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Hematodinium sp. (Alveolata, Syndinea) detected in marine decapod crustaceans from waters of Denmark and Greenland

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ABSTRACT: Five decapod crustacean species were examined for presence of the parasitic dinoflagellate *Hematodinium* spp. (Alveolata, Syndinea) by morphological methods (colour and pleopod methods) as well as by PCR and nested PCR with *Hematodinium*-specific primers. *Nephrops norvegicus*, *Pagurus bernhardus* and *Liocarcinus depurator* were sampled by trawling in Danish waters and *Chionoecetes opilio* and *Hyas araneus* were sampled by trapping off the west coast of Greenland. The existence of *Hematodinium* has not previoiusly been documented in Danish waters, but it was detected in all 3 decapod species examined in the present study. *Hematodinium* sp. was also detected for the first time in *H. araneus* and the existence of *Hematodinium* sp. in Greenlandic *C. opilio* was documented by PCR. Analyses of 26 *Hematodinium* sp. ITS1 sequences, including sequences from all 5 host species sampled, revealed more than 95% sequence similarity between 24 of the sequences. Two *Hematodinium* sp. ITS1 sequences from *C. opilio* were only 81% similar to the 24 other ITS1 sequences. The nested PCR approach resulted in the highest reported percentages of positive samples for *Hematodinium* sp.in the hosts investigated (between 45 and 87.5%). However, no decapods were found to be infected with *Hematodinium* sp. based on morphological methods. Consequently, *Hematodinium* sp. may be more common than previously believed, and, assuming that the DNA found originated from viable and infectious parasite cells, infections may not always be fatal. We suggest that the hosts investigated may have been subject to latent infections that could develop into a fatal disease only if the hosts were physiologically stressed due to other factors.

KEY WORDS: *Hematodinium* · Latent infections · ITS1 · Parasitic dinoflagellates · PCR detection

INTRODUCTION

Several crustacean decapods are parasitized by dinoflagellates of the genus *Hematodinium* (Stentiford & Shields 2005). This genus was first described by Chatton & Poisson (1931) with the type-species *H. perezi* infecting 2 different hosts, *Carcinus maenas* and *Liocarcinus depurator*, off the coast of France. *Hematodinium* spp. have since been recorded worldwide from a broad array of decapod hosts, including commercially and ecologically important species (Stentiford & Shields 2005). Infection with *Hematodinium* sp. is often referred to as bitter crab disease (BCD, Meyers et al. 1987) or pink crab disease (PCD, Stentiford et al. 2002).

Morphological examinations of parasite cells (Meyers et al. 1987, Field et al. 1992, Stentiford & Shields 2005) suggested the existence of several *Hematodinium* species and molecular and biochemical analyses have indicated that at least 2 groups of *Hemato-*

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dinium sequences may warrant species status (Small et al. 2007b,c, Jensen et al. 2010). Different *Hematodinium* genotypes were related to different host species (Small et al. 2007b, Hamilton et al. 2010, Jensen et al. 2010) and geographical regions (Hamilton et al. 2010). It is also clear, however, that a single *Hematodinium* genotype may infect more than one host species (Hamilton et al. 2007, 2010, Small et al. 2007a, Jensen et al. 2010). However, so far only one other species of *Hematodinium* has been formally described, namely *H. australis*, which was described on the basis of morphology, host species, and austral location (Hudson & Shields 1994).

In some host species, advanced infections lead to a discoloration of the carapace from reddish-brown to a reddish 'cooked' appearance (e.g. in *Nephrops norvegicus* and *Chionoecetes opilio*) and this may be used for detection of the disease (colour method; Meyers et al. 1987, Field et al. 1992). Rapid logarithmic growth of parasitic cells in the host's haemolymph causes a colour change from bluish-transparent to milky-white (e.g. Shields & Squyars 2000) and aggregation of parasitic cells occurs. In *N. norvegicus* and *Callinectes sapidus*, such aggregation can be seen when a pleopod is removed and observed under an inverted microscope (pleopod method, Field et al. 1992). Both morphological detection methods can only detect advanced infections and, therefore, underestimate parasite prevalence (Stentiford et al. 2001). For parasite detection, additional histological (Messick 1994, Messick & Shields 2000), immunological (Stentiford et al. 2001, Small et al. 2002) and molecular methods (Hudson & Adlard 1994, Gruebl et al. 2002, Small et al. 2006) are available that are also able to detect low-level (low parasite numbers in the host) infections. However, a staging of disease progression was only established for the pleopod method (Field et al. 1992, Field & Appleton 1995), which fails to detect latent and sub-patent infections (Stentiford et al. 2001). Therefore, an evaluation of disease status from low-level latent infections to apparent, patent peak infections is not yet possible.

