucleotide sequences and 1024 positions. Nodal supports (%) are indicated for ML (upper) and BI (lower, posterior probabilities), unless both were < 50%.

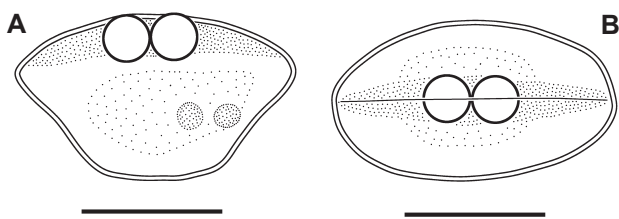


Fig. 2. Myxospores of Parvicapsulidae gen. sp. **A** – frontal view; **B** – apical view. Scale bar = 2 µm.

sprat. The description (measurements are in micrometres, N = 10) is based on myxospores from both hosts.

Spores ovoid in valvular view, wider than long; anterior end flattened, posterior more or less semicircular. Spores elliptical in apical view and bilaterally symmetrical along an inconspicuous sutural line. Two spherical polar capsules close to each other, in middle of most flattened side of spore. Spores smooth and thin-walled. Anterior end

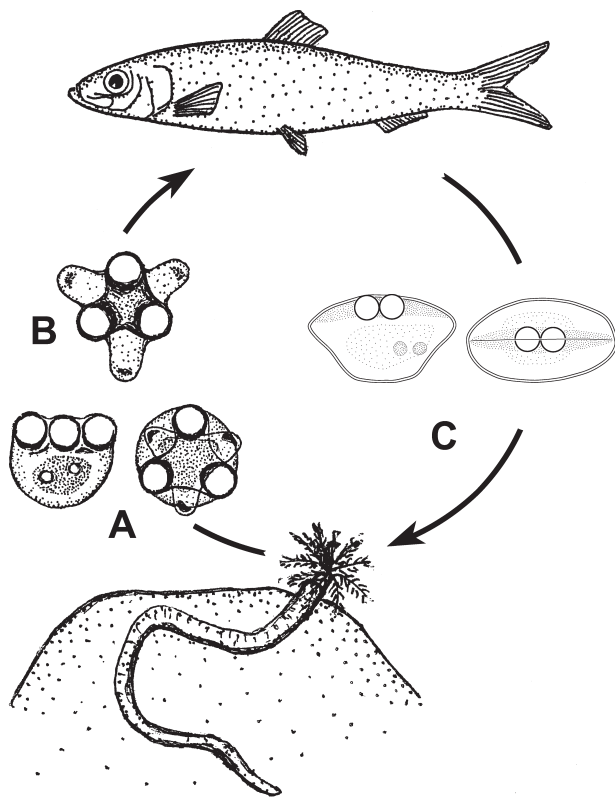


Fig. 3. Schematic illustration of the life cycle of Parvicapsulidae gen. sp. The polychaete *Hydroides norvegicus* (below) acts as the invertebrate host. The Atlantic herring, *Clupea harengus*, and sprat, *Sprattus sprattus* (above), act as the fish hosts. A – recently released actinospores; B – actinospore with slightly inflated episporous; C – myxospores, frontal and sutural views. Not to scale.

of spore slightly convex in valvular view; posterior side slightly angular. Spores 2.3–2.8 long, 4.0–4.4 wide and 2.3–2.6 thick. Polar capsules 0.6–0.7 in diameter. A thickening follows inconspicuous sutural line and attenuates close to slightly pointed ends of spore. Number of coils of polar filament unknown (coils not visible).

Fig. 3 shows a schematic diagram of the proposed life cycle of the parvicapsulid.

DISCUSSION

Partial SSU rDNA sequences of the myxosporean producing tetractinomyxon actinospores in the polychaete *Hydroides norvegicus* (see K oie 2002) were throughout several years compared with sequences of myxosporeans from potential fish hosts. A myxosporean sequence from a kidney sample from a sprat infected with *Ortholinea orientalis* (Shul'man et Shul'man-Albova, 1953) matched the sequences from the actinospores. However, we were unable to obtain the sequence from the actinospores in *H. norvegicus* in several *O. orientalis* – infected herring. Another and completely unrelated myxosporean sequence was consistently obtained from *O. orientalis* – infected

herring and sprat, and identified with that parasite (see Karlsbakk and K oie 2011).

Careful examination of a sprat individual, which was PCR positive for the parvicapsulid found in *H. norvegicus*, led to the discovery of the minute myxospores of Parvicapsulidae gen. sp. Subsequently, other clupeid specimens found infected with this parasite by microscopy were found PCR positive for the *H. norvegicus* myxosporean. In the large numbers of clupeids examined, there was no evidence for additional myxosporean infections in the kidneys.