Severely infected crabs are moribund and probably die due to oxygen depletion, organ dysfunction or secondary infections (Meyers et al. 1987). Due to seasonal prevalence of up to 85% (e.g. *Callinectes sapidus*, Shields et al. 2003) in specific areas, *Hematodinium* spp. can have significant effects on host populations and economic losses for commercial crustacean fisheries are thought to be considerable (e.g. Field & Appleton 1995, Wilhelm & Miahle 1996). Epizootics in commercially exploited stocks are documented for *Chionoecetes opilio* (Taylor & Khan 1995), *Chionoecetes bairdi* (Meyers et al. 1987), *Callinectes sapidus* (Newman & Johnson 1975), *Nephrops norvegicus* (Field et al. 1992), *Cancer pagurus* (Stentiford et al. 2002) and *Necora puber* (Wilhelm & Boulo 1988).

In the present study, 5 different decapod host species were examined for the presence of *Hematodinium* spp. DNA by PCR and nested PCR. Furthermore, samples of *Chionoecetes opilio* and *Nephrops norvegicus* were examined by the colour method and samples of *N. norvegicus* were also examined by the pleopod method to compare morphological and molecular methods. Positive PCR products were sequenced and the *Hematodinium* sp. sequences obtained were compared and examined for regional and/or host-related differences.

MATERIALS AND METHODS

Samples of *Chionoecetes opilio* and *Hyas araneus* were collected during a cruise between June 4 and 20, 2007, in the Sisimiut area off the west coast of Greenland (Fig. 1A). Crabs were caught by means of traps baited with cephalopods, with 10 traps in a line at each station. All samples of *C. opilio* (approximately 14 000) were examined by the colour method and 100 randomly selected crabs (5 stations with 20 crabs each) were also examined by nested PCR (Standard PCR, n = 40). Correlations between samples showing positive for *Hematodinium* sp. and sample stations of *C. opilio* were analysed with a chi-square test, and between percentages of *Hematodinium* sp.-positive samples and depth or water temperature by Spearman's ρ test. Statistical analyses were performed using the software package SPSS.

Samples of *Hyas araneus* (n = 20) were examined solely by nested PCR. Samples of *Pagurus bernhardus* (n = 10) were collected by trawling in The Sound, Denmark, on April 16, 2007 (Fig. 1B). *Nephrops norvegicus* (n = 72) and *Liocarcinus depurator* (n = 8) were caught by trawling on May 30, 2007, in the Kattegat, Denmark (Fig. 1B). All *L. depurator* and *P. bernhardus* samples were examined by nested PCR and *P. bernhardus* specimens additionally by standard PCR. Out of 72 *N. norvegicus* samples, 52 were examined by standard PCR and 20 by nested PCR, and all *N. norvegicus* samples were also examined by the colour and pleopod methods. For the pleopod method, the 3rd pleopod on the right side (ventral, caudal view) was removed and examined under an inverted microscope for aggregation of parasite cells (Field et al. 1992). For PCR, 1 ml of haemolymph and a piece of heart tissue (ca. 5 mm^3) were removed from each crab, stored in 300 µl of 2× 1% N-cetyl N,N,N-trimethylammonium bromide (CTAB) and frozen at –20°C until further treatment.

Cell destruction was performed with a TissueLyser (Qiagen Retsch) using silica beads (haemolymph sam-

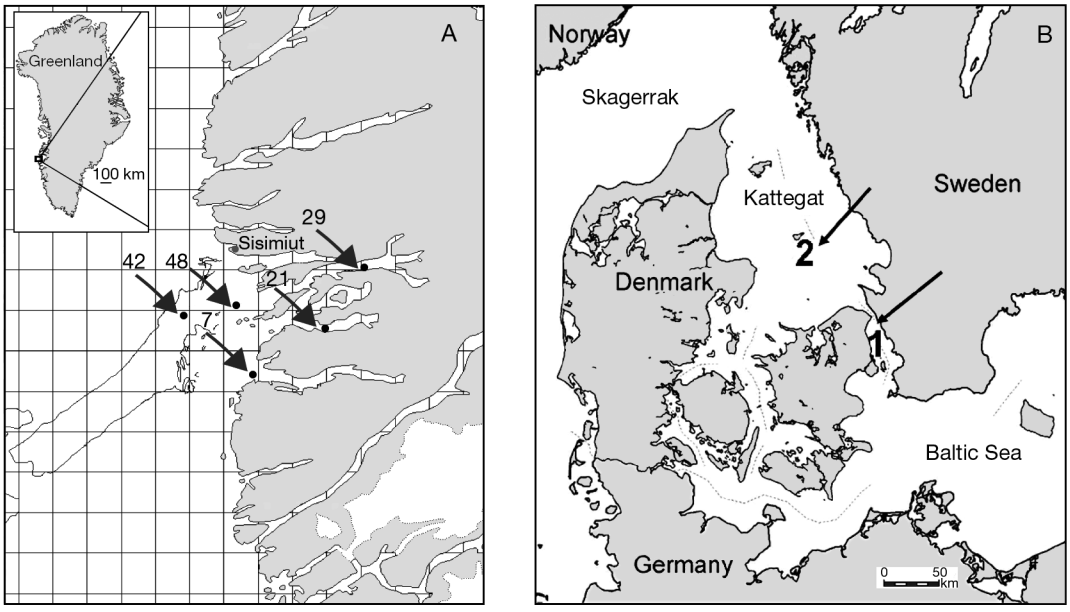

Fig. 1. Sampling sites to detect presence of *Hematodinium*. (A) Greenland: *Chionoecetes opilio* and *Hyas araneus*. (B) Denmark: *Pagurus bernhardus* (Stn 1), and *Nephrops norvegicus* and *Liocarcinus depurator* (Stn 2)

ples) or metal beads (heart tissue samples). DNA was extracted with the CTAB protocol (Doyle & Doyle 1987) and finally resolved in 30 µl TE-buffer (10 mM TrisHCl, 1 mM EDTA, MilliQ H_2O). For the PCR, the *Hematodinium*-specific primer pair 18SF2 and ITSR1 (Small et al. 2006) was used, which amplifies parts of the 18S rRNA gene and partial ITS1 (380 bp). The nested PCR was conducted using primers Hemat1487F (Gruebl et al. 2002) and 5′-CGC ATT TCG CTG CGT TCT TC R (Hudson & Adlard 1994) in the first PCR (~680 bp) and 18SF2 (Small et al. 2006) and Hem3R (5′-TAA CCC GAG CCG AGG CAT TCA) in the second PCR (480 bp). The primer Hem3R was designed based on known *Hematodinium* sp. sequences from GenBank. It anneals to the 5′ end of the 5.8S rRNA gene and, thereby, amplifies the entire ITS1 if used together with 18SF2. A search with the basic local alignment search tool (BLAST) showed that the Hem3R primer sequence only matched well with *Hematodinium* spp. sequences. The specificity of the primer was checked by performing a nested PCR on extract of the closest known relative of *Hematodinium*, *Syndinium turbo*. This reaction yielded no detectable product.

PCR reactions were performed using 2 µl of template DNA (or autoclaved, distilled water as negative control), 5 µl of each 10 µM primer, 0.1 µl *Taq*-polymerase (Ampliqon), 20 µl of 0.5 mM dNTP mix, 5 µl buffer (TrisHCl, pH 8.5, $MgCl_2$, NH_4SO_4, 2-mercaptoethanol), 5 µl tetramethylammonium chloride ($C_4H_{12}NCl$) and 8 µl autoclaved distilled water (total volume, 45.1 µl). PCR conditions were as follows: an initial denaturation step at 94°C for 1 min followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and chain extension at 72°C for 2 min. This was finalized with an extension step at 72°C for 6 min. Nested PCR reactions were done as described above, but 0.2 µl from the first PCR were used as template and the number of cycles was decreased to 30. Aliquots of 4 µl from each PCR reaction were checked for amplification products using 1.5% (w/v) ethidium bromide (EtBr)-stained agarose gels and a Kodak EDAS 290 apparatus for subsequent image analysis.

A crab was considered to be *Hematodinium* sp.-positive if either the PCR product of the haemolymph or the heart tissue sample, or both, showed a bright band of the appropriate length (standard PCR, 380 bp; nested PCR, 480 bp). Several amplification products from all examined hosts were purified using NucleoFast 96 PCR plates (Clontech-Takara Bio Europe) and bidirectionally sequenced by Macrogen (Korea), with the same primers as used for the PCR. All sequences were verified as being from *Hematodinium* sp. (BLAST searches) and then manually edited with BioEdit (Hall 1999). Sequence parts belonging to the 18S and 5.8S rRNA genes were removed after comparing with a sequence from the closest known relative of *Hematodinium* (*Syndinium turbo*, Skovgaard et al. 2005) and the remaining ITS1 sequences were compared pairwise using MEGA4 (Tamura et al. 2007). Sequence similarities were calculated as percentages of identical bases. Evolutionary distances between ITS1 sequences obtained in the present study and sequences taken

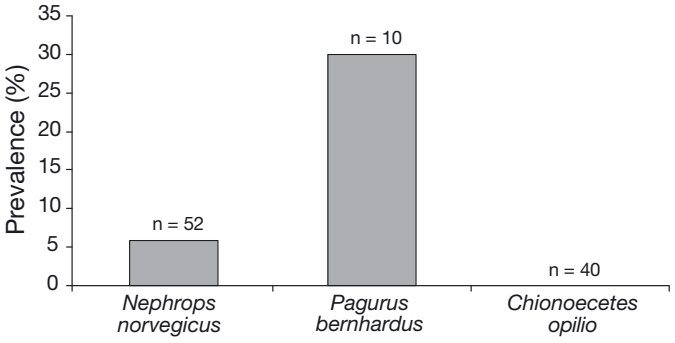

Fig. 2. *Hematodinium* spp. Percentages of *Hematodinium*-positive samples detected by standard PCR using primers 18SF2 and ITSR1