The present results suggest a high degree of intraspecific variation in the SSU rDNA of Parvicapsulidae gen. sp., which mostly can be allocated to two genotypes (B and S). The ten sequences from sprat are of type B, the three from herring are of type S. The occurrence of these genotypes in their respective host species significantly depart from random (Fisher's exact test, $P < 0.01$). The sequences from *H. norvegicus* show either B or S signal or a mixed B/S signal.

These observations suggest that the tetractinomyxon spores in *H. norvegicus* represent a myxosporean infecting herring and sprat. The only candidate is the myxosporean found herein producing minute spores in the kidney. While unsuitable fish hosts may be PCR positive for a myxosporean species following exposure to actinosporeans (see Holzer et al. 2013), clupeids were the only species found infected with Parvicapsulidae gen. sp. of the fish species common in  resund. These clupeid hosts have been also found infected over a large geographic area, during different times of the year, and infections revealed by PCR and sequencing are associated with a particular type of myxospores. Indeed, each clupeid species is infected with a characteristic genotype of the myxosporean. Hence the proposed two-host cycle is well supported (Fig. 3).

Observed divergence between the B and S type sequences is at 0.8%, and these genotypes are significantly associated with host-species. These observations suggest that these clupeids are infected with separate strains or possibly sibling species of Parvicapsulidae gen. sp. However, morphological differences were not apparent between the minute myxospores found in sprat and herring, or between actinospores of the B and S genotypes from *H. norvegicus*. This polychaete is obviously host to both the types that infect sprat and herring, but in one of the four examined worms a mixed B/S signal was observed. We interpret this as a mixed infection with the B and S types. The occurrence of such mixed infections in the annelid host without evidence for parasite hybridization may be taken as support for a status of independent species for the sprat and herring parvicapsulids. Since the vertebrate hosts are now known, these aspects may be examined experimentally in *H. norvegicus*.

The closest relative of the parvicapsulid reported here is *Parvicapsula minibicornis*. Interestingly, in that species, there is also evidence for fish-host species associated genotypes of the parasite, particularly clear in coho salmon *Oncorhynchus kisutch* (Walbaum) (Atkinson et al. 2011). The divergence between *P. minibicornis* SSU rDNA genotypes was found to be 0.1–1.1%. In the only other myxosporean found in the urinary system of herring and sprat, *O. orientalis*, a possible host-related sequence divergence was also noted (Karlsbakk and Køie 2011), but more sequences are needed.

Phylogenetic analyses place Parvicapsulidae gen. sp. in a well supported clade together with *P. minibicornis*, *Parvicapsula* sp. genotype X and *Sphaerospora testicularis* Sitjà-Bobadilla et Alvarez-Pellitero, 1990. The *Parvicapsula* sp. genotype X sequence was obtained from water samples and polychaete infections in the western USA, and its fish host and myxospores are unknown (Atkinson et al. 2011). However, the three known species show symmetrical spores, which set them apart from most other parvicapsulids (i.e. *Parvicapsula* spp.).

P. minibicornis, *P. pseudobranchicola* and *S. testicularis* have been found histozoic at other sites than the typical sites in kidney, pseudobranch and testes, respectively (Sitjà-Bobadilla and Alvarez-Pellitero 1990, Sterud et al. 2003, Bradford et al. 2010). The possibility that Parvicapsulidae gen. sp. may infect other sites than the kidney in herring and sprat deserves further study, because we found few myxospores in our kidney samples.

The minute spores of Parvicapsulidae gen. sp. can be distinguished from all described species by the small size and by morphology. It mostly resembles species of *Gadimyxa* Køie, Karlsbakk et Nylund, 2007, but it

apparently has not two morphological spore forms. Sequence comparisons and phylogenetic analysis show that *Gadimyxa* spp. and Parvicapsulidae gen. sp. are not closely related.

Parvicapsulidae gen. sp. appears to represent a new species of a new genus in the Parvicapsulidae, most closely related to *P. minibicornis* according to the SSU rDNA sequences. However, we refrain from erecting a new genus and species since the morphology, development and microhabitat of the novel parasite are insufficiently known.

Myxospores were found twice in *C. harengus* (prevalence 2%; N = 100) and three times in *S. sprattus* (1.5%; N = 200) from Øresund, Denmark using microscopy. However, prevalence is probably much higher because spores may have been overlooked. All the clupeids from northern Norway examined by PCR were positive for the parasite (N = 8), suggesting a high prevalence of infection there. The prevalence of the actinosporean developmental stages (Køie 2002) in *H. norvegicus* varied between 3 and 8% (about 50 specimens examined each year).

The present observations represent the sixth marine myxozoan for which both the polychaete and fish hosts are known (Køie et al. 2004, 2007, 2008, Rangel et al. 2009, Karlsbakk and Køie 2012). When these hosts are known, experimental studies may be performed in order to reveal details of the life cycles. Experimentally infected clupeids would ease the study of the very small myxospores and the description of this enigmatic myxosporean.

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