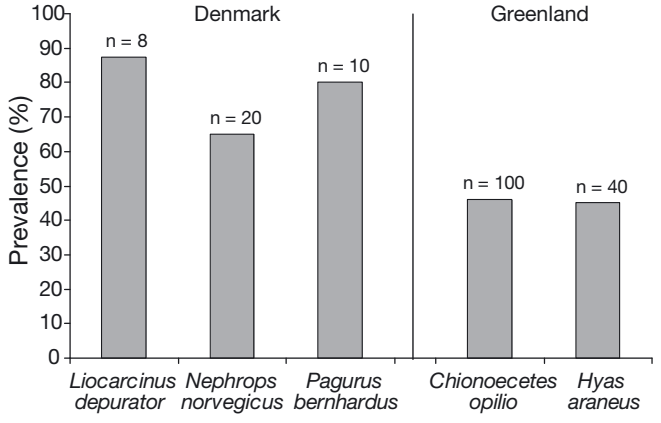

Fig. 3. *Hematodinium* spp. Percentages of *Hematodinium*-positive samples detected by nested PCR for the 5 host species

from GenBank (Clade B as defined by Jensen et al. 2010, and sequences from each subclade defined by Hamilton et al. 2010) were estimated with an alignment of 13 sequences (4 obtained in the present study and 9 taken from GenBank) using MEGA4 (Tamura et al. 2007), whereby positions containing gaps were eliminated from the data set. There were a total of 289 positions in the final alignment (excluding gap-containing positions). A pairwise alignment and comparison were made using BioEdit v. 7.0.9 in which positions containing gaps were included.

RESULTS

Colour and pleopod method

Based on the colour method, none of the collected *Chionoecetes opilio* (n = 14 000) and *Nephrops norvegicus* (n = 72) revealed indications of *Hematodinium* sp. infection. Samples of *N. norvegicus* were also examined by the pleopod method without detecting any parasite cells in the haemolymph.

PCR detection

With the standard PCR approach, prevalence of *Hematodinium* sp.-positive samples was 0% for *Chionoecetes opilio* (n = 40), 30% for *Pagurus bernhardus* (n = 10) and 5.8% for *Nephrops norvegicus* (n = 52) (Fig. 2). A few samples, e.g. samples 2b and 7a of *P. bernhardus*, produced weak double bands (see Fig. 4), which could not be accounted for. With the nested PCR

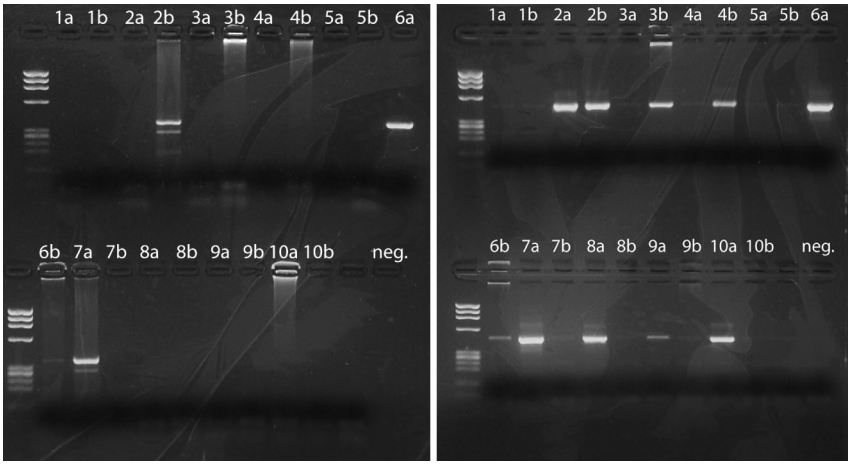

Fig. 4. *Pagurus bernhardus*. Comparison of standard PCR and nested PCR performed on the same 10 host individuals. The left gel shows the standard PCR and the right gel the nested PCR. Bands of molecular weight ladder represent 1353, 1078, 872, 603, 310, 281, 271, 234, 144 bp (from top to bottom). a: haemolymph sample; b: heart tissue sample; neg.: negative control

approach, the prevalence of *Hematodinium* sp.-positive samples was estimated as follows: 87.5% for *Liocarcinus depurator* (n = 8), 65% for *N. norvegicus* (n = 20), 46% for *C. opilio* (n = 100), 45% for *Hyas araneus* (n = 40) and 80% for *P. bernhardus* (n = 10) (Figs. 3 & 4). All *P. bernhardus* samples and 40 out of 100 *C. opilio* samples were examined with both PCR approaches. Samples of *N. norvegicus* were also examined with both PCR approaches, but in this case standard PCR and nested PCR were done on samples from different host specimens.

Distribution of *Hematodinium* sp.-positive samples in *Chionoecetes opilio*

There was a significantly higher detection frequency of *Hematodinium* sp.-positive samples detected by nested PCR in specimens of *Chionoecetes opilio* collected at inshore sample stations in comparison with offshore samples (chi-square test, p = 0.05): percentages of *Hematodinium* sp.-positive hosts were 3-fold higher at the inner stations (Fig. 1A, Table 1). The water depth at sampling stations ranged from 206 to 421 m and bottom temperature ranged from −1.0 to 2.3°C. There were no apparent correlations between percentages of *Hematodinium* sp.-positive samples and either depth (Spearman's ρ test: correlation coefficient = 0.4, p = 0.505, n = 5) or water temperature (Spearman's ρ test: correlation coefficient = −0.6, p = 0.285, n = 5) (Table 1).

Sequence similarities

A total of 31 PCR products of the appropriate length (ca. 480 bp, partial 18S, complete ITS1, partial 5.8S) were sequenced to determine whether they represented *Hematodinium* sp.; 26 of these sequences were without ambiguous positions and were deposited in GenBank. Five of the 31 sequences revealed several doubtful base pairs and were eliminated from further analysis. Five different ITS1 sequences were found (Fig. 5). Accession numbers, host species and sample location for the sequences are listed in Table 2.

Twenty-four ITS1 sequences (Fig. 5, sequence numbers 1, 3, 4, 5), including sequences from all 5 hosts, showed more than 95% similarity when compared pairwise. Two sequences from *Chionoecetes*

Table 1. *Hematodinium* sp. Sampling stations categorized as inner (i), edge (e) and offshore (o) stations with the respective depth and temperature. Prevalence of *Hematodinium* sp. is given as percentage of *Hematodinium* sp.-positive samples generated by nested PCR. All stations, n = 20

Station	*Hematodinium* sp. prevalence (%)	Temperature (°C)	Depth (m)
21 (i)	60	−1.0	261.5
29 (i)	85	1.2	347.5
7 (e)	40	−0.5	259
42 (o)	35	2.3	421
48 (o)	10	2.1	206

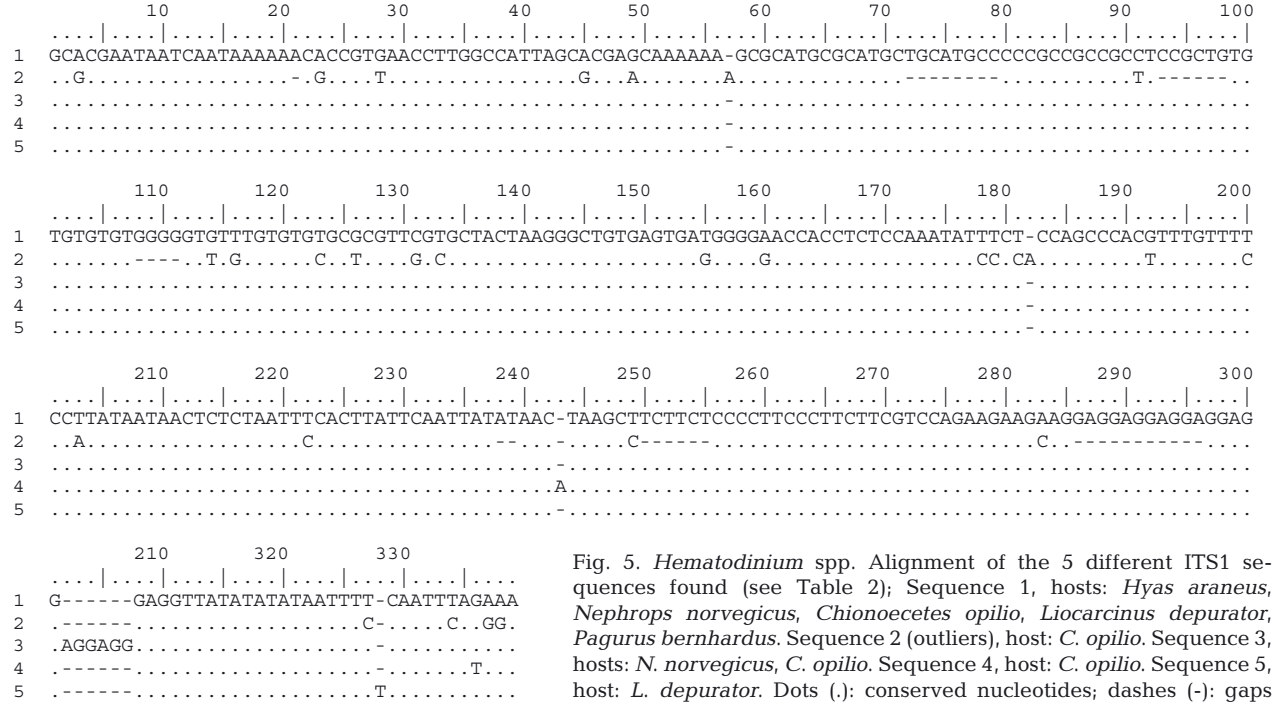

Fig. 5. *Hematodinium* spp. Alignment of the 5 different ITS1 sequences found (see Table 2); Sequence 1, hosts: *Hyas araneus*, *Nephrops norvegicus*, *Chionoecetes opilio*, *Liocarcinus depurator*, *Pagurus bernhardus*. Sequence 2 (outliers), host: *C. opilio*. Sequence 3, hosts: *N. norvegicus*, *C. opilio*. Sequence 4, host: *C. opilio*. Sequence 5, host: *L. depurator*. Dots (.): conserved nucleotides; dashes (-): gaps

Table 2. *Hematodinium* sp. ITS1 sequences. Host species, accession number, number of identical sequences obtained (n) and sample locations for the 5 different sequences numbered 1 to 5 in Fig. 5. Seq. no. = sequence number

Seq. no.	Host	Accession number(s)	n	Sample location
1	*Hyas araneus*	FJ172636, FJ172644	16	Greenland
	Pagurus bernhardus	FJ172637		Denmark
	Chionoecetes opilio	FJ172639–40 FJ172646–49		Greenland
	Liocarcinus depurator	FJ172654, FJ172660–61		Denmark
	Nephrops norvegicus	FJ172655–58		Greenland
2	*C. opilio*	FJ172642–43	2	Greenland
3	*N. norvegicus*	FJ172645	6	Denmark
	C. opilio	FJ172641, FJ172650–53		Greenland
4	*C. opilio*	FJ172638	1	Greenland
5	*L. depurator*	FJ172659	1	Denmark

opilio (FJ172642 and FJ172643) only showed 81% similarity (Fig. 5, sequence number 2) to the other 24 sequences. These 2 sequences were more than 99% similar to each other. Six sequences (from the hosts *Hyas araneus* and *C. opilio*) showed an insertion of an AGG AGG at position 302–307 (Fig. 5, sequence number 3). A single sequence from *C. opilio* (Fig. 5, sequence number 4) exhibited a single A as insertion at position 243 and a G–T substitution at position 336. Another sequence from *Liocarcinus depurator* (Fig. 5, sequence number 5) revealed an insertion of a T at position 328. There was no obvious relationship between the type of *Hematodinium* sp. ITS1 sequence and host species or sampling area. The estimation of evolutionary distance of 13 *Hematodinium* sp.-ITS1 Clade B (sensu Jensen et al. 2010) sequences showed a close relation between the first 9 sequences (≥94% similarity, Table 3) comprising the hosts *L. depurator*, *H. araneus*, *Pagurus bernhardus, Nephrops norvegicus, Carcinus maenas*, *Cancer pagurus* and *C. angulatus*. Sequences 10 and 11 (hosts *P. prideaux* and *Munida rugosa*) showed similarities ≥89% to the first 9 sequences, whereas sequences 12 and 13 (host *C. opilio*) showed only 77 to 81% similarity to the first 9 sequences and 76 to 78% similarity to sequences 10 and 11 (Table 3).

DISCUSSION

Morphological methods

Many surveys have relied on the colour method (e.g. Meyers et al. 1990, Taylor & Khan 1995) or the pleopod method (e.g. Field et al. 1992, Field & Appleton 1995) to estimate the infection level of *Hematodinium* spp. in crustaceans. However, comparison between the colour method and haemolymph wet smears did reveal that the colour method underestimates the apparent infection level and only detects late stage infections (Meyers et al. 1990). The pleopod method can detect earlier stages of infections (Field et al. 1992) and a classifica-

Table 3. *Hematodinium* sp. Estimates of evolutionary divergence between *Hematodinium* sp. ITS1 sequences. Results are based on the pairwise analysis of 13 sequences. Lower left below diagonal: The number of base differences per sequence from analysis between sequences in an alignment of all 13 sequences conducted in MEGA4. Positions containing gaps were eliminated from the data set. There was a total of 289 positions in the final data set. Upper right above diagonal: Sequence identity; sequences were aligned and compared pairwise with BioEdit v. 7.0.9. Positions containing gaps were included in the data set

GenBank no.	Host species	Table ID	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
FJ495187	*Pagurus bernhardus*	(1)		1.00	0.95	0.95	0.97	0.97	0.98	0.94	0.96	0.92	0.90	0.78	0.78
EU096208	*Nephrops norvegicus*	(2)	0		0.95	0.95	0.97	0.97	0.98	0.94	0.96	0.92	0.90	0.78	0.78
FJ172644	*Hyas araneus*	(3)	0	0		1.00	0.98	0.98	0.95	0.99	0.96	0.97	0.94	0.81	0.81
FJ172660	*Liocarcinus depurator*	(4)	0	0	0		0.98	0.98	0.95	0.99	0.96	0.97	0.94	0.81	0.81
EF675761	*Carcinus maenas*	(5)	0	0	0	0		1.00	0.97	0.97	0.98	0.94	0.92	0.80	0.80
FJ844426	*Chionoecetes angulatus*	(6)	0	0	0	0	0		0.97	0.97	0.98	0.94	0.92	0.80	0.80
EF031974	*N. norvegicus*	(7)	0	0	0	0	0	0		0.94	0.96	0.92	0.89	0.77	0.77
EF032012	*P. bernhardus*	(8)	1	1	1	1	1	1	1		0.96	0.96	0.94	0.80	0.80
FJ495186	*Cancer pagurus*	(9)	5	5	5	5	5	5	5	6		0.95	0.93	0.79	0.79
EU096218	*P. prideaux*	(10)	11	11	11	11	11	11	11	12	8		0.97	0.78	0.78
EU096217	*Munida rugosa*	(11)	19	19	19	19	19	19	19	18	14	8		0.76	0.77
FJ172643	*Chionoecetes opilio*	(12)	29	29	29	29	29	29	29	30	32	38	42		0.99
FJ172642	*C. opilio*	(13)	29	29	29	29	29	29	29	30	32	38	42	0	

tion system for the progress of the disease was established (Field et al. 1992, Field & Appleton 1995). However, in the present study apparent *Hematodinium* sp. infections were not detected with any of the applied morphological methods, despite the fact that many *Hematodinium* sp.-positive samples were detected by running PCR on samples derived from the same host individuals. Probably no crabs were infected heavily enough for detection by any of the morphological methods. In fact, all crab specimens examined appeared healthy and it is unknown whether they would subsequently reveal symptoms of *Hematodinium* sp. infection.

Prevalence of *Hematodinium* sp.

This is the first report of *Hematodinium* in Danish waters. With nested PCR, *Hematodinium* was detected in all 3 decapods examined from Danish waters. However, the presence of *Hematodinium* in Denmark was expected because infected *Nephrops norvegicus* have been found in waters off the nearby Swedish west coast (Tärnlund 2000). The present study is also the first report of *Hematodinium* sp. in *Hyas araneus*, but the detection of *Hematodinium* sp. in *H. araneus* was likewise not surprising, considering that *Hematodinium* spp. infect a broad array of decapods (Stentiford & Shields 2005). Furthermore, *H. araneus* and *Chionoecetes opilio* share habitats in Greenland, and Greenlandic *C. opilio* populations are already known to host *Hematodinium* sp. (A. Burmeister unpubl. data).

The 3-times higher prevalence of *Hematodinium* sp.-positive *Chionoecetes opilio* at inner stations compared with offshore stations along the west coast of Greenland is consistent with previous studies showing that *Hematodinium* sp. is most abundant in closed coastal habitats (e.g. Shields 1994, Wilhelm & Miahle 1996). Epizootics often occur in enclosed hydrological areas like fjords and poorly drained estuaries or other restricted areas (Shields 1994, Pestal et al. 2003, Shields et al. 2005). However, epizootics actually do occur in open areas (Meyers et al. 1996, Field et al. 1998, Briggs & McAliskey 2002, Stentiford et al. 2002), but in open ocean systems, e.g. the habitats of *C. opilio* and *C. bairdi* in the Bering Sea, the prevalence of *Hematodinium* sp. is generally low and variable (Meyers et al. 1996). One reason might be that sheltered areas have a lower water exchange and closed host populations, but these possibilities are speculative as long as the complete life cycle of *Hematodinium* is not known.

The present study reveals the highest prevalence of *Hematodinium* sp. for the respective hosts to date. Samples of *Chionoecetes opilio* (n = 40, Stns 42 and 48), in which no *Hematodinium* sp. DNA could be detected using the primer set 18SF2/ITSR1 (Small et al. 2006, Fig. 2 present study), exhibited 22.5% *Hematodinium* sp.-positive samples with nested PCR (data not shown), and 10 *Pagurus bernhardus* specimens revealed 30% *Hematodinium* sp.-positive samples with standard PCR versus 80% with nested PCR (Figs. 2 to 4). *Nephrops norvegicus* samples were not examined with both protocols, but one catch from May 30, 2007, comprised 40 crabs from which 20 were examined by standard PCR (5% *Hematodinium* sp.-positive) and the other 20 by nested PCR (65% *Hematodinium* sp.-positive), again indicating a higher detection sensitivity of the nested PCR. Unfortunately it was not possible to obtain samples from all host species in large numbers and in these cases prevalence of *Hematodinium* sp.-positive hosts can only be considered as estimates. However, data presented here suggest that *Hematodinium* sp. may be more common than previously believed, since high percentages of *Hematodinium* sp.-positive samples were observed in host populations that appeared to have low prevalence of *Hematodinium* sp. based on the lack of morphological symptoms of infection. It is likely that the actual percentage of *Hematodinium* sp.-positive samples was underestimated in the present study, due to the large amount of host DNA in the samples or the presence of undetected PCR inhibitors. The possible presence of such false negative samples was not addressed in detail. Juveniles of *N. norvegicus* and *C. opilio* typically show higher *Hematodinium* sp. prevalence than do adults (Field et al. 1992, Stentiford & Shields 2005). In the present study, only adults were examined and, moreover, sampling was conducted during periods of the year when infection levels in the respective hosts are thought to be low (Field et al. 1992, 1998, Stentiford & Shields 2005).

An infection with *Hematodinim* spp. is generally assumed to be fatal in many host species (e.g. Meyers et al. 1987, Stentiford & Shields 2005). However, assuming that the *Hematodinium* sp. DNA detected in the present study stems from viable and infectious parasite cells, with 45 to 87.5% of all examined crustaceans potentially hosting *Hematodinium*, this is unlikely, because no population declines associated with *Hematodinium* epizootics have been reported for the examined stocks (A. Burmeister unpubl. data). We hypothesise that hosts may be experiencing latent infections that will only lead to symptoms if the host is stressed or otherwise experiencing unfavourable conditions. For detection of latent infections several assays have been developed (Field & Appleton 1996, Stentiford et al. 2001). However, in these studies the term latent infections refers to low parasite numbers in the

host (i.e. infections cannot be diagnosed with the colour or pleopod methods), and it is assumed that all latent infections will develop into apparent infections. Widespread latent infections that do not necessarily develop into apparent infections are known from other alveolate parasites, such as *Toxoplasma gondii* (Jakubek et al. 2007), and represent a common phenomenon in the biology of parasites. Stentiford et al. (2002) reported that in 2000 to 2001 most *Cancer pagurus* individuals died during transportation from their fishing grounds in the UK to other European countries. All of these crabs were infected with *Hematodinium* sp. and the disease may have become fatal due to the stress associated with capture, handling and transportation. Also *Hematodinium* sp.-infected *Callinectes sapidus* crabs died if stressed by handling or capture (Shields et al. 2003). However, stock declines cannot be explained solely by stress on the crustaceans, but future research may benefit from taking into account the combined effects of *Hematodinium* sp. infections and other biological and abiotic factors in the decline of decapod populations.

Sequence comparison and evolutionary divergence

Comparisons of ITS1 sequences indicated that all 5 hosts investigated harboured DNA from the same phylogroup of *Hematodinium*. This suggestion was corroborated by the estimation of evolutionary divergence between the sequences (Table 3). However, conclusions regarding species status or phylogeny should not be drawn as long as ITS1 sequences are the only data available (Burreson et al. 2005, Logares et al. 2007). In our analysis, sequences from *Liocarcinus depurator* were identical to sequences derived from *Pagurus bernhardus*, *Chionoecetes opilio* and *Nephrops norvegicus*. This finding was surprising since a previous study (Small et al. 2007c) found *Hematodinium* sp. ITS1 sequences derived from *L. depurator* to be only ~50% similar to sequences derived from *P. bernhardus, C. opilio* and *N. norvegicus* (Clade B, Jensen et al. 2010). *Hematodinium* sp. ITS1 sequences from *L. depurator* were, on the other hand, very similar to sequences from *Callinectes sapidus* (i.e. Clade A, Jensen et al. 2010). In other words, representatives from both *Hematodinium* clades may be able to infect *L. depurator*. The present study is the first report that different *Hematodinium* phylogroups (Clades A and B) can be found in a single host species. Hamilton et al. (2010) suggested a host and geographically related distribution of Clade B *Hematodinium* sp. ITS1 sequences and generated a phylogenetic tree consisting of 3 subclades: NE Atlantic langustines (*N. norvegicus*), NE Atlantic crabs (*Carcinus maenas* and *Cancer pagurus*), and an all NE and NW Atlantic host species subclade. However, this grouping may not be universal considering that the sequence FJ495187 from the crab *P. bernhardus* is identical to sequences from the NE Atlantic langustines subclade (e.g. EU096208, Table 3). Furthermore, Jensen et al. (2010) found a sequence FJ844426 from *C. angulatus* from the NW Atlantic that is identical to sequences from the NE Atlantic subclade (e.g. EF675761, Table 3).

Similarities between ITS1 sequences suggest that 2 outlier sequences, FJ172642 and FJ172643, (Fig. 5, sequence number 2, and Table 3) derived from *Chionoecetes opilio* are affiliated with Clade B (Jensen et al. 2010). It is noteworthy that these 2 sequences were very similar even though they originated from 2 different host crustaceans, and it is, therefore, unlikely that these 2 aberrant sequences were results of methodological artefacts. Comparison of the last 103 bp of the 18S rRNA genes (adjacent to ITS1) reveals 99% similarity between members of Clades A and B (Fig. 6). On the other hand, the 2 outlier sequences (FJ172642 and FJ172643) showed only 93% similarity to 18S

```
                                0         10         20         30         40         50
FJ172655  (Clade B)              CAGTTTCTGG AAGTGGCAGC TGGAAGTTTA GTGAACCTTA TCACTTAGAG
EF153727  (Clade A)              .......... ...C...... .......... .......... ..........
FJ172642  (Outlier)              .......... .......... ......G... .C........ C.........


                               50         60         70         80         90         100
FJ172655 (Clade B)              GAAGGAGAAG TCGTAACAAG GTTTCCGTAG GTGAACCTGC GGAAGGATCA TTC
EF153727 (Clade A)              .......... .......... .......... .......... .......... ...
FJ172642 (Outlier)              .....T.... .......... .C........ ...G.A.... .......... ...
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Fig. 6. *Hematodinium* spp. Alignment of partial 18S rDNA sequences from Clades A and B (defined by Jensen et al. 2010) and the outlier sequence identified in the present study. Numbers in the left column are GenBank accession numbers

sequences of Clades A and B. It would have been desirable to obtain complete 18S sequences of these 2 outlier sequences before making a final conclusion on their relationship, but attempts to generate such sequences were unsuccessful. ITS1 is a region that is usually more variable than the 18S rRNA gene (Jorgensen & Cluster 1988). Thus, based on the partial 18S sequences of FJ172642 and FJ172643, it is likely that they belong to a unrecognised species.

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